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ROLE OF APOMIXIS IN PLANT BREEDING

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CERTIFICATE

This is to certify that the seminar report titled "ROLE OF APOMIXIS IN PLANT BREEDING " has been solely prepared by Ms. Sani George (2004-11-43), under my guidance, and has not been copied from any seniors, juniors or fellow students seminar reports.

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DECLARATION

I, Sani George, (2004- 11-43) hereby declare that the seminar entitled " Role of Apomixis in Plant breeding" has been prepared by me. after going through various references cited at the end and has not been copied from any of my fellow students.

Vellanikkara

Date: 26 -11-2005

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2004-11-43

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INTRODUCTION

Effective systems for the production of F1 hybrids have now been developed in a wide range of crop plants using, in the majority of cases, cytoplasmic male sterility and to a much lesser extent, self incompatibility while the present system of hybrid production have proved their value in field, and the resulting hybrid varieties have often substantially improved yield potentials, they suffer from a number of disadvantages. Here comes the role of apomixis.

However, before embarking on a systematic effort to develop apomictic crop genotypes it is important to understand the sorts of mechanisms that lead to asexual seed production in plants and which of these mechanisms are exploitable in agricultural systems. The aim of this paper is, therefore, to summarise current knowledge of the types of apomixis encountered in plants.

MODES OF REPRODUCTION IN PLANTS

The various modes of reproduction found in crop plants may be broadly grouped in to two categories. 1. Sexual 2. Asexual

Sexual reproduction

Sexual reproduction involves fusion of male and female gametes to form a zygote, which develops in to an embryo. Progeny will have both the characteristics of male and female parents.

Asexual reproduction

Asexual reproduction does not involve fusion of male and female gamete to form a zygote. Asexual reproduction is of two types

1.Vegetative reproduction 2 Apomixis

1. Vegetative reproduction

New plants arise from vegetative part of plant. This may occur through modified underground and sub–aerial stems and through bulbils.

Eg: Rhizome, setts.

2.Apomixis

Seeds may arise from embryo with out fertilization. Apomixis in flowering plants is defined as the asexual formation of seed from the maternal tissues of ovule avoiding the processes of meiosis and fertilization, leading to embryo development. Winkler introduced the term apomixis. Which means the substitution of sexual reproduction by an asexual multiplication process without nucleus and cell fusion.

Initial discovery of apomixis was on a solitary female plant of *Alchomea ilicif*olia, which sets seeds at Kew Gardens in England. The current usage of apomixis is synonymous with the term agamospermy (Richards, 1997).

PREVALENCE OF APOMIXIS

It has been described in over 400 flowering taxa, including representatives of over 40 families (Carman, 1997) and it is well represented among both monocotyledonous and eudicotyledonous plants, though it appears to be absent in gymnosperms Of the plants known to use gametophytic apomixis, 75% of confirmed examples belong to three families Asteraceae,Poaceae, and Rosaceae, which collectively constitute only 10% of flowering plant species Apomixis frequently is associated with the expression of mechanisms that limit selfertilization (autogamy) Many apomictic plants belong to genera in which sexual members predominantly exhibit physiological self incompatibility, dioecy, heterostyly or polyploidy In some cases it is clear that apomicts themselves have retained such mechanisms Dioecy is known in a number of apomicts, such as *Antennaria*, and *Coprosma* (Heenan *etal*., 2002). Similarly self incompatibility was demonstrated recently in apomictic species *Hieracium piloselloides* (Bicknel *etal*., 2003).

Apomicts are found commonly in habitats that are frequently disturbed or either where the growing season is short such as arctic and alpine sites, where other barriers are operate to inhibit the successful crossing of compatible individuals such as among

widely dispersed individuals within a tropical rain forest (Asker and Jerling, 1992). Gametophytic apomixis is known among herbaceous and tree species, but it is considerably more common among the former.

CLASSIFICATION OF APOMIXIS

Several systems have been proposed for the classification and description of this phenomenon.

Classification by Stebbins (1950)

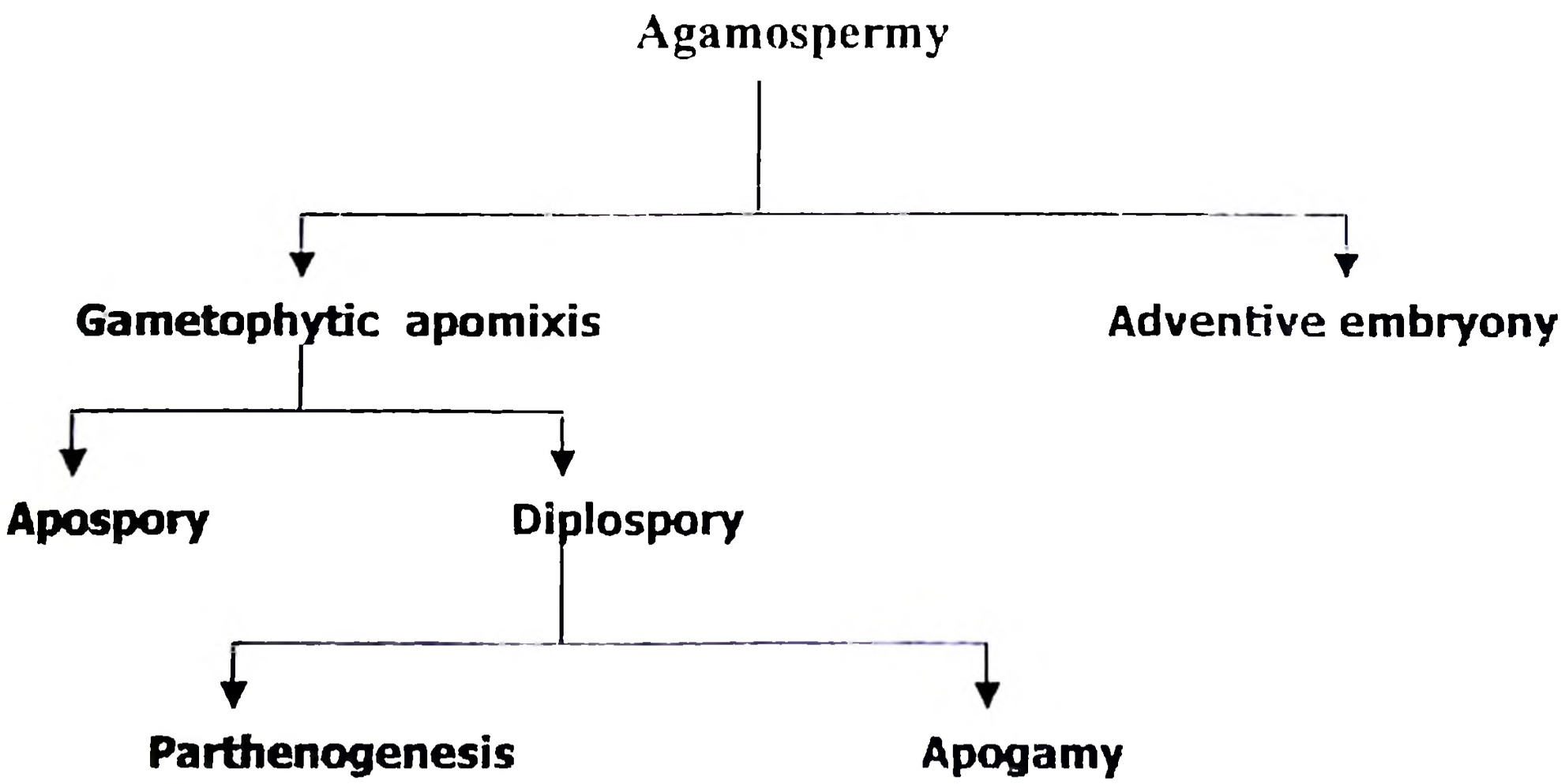
Under this scheme apomixis may be broadly classified in to two main types vegetative reproduction and agamospermy, asexual seed reproduction. As emphasized by Stebbins (1950) not all types of vegetative reproduction can be termed apomixis, but only those that are substitutes for the sexual method. These include the bulbils or other propagules, which replace flowers or inflorescence. This form of reproduction often incorrectly referred to as viviparv, has been reported in a number of genera including *Allium*, *Polygonum*, *Agave*, *Poa* and *Festuca*. However the term also covers reproduction by stolons,rhizomes, runners and the like where sexual reproduction are not functioning Several authors have argued against the inclusion of vegetative reproduction under the general heading of apomixis.

Agamospermy can in turn be divided into two main categories, adventitious embryony, and gametophytic embryony. In adventitious embryony the embryo develops directly from sporophytic cells in the ovule without any intervening formation of embryosacs and eggcells This phenomenon has also been called sporophytic embryony. Nucellar and integumental embryony are the sub divisions of sporophytic embryony.

In gametophytic apomicts the embryo develops without fertilization from a diploid gametophyte.As stressed by Stebbins (1940) gametophytic apomicts must involve two processes not found in sexual plants, substitute for meiosis and a substitute for fertilization . Modifications of meiosis fall into two types - apospory in which an unreduced embryo sac is formed from somatic cell in the ovule by a series of mitotic divisions. And diplospory, where unreduced embryosacs are formed from a cell of

archesporium by circumvention of modification of meiosis. So that a reduction in chromosome number doesn't occur. Unreduced embryosacs formed by apospory or diplospory may give rise embryos by parthenogenesis or pseudogamy.

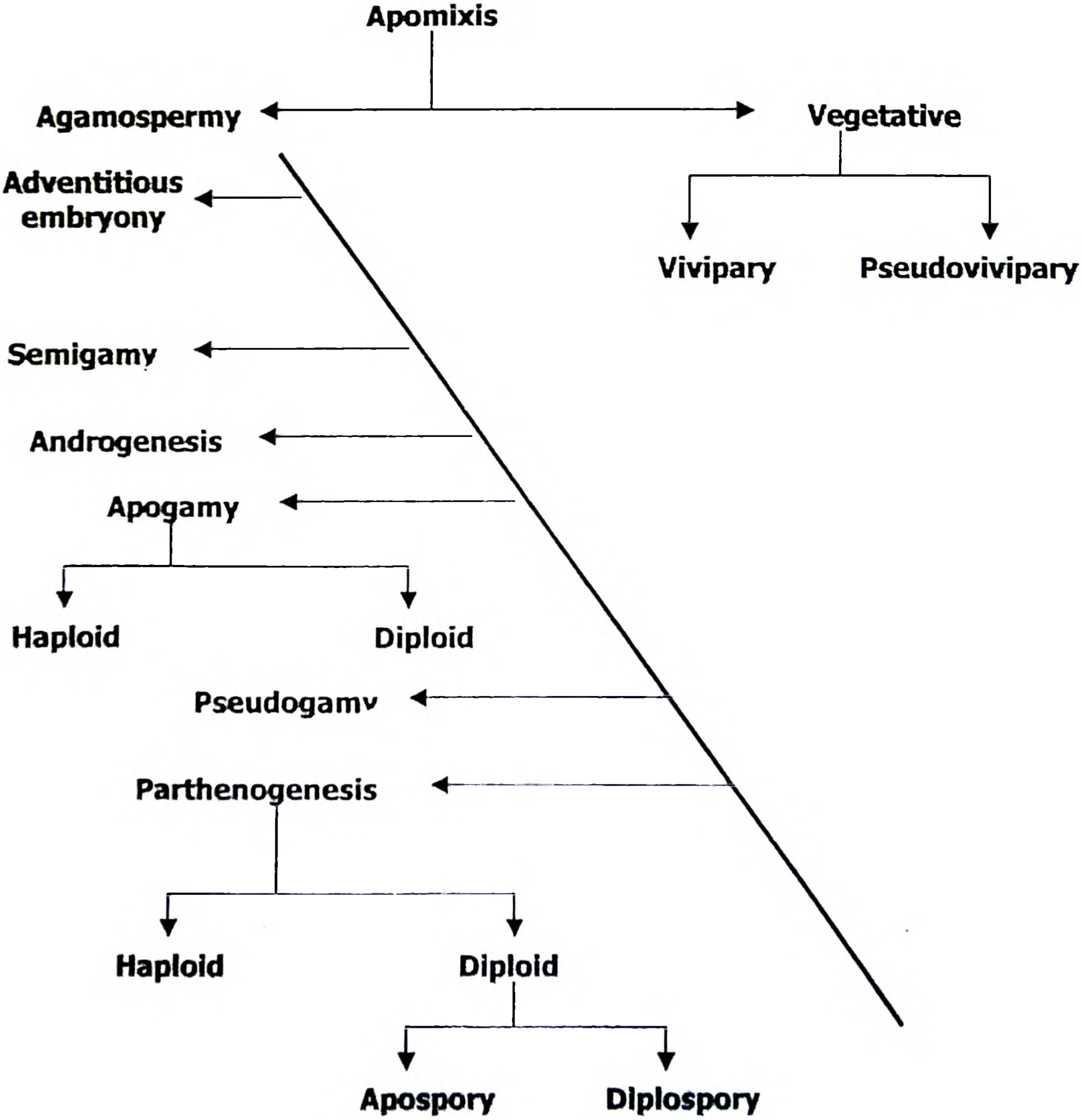
Gametophytic apomixis may be obligate or facultative. In obligate apomicts, the sexual process had been replaced completely by the asexual method of reproduction. In contrast in facultative apomicts, the sexual and asexual methods of reproduction coexist in one plant. Obligate apomixis is more useful in crop improvement programme.



(Stebbins ,1950)

CLASSIFICATION BY FRANKEL AND GALUN (1977)

This classification is widely used. Frankel and Galun classified the apomixis into two types agamospermy and vegetative apomixis as follows,



(Frankel and Galun, 1977)

AGAMOSPERMY

1.ADVENTITIOUS EMBRYONY

Embryo develops directly from sporophytic cells in the ovule without any intervening formation of embryo sacs and egg cells. It is also known as sporophytic apomixis.Two types of adventitious embryony are,

1.Nucellar embryony- Embryo develops from nucellar tissues. It is common in citrus and mango.

2. Integumental embryony- Embryos develop from integuments of ovule.

2.SEMIGAMY

Semigamous type of apomixis is closest to sexual process of reproduction. In *Rudbeckia* or these types of plants, normal pollination takes place, pollen tubes enter the embryo sac and male gametes are separated. One of male gametes going to polar nuclei and other entering egg cell. But fusion of male gamete with the egg cell nucleus does not take place. Thus binucleate zygote develops in which both the nuclei, from the egg cell and pollen cell, divide independently of each other. Embryo in this case is a conglomeration of cells with male and female nuclei

E.g. tomato, cotton, *Rudbeckia* (black eyed susan), variegated plants

3.PSEUDOGAMY

Normal pollination occur, the pollen tube grow and reach the embryo sac, one of male gametes fuses with the polar nuclei, and the other enter egg cell. The male gamete does not fuse with egg cell, but degenerates. The nucleus of egg cell starts further division to form embryo with only female nucleus. An essential condition for pseudogamous development of embryo is fusion of one of male gametes with the

central nucleus of the embryosacs, after which the endosperm develops. In such cases, the development of embryo is brought by the stimulating effect of endosperm.

Eg: *Potentilla sp.*, *Solanum*

4.ANDROGENESIS

The male gamete enters the egg cell, but degeneration of egg cell takes place, the nucleus of pollen is preserved and functions in cytoplasm of egg cell. Thus the embryo of male origin develops. Anther and pollen can also be cultured in tissue culture medium.

Eg: *Nicotiana* and rice.

5.APOGAMY

The egg cells as well as the other cells of the female gametophyte have the capacity for further development The embryos are formed along with the eggcell from synergids and from antipods Such apomictic development, in which the embryo develops not from the egg cell but from other cells of female gametophyte is called apogamy. Most commonly this occurs in the cells of egg apparatus where it is often difficult to establish, which of three cells is the egg cell and which are synergids In other cases synergids acquire the appearance of egg cells

Eg. orchids and Allium, liliaceae family

If the embryo develops in haploid embryo sacs it is called haploid apogamy. In diploid apogamy embryo develops in diploid embryo sacs

6.PARTHENOGENESIS

Autonomous development of an embryo from female gametophytes in the absence of pollination or any stimulus from male gametes.

Two types are,

1.Haploid parthenogenesis – embryo is developed from haploid egg cell in ovule (embryo sac is haploid).

Eg: tomato, potato, and wheat

2.Diploid parthenogenesis – embryo is derived from diploid egg cell in ovule (embryo sac is diploid). E.g.: meadow grass.

Diploid parthenogenesis is classified in to apospory and diplospory based on origin of diploid embryosacs.

Apospory

Diploid embryo sacs develop from somatic nucellar.or chalazal cell. It is common in poly ploid species.

Diplospory

Diploid embryosacs develop from megaspore mother cell (2n) without meiosis

a.haploid diplospory: embryo develops from haploid egg cell.

b.Diploid diplospory : embryo develops from diploid egg cell

Causes of parthenogenesis

- ✔ Early degeneration of sperm
- ✔ Very long style
- ✔ Schlerenchymatous style
- ✔ Short pollen tube
- ✔ Slow rate of pollen tube growth
- ✔ Inability of pollen tube to discharge the contents inside the embryo sac

Artificial induction of parthenogenesis

- By the atimulation of widely related pollen or foreign pollen
- By low temperature

- By pollinating with X- ray irradiated pollen
- By treatment with chemicals like Belvitin

VEGETATIVE APOMIXIS

The new plants are arised from modified floral organs. It is of two types.

1.vivipary: vegetative organs are formed instead of flowers. E.g.: *Poa*

2.pseudo vivipary: vegetative organs are developed along with flowers.

Eg: bulbils in *Allium* (leek).

TYPES OF APOMIXIS THAT ARE USEFUL IN PLANT BREEDING

The two broad categories of apomictic plants are, obligate apomicts those plants, which reproduce only through apomixis and facultative apomicts, those plants, which reproduce through sexual and apomictic reproduction. Obligate apomixis is more useful in plant breeding than facultative apomixis

Four processes that are useful in plant breeding are,

1. Diplospory
2. Apospory
3. Automixis or fusion of reduced cells
4. Semigamy

1. Diplospory

In diplospory , the megaspore mother cell differentiate like that of sexual ovules, but its nucleus doesnot undergo meiosis Instead the nucleus divides, mitotically or meiotically with First Division Restitution (FDR) In FDR the nucleus doesn't undergo the normally expected disjunctional separation of homologous chromosomes at anaphase I. Instead, the entire diploid complement divides meiotically, giving rise to a dyad with two unreduced spores. The two daughter nuclei are by and large similar; the embryo sac originates from such nuclei and carries the un reduced genome exactly similar to maternal tissue.

2. Apospory

Apospory differs from diplospory only in the origin of embryosac.Any cell from the nucellus forms the embryo sac. The types of divisions are similar to those in diplospory.

3. Fusion of reduced cells, Automixis

Fusion of two haploid nuclei in a meiotic embryo sac or restitution in the in the second division of meiosis also results in diploid reproductive cells. Such diploid cells will be completely homozygous and as such are useful in the practical exploitation of apomixis.

4.Semigamy

The male nucleus penetrates the eggcell but doesn't fuse with the egg nucleus, both nuclei divide independently and the embryo shows a co- existence of female and male nuclei. This phenomenon has been used for the production of haploids in cotton (Turcottle and Feaster, 1967)

GENETIC CONTROL OF APOMIXIS

The inheritance of gametophytic apomixis has been reported to be associated with the transfer of either a single locus or a small number of loci in most of the systems studied to date. In the aposporous grasses *Pennisetum* , *Panicum*, and *Brachiaria* (Valle *etal* , 1994) apomixis is reported to be simply inherited , with the trait conferred by the transfer of a single dominant factor Simple dominant inheritance also has been reported for apospory in the dicotyledonous genera *Ranunculus* and *Hieracium* (Bicknell *etal*., 2000). Among the diplosporous apomicts, independent inheritance of diplospory and parthenogenesis has been observed in the dandelion Taraxacum and in Erigeron (Noyes, 2000).

It is widely generalized that there is either one locus, or only a small number of loci, involved in the inheritance of apomixis in native systems. In almost all of cases mentioned, the inheritance of apomixis was recorded after crosses between closely

related sexual and apomictic species. Apomixis is a complex trait. Ideally it should be measured strictly through the production of genetically identical seedlings, coupled with an embryological study to confirm the reproductive mode of each progeny plant and an assessment of ploidy in all individuals. Recent studies also suggest that three unlinked loci are required for the inheritance of autonomous diplosporous apomixis in *Taraxacum officinale* (Van Dijk *etal.*,2003).

An apparent interspecific hybrid origin also is a common feature among apomicts, and the combination of polyploidy and hybridity is believed to have resulted in allopolyloidy in many gametophytic apomicts (Roche *etal.* 2001a).Hybridity between related species has been a key factor in the formation of many apomictic complexes and that different types of apomixis and related phenomena are all expected out comes of a theoretical model based on the disjunction of a relatively small number of key regulatory events.

There is growing evidence for the presence of supernumerary chromatin in several apomictic species, and it is clearly involved in the inheritance of apomixis in the grasses *Pennisetum squamulatum* and *Cenchrus ciliaris* (Roche *etal.* 2001b). It also has been proposed that the inheritance of apomixis may be favoured by the mediation of a diploid or polyploid gamete (Nogler, 1986). Apomictic polyploids often are capable of producing diploid progeny, typically through the operation of polyploidy a natural mechanism analogous to haploid parthenogenesis that often is observed in apomicts (Asker and Jerling, 1992).

DETECTION OF APOMIXIS

Apomictic reproduction is mostly observable in an offspring that is more uniform than expected, the uniform plants being similar to the mother plant. The percentage of uniform plants gives the degree of apomixis in the female plant. If use of own pollen is possible the lack of inbreeding depression in most of the progeny plants is another indication. An enhanced number of multiple seedling too refers to apomictic

behaviour, found 1- 2% twin seeds in the apomictic grass *Poa pratensis* in the cross fertilizing species *Lolium perenne* and in *Phleum pratensis* only 0.04%. An uneven chromosome number (triploid, aneuploid) combined with an undisturbed seed setting is typical of apomictic reproduction.

Embryological studies of young ovules can confirm apomictic behaviour. The occurrence of more embryo sacs in the young ovule is an important criterion.In some grasses the aposporous embryo sacs are not full grown but four nucleate. In diplosporous apomicts cytological studies should give information on whether the first division of a megaspore mother cell is meiotic or mitotic, which is not easy because of the small size and early age of ovule when meiosis occurs (Bashaw, 1980).

Embryological studies inform on the mechanism of apomixis, but progeny tests are required to determine the degree of apomixis. An aposporous embryosac itself will not guarantee a wholly or partly apomictic progeny, because it can fail to develop into a seed (Grazi *etal*, 1961). In many breeding programme only progeny test is used because the cytological work requires special equipment and expertise and is laborious. For highly reliable tests, many plants are needed and 60- 100 progeny plants are used. Other indications of apomixis are, Callose (β 1, 3- linked Glucan) depositions, observed during megasporogenesis in sexual species are nearly lacking in the developing apomictic embryosacs and uniformity of progenies from heterozygous or cross pollinated parents

INTRODUCTION OF APOMIXIS IN TO AMPHIMICTIC CROPS

Transferring the apomictic trait to desired varieties means fulfilling one of the most cherished dreams of plant breeders, to allow elite cultivars to reproduce with out losing hybrid vigour and or desirable traits. Overall two general approaches can be followed in introducing thee trait.

1. Introgression using classical methods.
2. *Denovo* engineering of apomixis through biotechnology.

Introgression using classical methods

An advantage of classical methods is that no prior knowledge of the molecular aspects or of the genes involved is needed. The introduction of apomixis may be done by any of the following three strategies.

1.Introgression of apomixis from an apomictic wild relatives

Wide crosses can be performed to introduce the apomictic trait from a wild species to the cultivated species. ARS-USDA staff and collaborating scientists followed this method where they transferred the trait from eastern gamagrass (*Tripsacum dactyloides*) to corn, there by producing the first apomictic corn in1998.

Eg. *Beta vulgaris*, a diploid cross breeding crop, can be hybridized with tetraploid apomictic forms of *Beta lomatogona* and *Beta intermedia*. From these crosses, triploid, tetraploid and pentaploid apomictic plants can be selected (Cleij *et al*, 1976). An apomictic *Beta* cultivar could not only fix heterosis but as the apomixis is of the autonomous type, pollination problems could be eliminated.

In self fertilizing crops, introduced apomixis could be used for the production of true breeding heterozygous cultivars. In *Triticum aestivum* apomixis could be introduced from the related *Agropyron scabrum*, which give fertile hybrids with *Triticum aestivum*. Hermsen (1980) suggests that in *Solanum tuberosum* possible apomictic cultivars could be used for raising potato from botanical seed instead of tubers.

2.*Generation of apomixis through hybridization of sexual progenitors* - this strategy is based on the genome collision theory for apomixis evolution which assumes that an apomictic hybrid can be obtained by crossing two reproductively divergent ecotypes.

3.*Combining reproductive mutants showing some aspects of apomixes* - in crosses between reproductive mutants that exhibit non –recurrent forms may be done to produce an apomictic cline.

***Denovo* engineering of apomixis through biotechnology**

In theory, it is possible to introduce apomixis to many species using conventional breeding approaches, it is more efficiently done using biotechnology or by

the combination of the two approaches. Biotechnology also is the only resort for plants that have no apomictic relative species.

The engineering of apomixis may be done at both gametophytic and sporophytic levels requiring several assumptions and concerted effort in threre maoin areas:

1. Identification and characterisation of candidate genes.
2. Isolation of promoters to allow precise control of the genes at both spatial and temporal levels.
3. Development of technologies to introduce and control transgenes and or perform targeted mutations.

APPLICATIONS OF APOMIXIS

1 Fixation of heterosis

2 Economic hybrid seed production

3 Propagation of hybrid seeds

4 Resistance against pathogens

5 Handling of propagation material

6 Increased reproduction efficiency

7 The production of pure homozygotes

8 The production of phenotypically stable population – vybrids

1.Fixation of heterosis

Completely true breeding F1 hybrids

Heterotic hybrid varieties of crop plants have several advantages over pure line varieties. They provide optimal performance under optimal growing conditions, provide phenotypic stability under stress, allow joint improvement of traits which are negatively associated through linkage or pleiotropy and allow the combination in one individual of harmonious set of traits from very diverse parents without necessarily disturbing the existing harmony (Griffing and Langridge, 1963). Cultivation of hybrid varieties,

however, is expensive, mainly due to high cost of seed that has to be purchased every year.

Apomixis of aposporous or diplosporous type results in the fixation of heterosis.Obligate apomixis result in complete fixation of heterosis. In addition to being inexpensive exploitation of heterosis through apomixis have the following advantages over the employment of conventional cytoplasmicgenic male sterility.

Advantages of apomixis in hybrid production

1 The production of several F1 hybrids through apomixis is more efficient than a conventional breeding programme using cytoplasmic genetic male sterility.

2.In apomixis breeding studies on testcrosses and combining ability are not needed. The evaluation of apomictic hybrids is more efficient than sexual F1's.

3.Incorporation of desirable characters like disease, insect and drought resistance is easier and quicker in apomictic material.

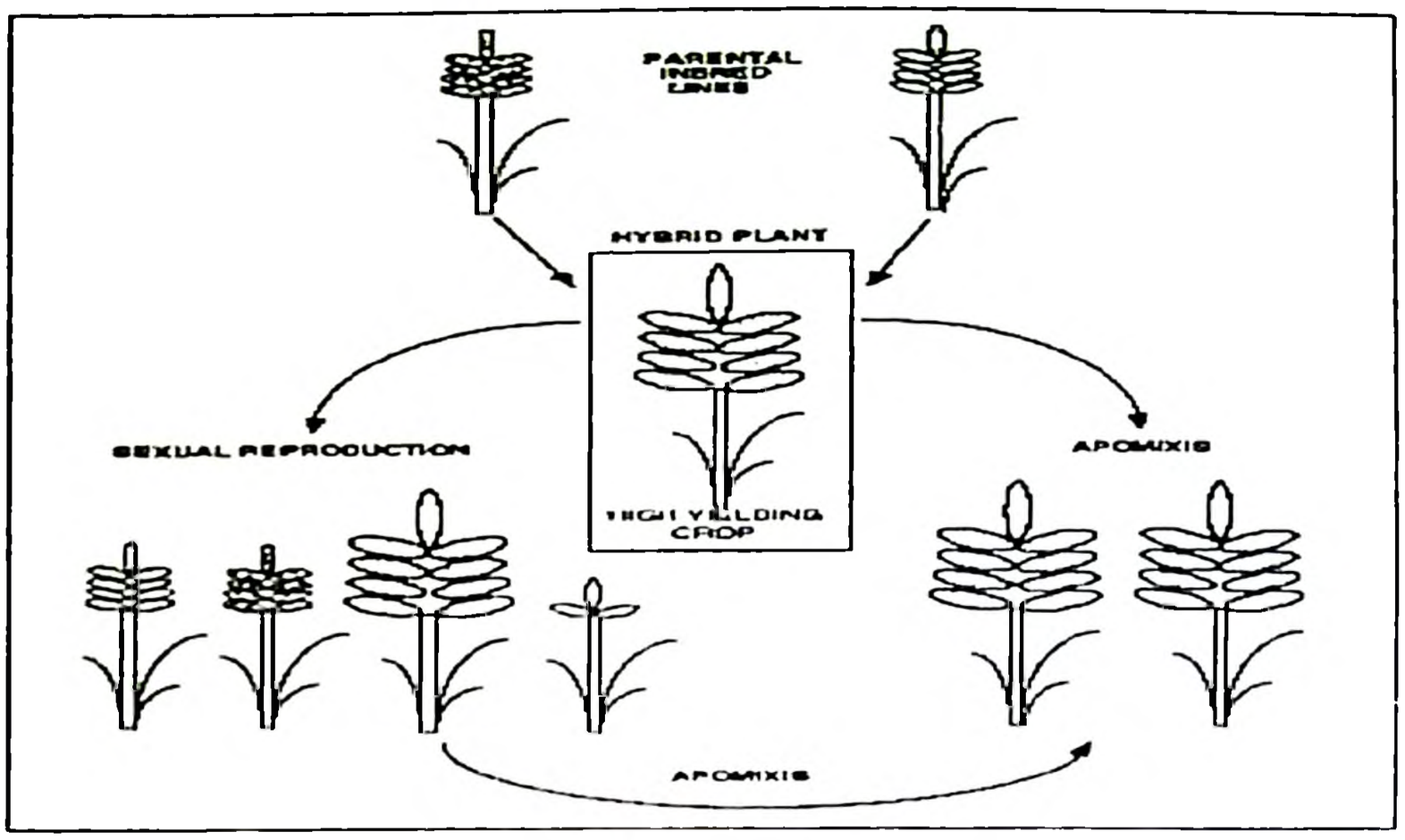
4.The genetic diversity that can be utilized is much more in apomictic material This is because the limitations in parental combinations imposed by use of cytoplasmic male sterility are absent

5.Seed stocks are easily maintained. An apomictic hybrid can be maintained as a single seed stock where as a conventional hybrid is maintained as three separate lines

6.The will be no problems of availability and dispersal of pollen in seed fields

7.Apomictic hybrids can be developed because of improved breeding efficiency

8.Vulnerability for epidemics with the use of a single cytoplasm can be avoided because of utilizing diverse materials



Picture showing the fixation of heterosis by apomixis

2.Economic hybrid seed production

The production of seed by inbred lines remains complicated by their reducing viability, laborious and expensive activities for preventing cross pollination So with apomixis the cost of hybrid seed production could be drastically cut

PRODUCTON OF VYBRIDS, PHENOTYPICALLY STABLE POPULATIONS:

The greatest advantage in the exploitation of apomixis for practical use among seed propagated plants has so far been made in sorghum and has resulted in the concept of Vybrids.

a) Apomixis in sorghum occurs to the extent of 30%under cross pollinated and up to 80%under self pollinated crops.

Apomixis in sorghum has the following three essential elements:

1. The production of unreduced and or diploid female sex cells.
2. The prevention of fertilization.

3. Embryo and endosperm formation from un reduced and or diploid sex cells without fertilization.

Apospory, diplospory, and synkaryogenesis are the three phenomena that result in the production of unreduced and diploid sex cells.

Cross sterility, the in ability to set seed on crosspollination, prevents fertilization. Seed making ability in emasculated spikelets of apomictic sorghum is under genetic control. Apomixis in sorghum is pseudogamous, that is, a stimulus of pollination is needed to produce viable embryos and endosperm. Single plants from advanced generation progenies of sexual apomictic cross have been found to induce seed set on emasculated spikelets of facultative apomicts (Murty and Rao, 1979).

THE CONCEPT OF VYBRIDS

Heterozygous F1 individuals with facultative apomixis produce a population containing two types of offspring. Those with F1 genotypes derived through apomixis and those with F2 types obtaining through sexuality. The frequency of F1 genotypes will be equal to the frequency of apomixis. If the next generation is deriving through the F1 genotypes plants only, then the population mean will remain the same. If the procedure is repeated it is possible to maintain heterozygotic vigour in subsequent generation and to keep the mean a constant. Such populations are known as Vybrids. Vybrids are the progenies obtained from crossing two facultative apomicts, which reproduce through facultative apomixis. They are super varieties with yield levels remaining constant

PROCEDURAL DETAILS FOR THE PRODUCTION OF VYBRIDS

1. Select desirable genotypes and cross them with one or more facultative apomicts with the latter as females

2. Grow F1 generation. Build B2 and F2 generation of heterotic F1's.

3.Grow F1, F2, and B2 generation side by side. Select vigorous F1 like plants from either the F2 or B2. Vigorous plants occur in greater frequency in B2 tag the plants at the time of anthesis. Test for cross sterility by crossing a portion of ear to any other pollen parent. At maturity harvest only the selfed seed of the cross steriles.

4.Grow the progeny of cross steriles. Make crosses between desirable plants from different crosses.

5.The seed from such crosses give both sexuals as well as facultative apomicts. Reject completely segregrating sexuals. Select only vybrids that have a high frequency of F1 like vigorous plants.

6.Yield test of good vybrids. Good vybrids should have a) A high frequency of apomixis. b) A high degree of yield heterosis and c) A minimum of negative transgressive segregration.

Murty, *etal.* (1985) concluded that in situations where sorghum are grown for both fodder and grain, the vybrids represent optimal phenotypes exhibiting greater stability of performance over diverse environments

A heterozygous genotype may possess individual buffering, and a heterogenous population will possess population buffering. According to them the single genotype would have the property of individual buffering resulting from its heterozygosity,while the composite of genotypes would have the property of population buffering resulting from both heterogenity as well as heterozygosity. The vybrids combine the attributes and hence appear more stable than F1 hybrids

THE PROBLEMS OF APOMIXIS BREEDING

Problems associated with apomixis breeding are,

1.The estimation of the level of apomixis

2.The environmental effects on apomixis frequency

3. The difficulties encountered in maintaining genetic stocks and

4.The complicated nature of its inheritance.

1.The estimation of the level of apomixis

The frequency of apomixis has two components they are, frequency of apomictic embryosacs and the frequency of apomictically formed offspring. But true apomictic frequency is the frequency of progeny obtained without the fusion of male and female gametes.

a) Study of serial sections of ovules:

The classical procedure for the estimation of the frequency of diploid embryosacs involves cytological screening of sectioned ovules during megasporogenesis. It should also be realized that the relative frequency of apomictic embryosacs might not accurately reflect the apomictic seed, particularly when pollination is uncertain or there is marked zygotic competition (Barlow, 1958). The classical microtome methods being tedious and time consuming, from time to time simple procedures were being applied. These included methods for observing whole embryosacs (Herr 1971, Young *etal*, 1979) and acetocarmine squashes.

b) Progeny tests

Progeny test provide a direct measure of relative frequency of apomictic seed. These methods allow for scoring of large numbers of individual plants. Progeny tests have disadvantages. The frequency of apomixis could be biased upward due to accidental selfing and tight linkage of marker genes. Moreover, the frequency of apomixis is not constant and is subject to environmental effects.

2.Enviornmental effects on apomixis

The frequency of apomixis, especially in facultative, is subject to environmental influences like, locations, seasons and years. The sorghum Line R473 was investigated one at tropical (India) and one at temperate location (Texas). It showed different frequencies of apomixis in two environments. In Kentucky blue grass, there exists a delicate balance in the competition for survival of the sexual and apomictic embryo sacs within the ovule. Sometimes environment can upset their balance in favour of one of these gametophytes.

3.Maintenance of pure genetic stocks

In the absence of morphological markers linked with apomictic reproduction, maintenance of stocks become difficult. This is especially true with facultative apomicts. To determine the reproductive status of an accession it becomes necessary for periodical progeny tests or embryological analysis.

4.Genetics of apomixis

A thorough knowledge of the inheritance of apomixis is necessary for the successful manipulation of apomixis in plant breeding. However, precisely this information is lacking in many apomicts. The principal reason for this is the apomixis in most plants is not a single event but is made up of different elements, each of which may or may not be under genetic control of one or a different kind.

ACHIEVEMENTS IN APOMIXIS BREEDING

'Adelphi' Kentucky blue grass is a first generation highly apomictic hybrid developed from the cross 'Bellevue' x 'Belturf' kentucky blue grass. An unreduced, egg of 'Bellevue' was fertilized by a reduced gamete from 'Belturf' resulting in a facultative apomictic hybrid possessing approximately 800 chromosomes (Funk *etal*., 1973).'Bonnie blue' is another highly apomictic first generation hybrid developed by crossing'Bellevue' kentucky blue grass with 'Penstar' kentucky blue grass. An unreduced egg of' Bellevue' was fertilized by a reduced gamete from'Penstar', resulting in a hybrid possessing approximately 94 chromosomes.

Significant efforts have been made to transfer apomixis from wild to cultivated species in wheat, maize and pearl millet. The BC program in pearl millet has progressed to the BC7 resulting in tetraploid (2n=4x=28 or 29) apomictic pearl millet like plants. The pearl millet program has an advantage over the other genera in that the *Pennisetum* genus has a number of different apomictic poly ploid species to use as the donor species. A major problem to overcome in the conventional BC approach is poor

seed set on derived apomicts. This has been partially overcome in pearl millet program by BC to genetically diverse tetraploids.

A new blight tolerant buffel grass cultivars AN – 17 –PS developed by crossing between the sexual clone TAM—CRD Bls and Zarago –za-115 (apomictic). This selected genotype has shown as excellent forage yield potential, good seed quality, blight disease tolerance, and an out standing cold tolerance. The protocols for obtaining obligate apomictic hybrids were derived and implemented. The source of the obligate apomixis was a biotype *Cenchrus ciliaris* L.

Since 1977, Prof. Jiansan Chen has been dedicated in fixation of heterosis in rice hybrids. By crossing facultative apomictic hybrid varieties *Oryza sativa* to *Oryza longistaminata*, Chen selected and later released stable and uniform varieties from the F3and F2 generations. These rice varieties, called vybrids (synkaryogenesis, Fn) following the mechanism of asexual duplication, can fix the heterosis of interspecific, inter sub specific and intervarietal crosses and surpass the best 3- line (cytoplasmic male sterile, maintainer and restorer) F1 hybrid rice cultivars in yield and are superior in rice quality

From intersubspecific crosses of these varieties, the additional varieties (*'Zhongwu No.1'*, *'Zhongwu No.2'*, and *'Guyou No.5'*,) have been selected. These varieties are being grown on 90,000 ha in China, combing the advantages of both hybrid rice and conventional rice and characterize a good rice quality, multi-resistance and wide adaptability, and per ha yield 9000–12500 kg, being 10-25% higher than that of the conventional varieties

The new ***vybrid*** rice breeding method is better than the 3-line hybrid rice system in that any high-yielding good-quality varieties can be used as parents of ***vybrids***. Furthermore, the price of ***vybrid*** seeds is low because this approach eliminates the need to annually produce hybrid seeds in crossing blocks, and ***vybrid*** breeding takes only 1/3 of the time to release new varieties compared to inbred rice breeding. This breeding method bypasses the dilemma of unavailability of both good quality and high yield, and both early-maturity and high yield, simultaneously. The ***vybrid*** breeding technique may

be the beginning of a second " green revolution " due to its capability to rapidly "pyramiding" genes for yield, quality, and abiotic stress tolerance into one variety. If this methodology can be demonstrated and documented widely, its adoption will greatly increase both the rice production and nutritional value to better meet the needs of an ever-increasing population in the world.

GuYou No.20 by crossing facultative apomixis (3027 * IR72 * Zhongzu No.300) F2 was selected in 2000. It has significant heterosis, strong tillering ability, good plant type, plant height 110cm, panicle length 32~38 cm, fertility 92% with fertility grains 230, 1000 grains weight 28g, transparent grains, good color on ripe plants, fertilizer resistance, high lodging resistance, strong disease resistance, and can be used in many generations without seed reproduction every year, and adaptable to Yangzi river valley plain with whole growth period 135 days on single cropping with per hectare yield 1300 kg.

Current research on apomixis

Apomictic species/approach	Organization	Location
Wheat	Institute of Plant Genetics	Gatersleben, Germany
Hieracium	C&FR	Christchurch, New Zealand
Evolution of apomicts	Utah State University	Logan, UT, USA
Histology of apomixis	Jagellonian University	Kraków, Poland
Taraxacum	NIOO	Heteren, the Netherlands
Pennisetum	USDA-ARS	Tifton, GA, USA
Molecular tools for apomixis	CAMBIA	Canberra, Australia
Allium	Kyushu National Agricultural Experimental Station	Miyazaki, Japan

Rice	Academia Sinica	Bejing, China
Hieracium	CSIRO	Adelaide, Australia
Pennisetum	University of Georgia	Tifton, GA, USA
Paspalum	IBONE	Corrientes, Argentina
Tripsaccum dactyloides	CIMMYT	Mexico, Mexico
Brachiaria	CIAT	Cali, Colombia
Somatic embryogenesis	Wageningen Agricultural University	Wageningen, the Netherlands
Cassava	University of Brasilia	Brasilia, Brazil
Arabidopsis mutagensis	CSIRO	Canberra, Australia
	University of California	Berkeley, CA, USA
	Cold Spring Harbor Laboratory	Cold Spring Harbour, NY, USA
	CPRO-DLO	Wageningen, the Netherlands
	Harvard University	Cambridge, MA, USA

Patents related to apomixis

Publication number (Filing date)	Title	Abstract of claims	Applicant(s)
WO9743427 (13 May 1996)	production of apomictic seed	expression of a gene which renders the embryogenetic production of embryo sac tissue	Novartis
US5710367 (22 Sept 1995)	apomictic maize	maize/*Tripsacum* hybrids used to introgress apomixis into a maize background	USDA

US5811636 (22 Sept 1995) WO9710704 (23 Sept 1996)	apomixis for producing true-breeding plant progenies	gene(s) transferred from *Pennisetum squamulatum* into cultivated plants resulting in apomictic progeny	USDA
WO8900810 (9 Feb 1989) EP329736, AU629796, CN1040123, AU2255288	asexual induction of heritable male sterility and apomixis in plants	induction of male sterility and apomixis through the introduction of transmissible male sterility factors present in extracts of male sterile alfalfa plants.	Maxell Hybrids Inc.
WO9836090 (17 Feb 1998) AU6405498	means for identifying nucleotide sequences involved in apomixis	genes in sexual species of *Gramineae* known to be orthologous to equences associated with apomixis in related species. Isolation and modification of those sequences	CIMMYT-ABC
CN1124564 (19 June 1996)	hybrid vigor fixing breeding process for rice apomixis	breeding and selection strategies for isolating apomixis in rice and for developing apomictic rice varieties	Chen Jiansen
WO9828431 (24 dec 1997)	transcriptional regulation in plants	meiosis specific promoter that will direct gene activity to tissues associated with gamete formation in plants	John Innes Centre Innovations Ltd
WO9808961 (5 March 1998)	endosperm and nucellus specific genes, promoters and uses thereof	endosperm specific and a nucellus specific promoter	C Linnestad, O A Olsen, D N P Doan
Sources: http //patent womplex ibm com/patquery, http //ep espacenet com/			

FUTURE THRUST

Significant effort is required on a number of fronts before we can hope to successfully engineer a controlled, commercially viable form of apomixis in a wide

range of target crops. We have no knowledge of cues and genes that enable cells in the ovule to switch to an apomictic pathway. The inter action between embryo and endosperm development in apomict is poorly understood, as in the capability of apomicts to tolerate parental genome imbalancing during endosperm development that results in seed sterility in sexual plants. The continued investigation of both native apomicts and sexual systems will provide the best opportunity to elucidate the genetic and physiological factors that contribute to control of apomixis and that this ultimately will permit the controlled formation of maternally derived, genetically identical seeds in flowering plants (Bicknell and Koltunow, 2004).

Apomixis in India has been targeted on two strategies, inducing apomixis in cereals such as sorghum to harness benefits of heterosis, and disrupting apomixis in forage grasses to enable effective breeding strategy through hybridization in crops such as wheat, rice, barley and oats, hybrids are not as widely used and in some crops , hybrids are not now possible on a commercial basis. Apomixis in these crops would make it possible to use F1 hybrids. In potato, apomictic propagation holds a great promise for the new technology of growing of potatoes from botanical seeds instead of tubers

CONCLUDING REMARKS

Apomixis is of interest to plant breeders in fixing heterozygosity and maintenance of heterogeneity in crop plants. Apomixis results from changes in a small number of key events typical of sexual reproduction apomixis occurs in a variety of ways, only four different types of apomixis , diplospory , apospory, automixis and semigamy are useful in plant breeding. In addition to necessitating a reorientation of breeding and seed certification procedure , the estimation of frequency of apomixis, its modification by different environmental conditions and its complex mode of inheritance in most cases, causes serious problems in the utilization of apomixis.However, the advantages of apomixis breeding out weigh the disadvantages and breeders are

continuously looking apomictic strains in different crops. With the gradual accumulation of accurate information of the various aspects of apomixis, it is likely for apomixis breeding to become a powerful tool in plant breeding for the exploitation of heterosis especially in crops where cytoplasmic genetic male sterility has not been developed.

DISCUSSION

1. What are vybrids?

Vybrids are the progenies obtained by crossing two apomictic lines. They are more stable and give good performance even under adverse conditions.

2. In which crops vybrids are more exploited?

In sorghum and rice.

3. Are there any vegetables in which apomixis is common?

Tomato and potato

4. How apomixis is helpful in reducing diseases in vegetatively propagated crops?

In vegetatively propagated crops the spreading of virus and viroids inoculum to next generation is high. But through asexual seeds the spreading is very less.

5. What are recurrent and non-recurrent apomixis?

Recurrent apomixis inherits from one generation to next generation. It is common in obligate apomixis. But in non-recurrent apomixis apomictic nature will not transfer from one generation to next generation. It is common in facultative apomixis.

6. What are crops in which apomixis is more exploited?

Sorghum, rice, pearl millet, fodder grasses etc.

7. Is there any varieties released in rice?

Gu you No.20 is a variety released in rice in 2000. But it is not commercially exploited.

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KERALA AGRICULTURAL UNIVERSITY
DEPARTMENT OF PLANT BREEDING AND GENETICS
COLLEGE OF HORTICULTURE, VELLANIKKARA.
PbGen 651 -seminar

TOPIC: ROLE OF APOMIXIS IN PLANT BREEDING

Name. Sani George
Admission No: 2004-11- 43
Major Advisor:Dr. Dijee Bastian

Venue: Seminar Hall
Date: 22-10-05
Time:10.00 – 10.45 am

ABSTRACT

Knowledge on the floral biology and pollination behaviour of plants is essential for a plant breeder before embarking on a crop improvement programme. In plants mode of reproduction may be classified as sexual and asexual.

Sexual reproduction involves fusion of male and female gametes, while asexual reproduction does not involve the fusion of male and female gametes. Asexual reproduction involves vegetative reproduction and apomixis. Vegetative reproduction is by means of vegetative organs like rhizome, bulbs etc.

Apomixis is seed formation without the fusion of male and female gametes Apomixis is prevalent in more than 400 flowering taxa and over 40 families (Carmen, 1997). It is common in families like Poaceae, which include many grasses like, meadow grass and buffel grass (*Cenchrus ciliaris)*, Rosaceae, which include *Malus, Rubus* and *Potentilla* and Compositae includes *Crepis, Rudbeckia* and *Dandelions*. Most apomictic species are allopolyploids but apomixis is also demonstrated to occur in several diploids

Apomixis is mainly classified into vegetative apomixis and agamospermy. Agamospermy is also used synonymous with apomixis. Here, seeds are formed without fertilization. The embryo sacs in apomictic plants may be haploid or diploid. The embryo formation in apomictic plants is possible either by parthenogenesis, apogamy, adventitious embryony, semigamy or androgenesis.

Apomixis that are useful in plant breeding are apospory, diplospory, automixis and semigamy (Mandal *et al.*, 1993) The apomixis can be introduced into crop plants either by hybridization with apomictic species, mutations or by biotechnological tools.

Apomixis is an attractive trait for the enhancement of crop species because it mediates the formation of large genetically uniform population and perpetuates hybrid vigour through successive seed generations. The continued comparative analysis of apomictic and sexual reproduction at the fundamental level in appropriate model systems remains essential for the development of successful strategies for the greater application and manipulation of apomixis in agriculture (Bicknell and Koltunow, 2004).

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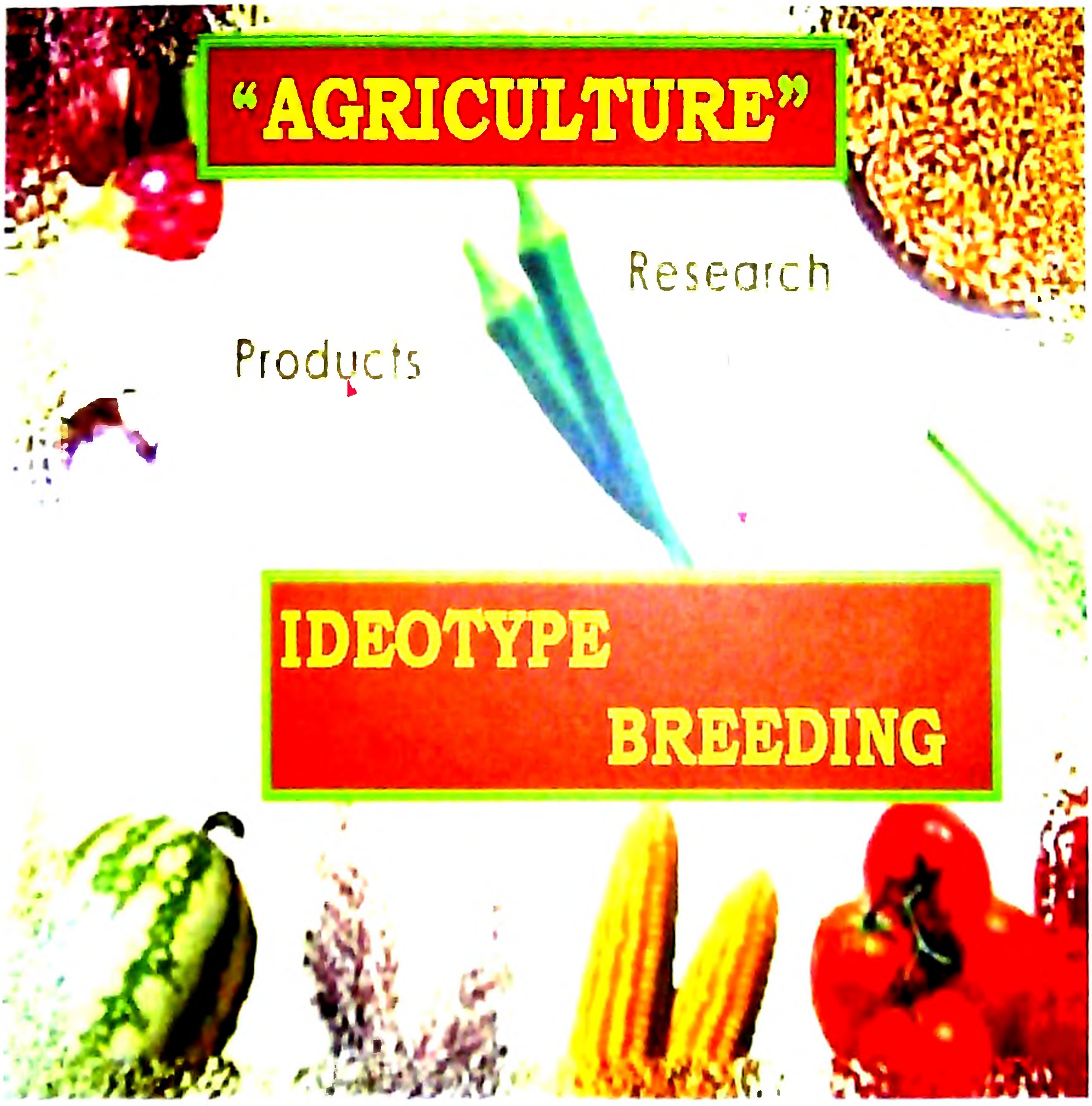
IDEOTYPE BREEDING IN CROP IMPROVEMENT



Presented by;
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"IDEOTYE BREEDING IN CROP IMPROVEMENT"

SEMINAR REPORT

BY

M.Marimuthu
(2004-11-51)

Submitted in partial fulfillment for the
Requirement of the course
PB & GENETICS 651 – Seminar

DEPARTMENT OF PLANT BREEDING AND GENETICS
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DECLARATION

I, Marimuthu, (2004 – 11 – 51) here by declare that this seminar report entitled **"IDEOTYPE BREEDING IN CROP IMPROVEMENT"** have been prepared by me independently, after going through the various references cited herein and has not been copied or adopted from any of the fellow students or previous seminar reports.

Vellanikkara
Date

M.Marimuthu
(2004 – 11–51)

CERTIFICATE

This is to certify that the seminar report titled **"IDEOTYPE BREEDING IN CROP BREEDING"** has been solely prepared by Mr. M.Marimuthu(2004 – 11 – 51), under my guidance, and has not been copied from any seniors, juniors or fellow student's seminar reports.

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INDEX

INTRODUCTION:

In broad sense an ideotype is a biological model, which is expected to perform or behave in a predictable manner within a defined environment. More specifically, crop ideotype is a plant model, which is expected to yield greater quantity of grains, fibre, oil or other useful product when developed as a cultivar. Donald first proposed the term ideotype in 1968 working on wheat.

The main points about ideotypes are given below:

- Crop ideotype refers to model plants or ideal plant type for a specific environment.
- Ideotype is a moving goal, which changes according to climatic situations, type of cultivation, national policy, market requirement etc. In other words, ideotypes have to be redesigned depending upon above factors. Thus, development of crop ideotypes is a continuous process.
- Ideal plant type or model plant type also varies from species to species. Moreover, this is a difficult and slow method of cultivar development because various morphological, physiological and biochemical characters have to be combined in single genotype from different sources.

IDEOTYPE BREEDING:

Ideotype breeding or plant type breeding can be defined as a method of crop improvement which is used to enhance genetic yield potential through genetic manipulation of individual plant character. Each character plays a definite role in the enhancement of yield. In other words, plant characters are chosen in such a way that each character contribute towards increased economic yield. Main features of ideotype breeding are briefly discussed below:

- **Emphasis on individual trait:** In ideotype breeding, emphasis is given on individual morphological and physiological trait, which enhances the yield. The value of each character is specified before initiating the breeding work.
- **Includes yield-enhancing traits**:Various plant characters to be included in the ideotype are identified through correlation analysis. Only those characters, which exhibit positive association with yield, are included in the model.

- **Exploits physiological variation**: Genetic differences exist for various physiological characters such as photosynthetic efficiency, photorespiration, nutrient uptake, etc. Ideotype breeding makes use of genetically controlled physiological variation in increasing crop yields, besides various agronomic traits.
- **Slow progress**: Ideotype breeding is a slow method of cultivar development, because incorporation of various desirable characters from different sources into a single genotype takes long time. Moreover, sometimes-undesirable linkage affects the progress adversely.
- **Selection**:In ideotype breeding selection is focused on individual plant character, which enhances the yield.
- **Designing of model**: In ideotype breeding, the phenotype of new variety to be developed is specified in terms of morphological and physiological traits in advance.
- **Interdisciplinary approach**.Ideotype breeding is in true sense an interdisciplinary approach, it involves scientist from the disciplines of genetics, breeding, physiology, pathology, entomology etc.
- **A continuous process**: Ideotype breeding is a continuous process, because new ideotypes have to be developed to meet changing and increasing demands. Thus development of ideotype is a moving target

TYPES OF IDEOTYPE:

In 1976, Donald and Hamblin proposed the concepts of isolation, competition and crop or communal ideotypes, with special reference to cereals, these concepts are briefly described, below

- **Isolation ideotype**

It is the model plant type that performs best when the plants are space-planted. In case of cereals, isolation ideotype is lax, free tillering, leafy, spreading plant that is able to explore the environment as fully as possible. It is unlikely to perform well at crop densities

- **Competition ideotype**

This ideotype performs well in genetically heterogeneous populations, such as, the segregating generations of crosses. In case of cereals, competition ideotype is tall, leafy, free-tillering plant that is able to shade its less aggressive neighbors and thereby, gain a larger share of nutrients and water. In case of annual seed crops, such an ideotype will

include the following features: annual habit, tallness, leafy canopy, tillering or branching, seed size, speed of germination and root characters.

➢ **Crop ideotype**

This ideotype performs best at commercial crop densities because it is a poor competitor. It performs well when plants of the same form surround it. But it performs less well when plants of other forms, e.e., competition ideotype, and also in isolation surround it. In case of cereals, a crop ideotype or communal ideotype is erect, sparsely-tillered plant, with small erect leaves and is able to survive in the highly competitive situation of being surrounded by the plants of the same form. The concept of 'week competitor' is the central theme of this ideotype.

In addition, Donald (1968) had proposed several other ideotypes that include traits concerned with specific features. These are given below;

- Market ideotype includes traits like seed colour, seed size, cooking and backing quality etc. since these traits determine the market acceptability of the produce.
- Climatic ideotype includes traits important in climatic adaptation, e.g., early maturity, thermo period-insensitivity, heat and cold tolerance, drought tolerance, photoperiod-insensitivity etc
- Edaphic ideotype (traits salinity tolerance, mineral toxicity/deficiency tolerance etc)
- Stress ideotype (traits resistance to the concerned abiotic and biotic stresses),
- Disease/pest ideotype (traits resistance to the concerned diseases and insect pests) etc

OBJECTIVES OF PLANT BREEDING:

The prime objective of plant breeding is to develop superior plants over the existing ones in relation to their economic use. The objectives of plant breeding differ from crop to crop. However, there are some objectives, which are common in majority of field crops. A brief account of some important objectives is given below:

⇒ **Higher Yield**

The ultimate aim of plant breeder is to improve the yield of economic produce. It may be grain yield, fodder yield, fibre yield, tuber yield, cane yield or oil yield depending upon the crop species. Improvement in yield can be achieved either by evolving high yielding varieties or hybrids.

⇒ **Improved Quality**

Quality of produce is another important objective in plant breeding. The price of produce id determined by its quality. Again quality differs from crop to crop. It refers to cooking quality in rice, baking quality in wheat, malting quality in barley, fibre length, strength and fineness in cotton, nutritive and keeping quality in fruits and vegetables, protein content in pulses, oil content in oil-seeds and sugar content in sugarcane and sugar beet, etc.

⇒ **Biotic Resistance**

Crop plants are attacked by various diseases and insects, resulting in considerable yield losses. Genetic resistance is the cheapest and the best method of minimizing such losses. Resistant varieties are developed through the use of resistant donor parents available in the gene pool.

⇒ **Abiotic Resistance**

Crop plants also suffer from abiotic factors such as drought, soil salinity, heat, wind, cold and frost. Breeder has to develop resistant varieties for such environmental conditions.

⇒ **Earliness**

Earliness is the most desirable character, which has several advantages. It requires less crop management period, less insecticidal sprays, permits double cropping system and reduces overall production cost. Thus earliness is an important objective in plant breeding programmes. Determinate growth habit has close association with earliness.

⇒ **Photo and Thermo insensitivity**

Development of varieties insensitive to light and temperature helps in crossing the cultivation boundaries of crop plants. In maize, rice and potato now varieties are available which can be grown during summer as well as winter season. Evolution of photo and thermo insensitive varieties permits their cultivation in new areas outside the boundaries of cultivation of a crop species.

⇒ **Synchronous Maturity**

It refers to maturity of crop species at one time. This character is highly desirable in crops like green gram, cowpea, and cotton where several pickings are required for crop harvest.

⇒ **Desirable Agronomic Traits**

It includes plant height, branching, tillering capacity, growth habit etc. Usefulness of these trails also differs from crop to crop. For example, tallness, high tillering and profuse branching are desirable characters in fodder crops, whereas dwarfness is a desirable character in wheat, rice, Sorghum and pearl millet. Dwarfness confers lodging resistance in these field crops, in addition to better fertilizer response.

⇒ **Removal of Toxic Compounds**

It is essential to develop varieties free from toxic compounds in some crops to make them safe for human consumption. For example, removal of neurotoxin in Khesari, which leads to paralysis of lower limbs, erucic acid from Brassica which is harmful for human health, and gossypol from the seed of cotton is necessary to make them fit for human consumption.

⇒ **Wider Adaptability**

Adaptability refers to suitability of a variety for general cultivation over a wide range of environmental conditions. Adaptability is an important objective in plant breeding because it helps in stabilizing the crop production over regions and seasons.

⇒ **Some Other Characters**

In some crops such as green gram, black gram and pea, seeds germinate in the standing crop before harvesting if rains are received. A period of dormancy has to be introduced in these crops to check loss due to germination. In *arboreum* cotton shedding of kapas after boll bursting is a serious problem. Locule retentive varieties have to be developed in this species of cotton. The serious problem in green gram. Hence resistance to shattering is an important objective in green gram.

IDENTIFICATION OF TRAITS FOR ANALYSIS:

The various traits of crop plants that limit growth and yield can be grouped into the following 4 classes (1) morphological and anatomical, (2) compositional, (3) process rates, and (4) process controls (Austin, 1993).

⇒ **Morphological and Anatomical traits**

Examples of such traits are plant height, leaf size and orientation, stomata frequency etc. They are most commonly used by breeders as they are easily identified and measured, and are often highly heritable, and usually they do not change in quantity in the short-term, i.e., are stable. However, their importance for growth and yield is not well understood and they are likely to show important interrelationships with other morphological/physiological traits.

⇒ **Compositional Traits**

Such traits concern the concentrations of specific biochemicals in plant tissues and organs, e.g., levels of proline, ABA (abscisic acid), Na and protein etc. Compositional traits of growing vegetative tissues generally change with age and environment; therefore, appropriate standardization is necessary in such case. But the traits are relatively stable for the parts harvested as economic yield, e.g., in case of quality traits.

⇒ **Process Rate Traits**

Process rates that limit growth and yield relate to photosynthesis, respiration, photoperiodic response, winter hardiness etc. Process rates are very sensitive to current and previous environments. Therefore, careful standardization is essential if meaningful genetic comparisons are to be made. Screening for photosynthetic and photorespiration rates is feasible among different species as the differences are usually large. However, relatively smaller within species differences are not detected with confidence. In addition, estimation of such traits demands infrastructural facilities, funds, and technical expertise

⇒ **Process Control Traits**

Examples of such traits are activities of Calvin cycle enzymes, stomatal aperture etc , these traits are considered to control or limit photosynthesis Similarly, nitrate reductase activity is thought to limit nitrate assimilation Most enzymes are extremely sensitive to environment, there activities change dramatically even in short-term, and it is difficult to relate these activities to plant performance

DIFFERENCES (IDEOTYPE& CONVENTIONAL BREEDING):

⇒ **Conventional breeding**	⇒ **Ideotype breeding**
❖ The main objective is defind before initiating the breeding work ❖ Selection is focused on yield and some other characters. ❖ It usually includes various morphological and economic characters ❖ Value of each trait is not fixed in advance. ❖ This is a simple and rapid method of cultivar development ❖ The phenotype of new variety is	❖ The conceptual theoretical model is prepared before initiation of breeding work. ❖ Selection is focused on individual plant character ❖ It includes various morphological, physiological, and bio chemical plant charters. ❖ Value of each trait is defind in advance. ❖ This is a difficult and slow method of cultivar development.

not specified in advance.	❖ Phenotype of new variety to be developed is specified in advance

STEPS IN IDEOTYPE BREEDING:

Ideotype breeding may be viewed as consisting of the following four steps: (1) development of a model plant type, i.e., ideotype, (2) creation of adequate genetic diversity for the concerned traits, (3) selection of plants/lines with the desired phenotypes, (4) evaluation of the phenotype in several genetic and cultural backgrounds.

⇒ **Defining the Phenotypic Goals:**

The first step in ideotype breeding consists of defining the traits that are to be modified and then the level of phenotypic expression that is to be achieved must be specified. This step is based on and consists of the following.

- The roles of individual traits in determining yield are determined.
- A hypothesis regarding the role and the importance of the trait, that is proposed to be modified, in determining yield is developed.
- A decision has to be then made whether or not to proceed with a breeding effort
- Often the available information may be rather limited to allow confident decisions. A breeder may usually opt for traits that are easy to score. But incorporation of traits that are difficult or costly to measure in a segregating population may enhance yield in progeny even if such a trait is not selected directly

⇒ **Creation of Adequate Genetic Diversity:**

A sufficient genetic diversity for the trait to be modified must be present to justify breeding effort. The genotypes to be used as parents in hybridization should have broad genetic base and wider adaptability. As far as possible, they should belong to the elite gene pool. If the desired genes are available only in the unimproved gene pool, they should be first introgressed into lines having good agronomic value; these lines should then be used in crossing programmes.

⇒ **Selection of Lines Having Desirable Phenotype:**

The segregating generations are handled according to a suitable breeding scheme to isolate a number of lines, which exhibit the desired level of the trait under modification.

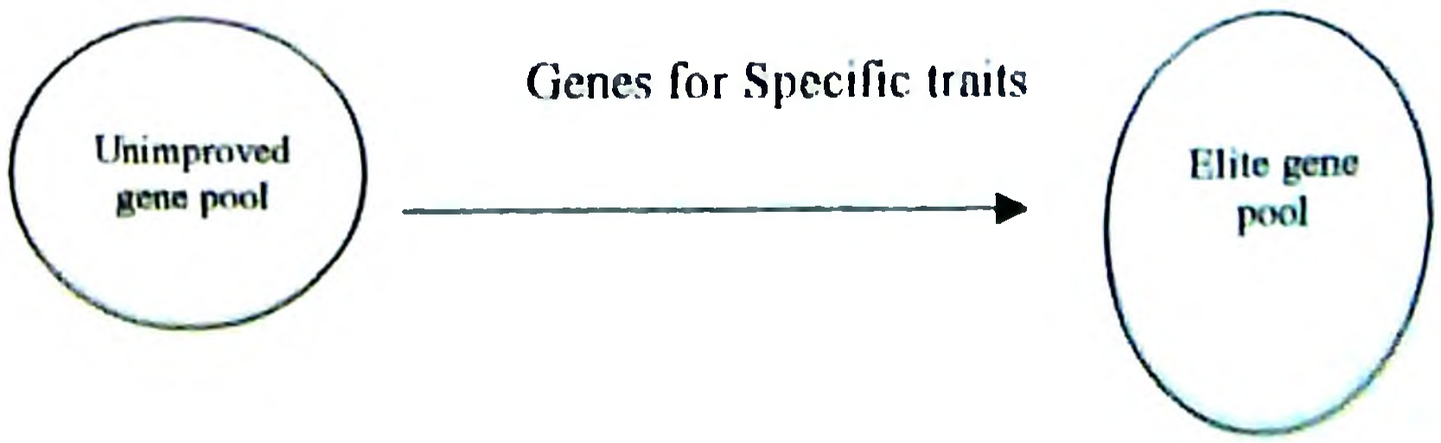
⇒ **Continued Breeding Effort**

An ideotype breeder must be prepared for continued breeding and evaluation effort. In order to increase the likelihood of the modified trait leading to increased yields, it may be necessary to conduct several cycles of breeding to break undesirable linkage, and to try the trait in question in different genetic backgrounds and, possibly, under different cultural practices.

Rationale for (Merits of) Ideotype Breeding:

The rationale for ideotype breeding may be summarized as follows (Rasmusson, 1987).

- In the past, yield has been enhanced by selecting for individual traits associated with yield. For example, reduced plant height in wheat and reduced plant height coupled with erect leaf in rice are well known cases. In U.S.A., changed maturity of soybeans and reduced height of sorghum have dramatically enhanced the range of their adaptation and productivity.
- Grain yield is the direct or indirect product of individual plant traits. Substantial diversity is, therefore, generated for such traits that are considered to contribute to yield. This is likely to generate such gene combinations that would favorably change productivity.
- The primary gene pool of a crop may be divided into improved and unimproved gene pools. The improved or elite gene pool consists of lines developed by several cycles of breeding that are commonly used as parents in breeding programmes. The unimproved gene pool is occasionally used for the transfer of specific traits. In ideotype breeding, genes for specific traits would be introgressed from the unimproved into the improved gene pool in order to generate the desired level of variability for the trait. Thus ideotype breeding serves as a bridge between the two gene pools. It also complements the conventional breeding efforts by providing genetic diversity obtained from little used or inaccessible germplasm.



- It encourages development and evaluation of hypotheses regarding how yield is achieved. It requires the selection objective to be defined in terms of the phenotypic goals for each of the traits that are postulated to contribute to yield, i.e., the ideotype. The objectives are tested and revised based on the results. This is expected to lead to a more effective breeding strategy.
- Perhaps the strongest argument in favour of ideotype breeding emerges from the features of crop and other ideotypes. A selection based on either visual evaluation or yield data in the segregating generations of crosses is not likely to identify crop ideotypes. Therefore, a conscious selection for the traits constituting the crop ideotype has to be exercised during the segregating generations.
- Even when conventional breeding efforts include specific morphological/physiological traits in their selection criteria, the breeder does not, ordinarily, specify the goals to be achieved. In such a situation, exploitation of a trait over time is likely to be haphazard, i.e., in opposing directions, and inefficient. Therefore, it is important that the goals for specific traits are established with a view to maximize their effects on yield.

PRACTICAL ACHIEVEMENTS:

Ideotype breeding has significantly contributed to enhanced yields in cereals (wheat and rice) and millets (Sorghum and pearl millet) through the use of dwarfing genes, resulting in green revolution. Semi dwarf varieties of wheat and rice are highly responsive to water use and nitrogen application and have wide adaptation. These qualities have made them popular throughout the world. Spontaneous mutations have played significant role in designing new plant types in wheat and rice. The **Norin- 10** in wheat and **Dee-geo-Woogen** in rice are the sources of dwarfing genes. These sources of dwarfing genes were obtained as a result of spontaneous mutations. Several high yielding semi dwarf varieties have been evolved in wheat and rice through the use of respective dwarf mutant. The Norin 10 dwarfing gene in wheat, the Dee-geo-woogen dwarfing gene in rice and the genic cytoplasmic male sterile systems in *Sorghum* and pearl millet laid the foundation of green revolution in Asia. Thus, ideotype breeding has been more successful for yield improvement in cereals millets than in other crops.

- In rice, the improved plant type includes (1) erect, short and thick leaves, (2) dwarf stature (short and thick straw), (3) light leaf shealth, (4) high tillering capacity, (5) responsiveness to high levels of nitrogen, and (6) high harvest index. Examples of semidwarf varieties of rice are IR 8, IR 20, and TN- 1 etc. The Chinese variety

  Dee-geo-Woogen is the source of dwarfing gene in all these varieties.
- In wheat, the improved plant type included (1) short and still straw (culms), (2) insensitivity to photoperiods, (3) high response to nitrogen application, (4) high harvest index, and (5) resistance to different rusts. The semidwarf Japanese variety Norin 10 was used as source of dwarfness in the development of semi dwarf Mexican varieties of wheat.
- In *Sorghum* and pearl millet, short statured hybrids have been developed through the use of dwarfing genes. The dwarf hybrids have made machine harvesting possible in these crops. In *Sorghum*, combine harvesting has reduced the labour requirement by 1/8.
- In cotton, as a result of high selection pressure for earliness, short stature and compactness in the past, there has been a gradual reduction in the overall plant size. The earlier varieties were late maturing, till growing and spreading types leading to bushy appearance. The target shifted to development of varieties with medium height, medium maturity and semi spreading habit. Now major emphasis is to evolve varieties with sort duration, short stature and compact plant type

EXAMPLES OF THE IDEOTYPE APPROACH:

The crop ideotype consists of several morphological traits and physiological traits which morphological traits and physiological contributes for enhanced yield or higher yield than currently prevalent crop cultivars. The morphological and physiological features of crop ideotypes differ from crop to crop and sometime within the crop also depending upon the whether ideotype required for irrigated or rain fed cultivation. Ideal plant or model plants have been discussed in several field crops, although examples in horticulture crops (fruit crops), as well as forage crops.

⇒ Field crops (Rice, Cotton, Maize, Wheat, chick pea).
⇒ Fruit crops (Apple, Tamarind).
⇒ Forage crop.

RICE IDEOTYPE BREEDING

(A SUCCESSFUL BATTLE ON RICE)

Ideotype for Higher Yield

Since the evolution of dwarf rice varieties with increased yield, rice breeders have made no significant achievement worldwide over last three decades in breaking the yield plateau of rice. Rice scientists have been engaged in designing a rice ideotype for achieving a quantum jump in yield. On the strength of experimental findings and experience, many rice scientists particularly at IRRI still believe that rice yield could be raised by 20-25 per cent through selective improvement of major yield components.

Proposed rice ideotype based on the cultivation ecosystem. Rice is usually grown under different type of eco system throughout the world and given below;

Lowland (Irrigated) ecosystem:

It is cultivated under irrigated condition. It accounts for 55 percent of world harvested rice area and contributed to 76 percent to global production. Irrigated ecosystems are concentrated in semi-arid and sub- humid subtropics of Asia.

Upland (Rainfed) ecosystem

Under this ecosystem, rice is direct seeded in non-flooded, well-drained soil on level to steeply sloping field. Crop suffers from lack of moisture and inadequate nutrition, so yield of upland rice are generally very low. Upland rice ecosystem is dominant in Africa and South America but relatively less important in Asia.

Deepwater ecosystem:

It is also known as "bao" rice in Assam. It refers to that ecotype which are planted in the crop field when the standing water for a certain period of time is more than 50 cm and when the water depth varies between 51 and 100 cm for more than half of the growth duration.

LOW LAND (IRRIGATED) ECOSYSTEM:

A rice ideotype with a potential yield of 13-15 t/ha under irrigated situation has been designed with the following morphological and physiological features:

- **Morphological Features**
  - Medium maturity duration (100 to 130 days)

- 3-4 large panicles with high grain number (200 to 250 grains/panicle) and with high grain weight (more than 25 g for 1000 grains); no unproductive tiller
- High tillering ability under transplanted condition but low tillering ability (4 to 5 tillers) under direct seeding

⇒ **Physiological Features**

- Faster foliar growth during crop establishment with reduced tillering
- Slower foliar growth and enhanced translocation of assimilates from foliage to stem during late vegetative and reproductive stages
- Expanding capacity of stem to store assimilates
- Increased harvest index (usually 0.6 or more)
- Improved starch accumulation capacity in grain along with longer ripening period
- Descending concentration of N from top to bottom in the crop canopy

Physiological Needs for Higher Yield

Depending on the knowledge of crop photosynthesis, mineral nutrition and yield components, Yoshida (1981) suggested the following physiological needs for achieving high yields of rice

- The plant must have a short and stiff stem
- Erect leaves must be present at upper canopy levels whereas droopy leaves at low canopy levels
- A LAI (leaf area index) of 5 to 6 is essential for maximum photosynthesis during reproductive stage
- During grain filling stage, increased number of active green leaves must be maintained
- Planting time should be so chosen that the reproductive or ripening stage, whichever is more critical to yield, is exposed to high solar radiation
- All essential nutrients must be supplied through fertilizers to meet the crop's requirements
- During ripening process, about 70% of the nitrogen absorbed by the straw is translocated to grain.

It is essential to maintain high level of leaf nitrogen to produce high yields either by nitrogen absorption after flowering or by supplying already absorbed nitrogen before flowering from vegetative parts.

⇒ **Ideotype for Upland Ecosystem**

Scientists at IRRI formulated the rice ideotype for upland areas (IRRI highlights, 1987). Such ideotype must have the following features:

- ❖ Intermediate plant height with moderate tillering to improve nitrogen responsiveness
- ❖ Droopy lower leaves to shade out weeds and conserve soil moisture
- ❖ Erect upper leaves for efficient light penetration
- ❖ Deep thick root system for drought resistance
- ❖ Blast resistance and acid soil tolerance

Ideotype for Flood Prone Ecosystem

Under flood prone ecosystem the rice plant must have the following characteristics:

- ❖ Intermediate plant height with thick culm and well-developed sclerenchymatous band in the culm
- ❖ Long, narrow, medium-thick, dark green leaves with high sugar and protein content
- ❖ Deep root system with ability to withstand short drought spell when flood water recedes
- ❖ Higher and rapid photosynthesis under low light intensity of submergence condition
- ❖ Higher cytochrome oxidase activity to carry out functional activity at low O_2 concentration
- ❖ Higher tensile strength of the root, stem and leaves to withstand the mechanical force of flood water
- ❖ Regeneration ability of the shoot after recession of flood

⇒ **Ideotype for Deepwater Ecosystem**

The rice ideotype for deepwater ecosystem must process some specific genetical and morphological traits e.g.

Genetical traits:

- ❖ High photoperiod sensitivity with late maturity.
- ❖ Higher seedling vigour.
- ❖ Submergence tolerance.
- ❖ Stem elongation ability with rising water level.

- Increased grain dormancy.

Morphological traits:

- Seed germination under submergence condition with rapid elongation of plumule for direct seeding
- Long, thin, droopy leaves with pubescence
- Few tillers with nodal tillering ability at the water level
- Rapidly spreading tillers with kneeing ability
- Culm with elongated internodes, and air space for floating (aerenchymatous tissue)
- Shallow and thin basal roots as well as nodal rooting ability
- Only 1 to 4 photo synthetically active leaves at maturity

Ideotype for High Density Grain

High-density grains refer to the heavier grains in the panicle. Rice scientists at IRRI have formulated the ideal ideotype for produced high-density grains (IRRI highlights, 1987). The essential features of the ideotype are mentioned below:

- Low tillering with only primary tillers developing.
- Large panicles to compensate for low tillering.
- Thick culm with more vascular bundles to lessen lodging, to support a larger panicle and to promote carbohydrate accumulation.
- Panicles with only primary branches to increase the percentage of filled grains.
- Larger pedicel led vascular bundle to promote transport of assimilates.
- Medium-size grains (about the size of IR8 grains) with less white belly.
- Erect, thick leaves to give better light distribution and higher photosynthetic rate
- Higher photosynthesis under low photo synthetically active radiation, so that carbohydrate supply will not be limited during rainy season
- Low maintenance respiration to increase net assimilation rate, higher shoot: root ratio might decrease root maintenance respiration.
- Medium growth duration, carbohydrate accumulation before heading can be used to produce larger panicles and heavier grains.
- Intermediate plant height, a harvest index of 0.55 will make the plant lodging resistant, decrease maintenance respiration and provide optimum partitioning of carbohydrate to the grain.

Development of Ideal Plant-type:

The ideal plant-type must possess erect, thick and dark green narrow leaves and short culms. A single plant is expected to have 6-10 culums - all of them productive - which bear large panicles, each with 200-250 grains. Thicker and sturdier culms support the larger panicles and with vigorous roots, prevent the plant from lodging. Thicker, dark green and erect leaves catch more sunlight and use it more efficiently. Short culms lower the consumption of assimilates (Sasahara, 1994). The ideal plant-type can be developed in two ways:

- **Agronomic practice**: Agronomic practice, especially manipulation of fertilizer application, can produce an ideal plant-type. A reduction in nitrogen content in rice plants ca. 30 days before heading is intrinsically associated with development of ideal plant type at grain filling stage (Matsushima, 1957). The nitrogen reduction in the plant at this growth stage results in prevention of the overgrowth of the flag leaf, the penultimate 2 to 3 leaves and the lower internodes and hence favours establishment of the ideal plant type. But the level of reduction in nitrogen content in the plant should be limited to allow formation of enough grains per unit l and area to ensure a high rice yield.
- **Breeding approach** The ideal plant-type has been developed by rice breeders through the development of short-statured plants having semi-dwarfing genes (DGWG gene). The semi-dwarf genes decrease the overgrowth of lower internodes, and of the flag leaf and penultimate leaves (Futsuhara, 1968). Exploitation of semi-dwarf genes has coincided with the ideal plant-type concept proposed by plant breeders (Tsunoda, 1959)

Increasing Yield Potential

Yield potential of present day high yielding semi-dwarf rice varieties under the best conditions in the tropics is 10 tons per hectare during dry season and 6.5 tons per hectare during wet season (Khush, 1996). Plant physiologists have suggested that the physical environment in the tropics is not a limiting factor to increase rice yields.

Yoshida (1981) estimated the maximum yield potential of rice as 15.9 tons and 9.5 tons per hectare during the dry season and the wet season, respectively. Maximum rice yield potential can be realized through idetype breeding. This approach is development of New Plant Type.

Development of New Plant Typ

Development of the plant type of modern high yielding semi-dwarf rice varieties in the mid-1960s almost doubled the rice yield. This plant type is short statured and possesses sturdy stems, dark green erect leaves and high tillering ability. Number of tillers in this plant type varied from 20 to 25 with nearly 14 to 16 tillers bearing panicles but the remaining tillers are unproductive. More than 60% of world's rice area is now under varieties of this semi-dwarf plant type.

During the last 30 years, no significant increase in yield potential was achieved. This is because rice improvement efforts at IRRI were directed towards incorporation of disease and insect resistance, early maturity and improved grain quality in semi-dwarf varieties. For another quantum jump in rice yield, the yield potential must be enhanced either by increasing the harvest index (HI) or biomass (total dry matter production) or both in a new plant type.

❖ **Increasing Harvest Index (HI)**

Harvest index is defined as the ratio of economic yield (grain) to biological yield (straw) excluding the roots. In other words, harvest index is the grain to straw ratio. The HI of modern high-yielding cultivars varies from 0.45 to 0.53 depending upon the season. The HI is higher during dry season than that of wet season (Vergera *et al*., 1977). Harvest index should be increased to around 0.60 in the rice varieties with new plant type for increased yield potential. The maximum possible HI in wheat was estimated to be 0.63 (Austin *et al*., 1980). The HI of 0.63 may also be considered as the maximum harvest index for rice assuming the same biomass production of the modern semi-dwarf plant type.

For increasing the harvest index, the following plant characteristics need improvement (Khush, 1994).

⇒ Increased sink size
- Large spikelet number per panicle
- Greater partition of assimilate in spikelet formation

⇒ Increased spikelet filling
- Reducing canopy senescence
- Higher proportion of high-density (heavy weight) grains
- Maintaining healthy root system
- Increased lodging resistance

❖ **Increasing Biomass**

Yield is a function of biomass or total dry matter production. Increasing biomass production will increase rice yield. Biomass production can be increased by growing rice crop in a high solar environment similar to that of dry season in the tropics provided there is sufficient nitrogen supply to the rice crop (Akita, 1989). In general, increased biomass production produces tall culms, which result in lodging and mutual shading. As a result of mutual shading, humidity in the lower canopy increases and it helps in increasing disease incidence consequently reducing grain yield (Vergara, 1988). If lodging and disease problems can be solved, increased biomass production could increase yield potential in tropical environments.

Biomass production can be enhanced by improving the following plant characteristics:

⇒ Establishment of desirable canopy structure

- Rapid leaf area development
- Rapid nutrients uptake
- Reducing carbon consumption.

❖ **Phenotype of New Plant Type**

For achieving high harvest index and high biomass production, a new plant type (ideotype) has been conceived with the following characteristics (Khush, 1994):

- Low tillering capacity (3-4 tillers for direct seeding and 6-8 tillers under transplanting)
- No unproductive tillers
- 200-250 grains (spikelets) per panicle
- 90-100 cm tall
- Very sturdy stems
- Dark green, thick and erect leaves
- Vigorous root system
- 100-130 days growth duration (i.e. seed to seed duration)
- Acceptable grain quality
- Multiple disease and insect resistance
-

❖ **Distinct Plant features for Increased Yield Potential:**

⇒ **Plant height and stem thickness**: A decrease in plant height reduces total biomass production and resists lodging. But short culms increase harvest index and require less maintenance respiration. Reduced respiration of short culms leads to an increased balance between photosynthesis and respiration (Tanaka *et al.*, 1966), which results in more storage of photosynthate. A plant height of 90-100 cm is considered ideal for maximum yield. Plant height less than 90 cm considerably

reduces total biomass, thereby reducing the grain yield. Thicker stems accumulate more assimilates and resist lodging. Thicker stems also have more vascular bundles which enhance photosynthate translocation.

⇒ **Low tillering and large panicles**:

Modern semi-dwarf rice varieties produce nearly 20-25 tillers under favourable environment but of these, only 14-15 tillers bear panicles. Other tillers are unproductive and compete with the productive tillers for photosynthates, solar energy, mineral nutrients (particularly nitrogen) and space. High tillering produces a dense canopy which restricts the penetration of sunlight and creates a shady humid microenvironment favourable for disease, particularly for the endogenous pathogens like sheath blight (*Rhizoctonia solani*) and stem rot (*Sclerotium oryzae*).

Elimination of unproductive tillers through breeding will direct more nutrients for the grain production resulting in increased yield. Low tillering genotypes are characterized by synchronous flowering and maturity and more uniform panicle size which help in harvesting. The proportion of high-density grains (heavy weight grains) is high in a low tillering genotype

High tillering produces some unproductive tillers which lead to high leaf area index (LAI) and vegetative growth but produces more unfilled grains. The new plant type must have a few tillers, each with large panicle. Traditional rice varieties possess fewer tillers than the modern semidwarf varieties. Large panicle will compensate for small number of panicle in a low tillering genotype

⇒ **Grain characteristics**. The proportion of heavier, high density grains is the greatest at the top of the panicle and the smallest in the inferior spikelets of the lower panicles. A thousand grain weight of 25 g is ideal for rice. Larger grains (i.e. higher thousand grain weight) are chalky and thus have lower market value. Large variation in the weight of single rice grains was reported by Venkateswarly *et al.* (1986)

The proportion of high density grains is higher in the primary tiller than in the secondary or tertiary tiller (Rao, 1987b). Low tillering genotypes produce more primary tillers per unit land area and hence more high density grains per unit land area. High density grains contribute to increased yield potential.

⇒ **Root system:** Thicker and deeper roots are desirable in an ideal plant. These roots absorb water and minerals more efficiently from soil. In addition to this,

the deeper roots provide better anchorage of rice plant to soil and reduce lodging. Healthy roots are necessary during grain filling period for better nutrient supply. Variation in rice varieties for root characteristics namely root length, root volume, root thickness and degree of branching has been observed. Such variation could be exploited for increasing rice yield potential.

⇒ **Growth duration:** Seed to seed growth duration of 120 days is considered ideal for maximum yield potential of rice in the tropics. Variation in growth duration among rice varieties is mainly due to the difference in the vegetative growth period (Vergera *et al.*, 1969). Studies have also indicated that panicle growth period (from panicle initiation to heading) also differs in early-maturing and late-maturing varieties. In general, an early maturing variety has relatively short panicle growth period than a late-maturing variety. Short panicle growth period than a late-maturing variety. Short panicle is often accompanied by decreased grain yield. Thus, increasing yield potential may be explored by extending the panicle growth period and also possibly by increasing the grain filling period independent of the total growth duration (Khush, 1996)

⇒ **Leaf and canopy characteristics:**

- In a high yielding variety, the leaf must be small, thick, erect and dark green in colour. V-shaped leaf is preferable to flat leaf for increased photosynthesis due to improved CO_2 penetration. Light is used more efficiently at a high leaf area index (LAI) in an erect-leaved canopy (Yoshida, 1976). Carbon assimilation of a single leaf exposed to light on only one side is lower than when the leaf is exposed to light on both sides and this difference is the greatest when leaves have a high nitrogen content and greater thickness.
- Long, thin leaves of the traditional rice varieties are droopy or horizontally oriented. These leaves reduce sunlight penetration and air movement. As a result, the canopy becomes shady and humid. High humidity, in turn, favours the growth of endogenous pathogens likes sheath blight and stem rot, consequently decreasing rice yield.
- Thicker leaves are not associated with yield potential but positively correlated with leaf photosynthetic rate. Thicker leaves are thought as

desirable trait and can be visually selected with ease as a criterion for enhancing yield potential.

⇒ **Disease and insect resistance:**

The prospective new plant type with increased yield potential adapted to tropical conditions must have resistance to major diseases like blast, bacterial blight, sheath blight and several viral diseases and also to major insects like stem borers, leaf hoppers and plant hoppers (Khush, 1996).

Wheat

An ideotype for wheat was first proposed by Donald (1968) for an optimum environment that is endowed with adequate nutrient and water supply. All the traits included in this ideotype were morphological traits, but their inclusion was based on physiological considerations. The main features of this ideotype are briefly described below

1. Short Strong Stem. In reduces the risk of lodging, and would also reduce the amount of photosynthates invested in stem production. But the stem should not be excessively short as it may lead to mutual shading of leaves.

2. Erect Leaves In a dense plant population, near vertical leaves will permit adequate illumination of a greater area of leaf surface than would long horizontal/drooping leaves. This would be applicable to all such species in which the leaf is nearly saturated for photosynthesis at a light intensity substantially below the prevailing light intensity, e g , in rice, wheat, barely. Vertical leaves are associated with higher productivity, low competitive ability, and greater tolerance for crowding

3. Few Small Leaves The number of leaves on the main shoot of wheat ranges from 7 to 20 or more, the axiv of lower leaves give rise tillers. In a monoculum plant, leaves serve mainly as photosynthetic, respiring and transpiring surfaces. Therefore, few small leaves per uniculum plant are desirable as the planting density will be high.

4. Large Ear. This would mean large number of fertile florets per ear, i e., per unit of dry matter of above ground parts. This is highly desirable since in wheat ear is normally a limiting sink for photosynthates.

5. Erect Ear. This will ensure illumination of ears from all sides and, thereby, greater photosynthesis. This is a common trait in commercial varieties.

6. Presence of Awns. Awns provide substantial photosynthates to the developing grains (usually more than 10% of grain dry weight), especially, under semiarid conditions. This is because awns are xerophytic structures.

7. Single culm. In tillering genotypes, at least some of the tillers do not produce ears; this represents a waste of resources and additional useless competition. This problem will not be encountered in the uniculm genotypes. Nontillering mutants are available in barley and monoculm lines have been developed in wheat.

8. Other Traits. The parents used for developing the proposed ideotype must include high yielding and locally adapted cultivars; they should also include resistance to the diseases prevalent in the locality. Other desirable traits may be early flowering longer grain-filling period and, possibly, heavy accumulation of sugar in the stem followed by the maximum translocation to the growing grain.The ideotype was proposed for an agronomic practice having the following main features: (1) suitably increased planting density, (2) heavy nutrient supply, (3) narrower rows, (4) effective weed control, and (5 assured moisture availability.

Maize

An ideotype for the optimal production environment was proposed by Mock and Pearce (1975). The environment is presumed to include adequate moisture, favourable temperature throughout the growing season, adequate fertility, high plant density, narrow row spacing and early planting dates. The main features of the proposed ideotype are summarized below

- ⇒ **Stiff-vertically-oriented leaves above the ear:** the leaves below the ear should be horizontally oriented
- ⇒ **Maximum photosynthetic efficiency**. It seems that selection for high photosynthesis rates in maize is possible
- ⇒ **Efficient translocation of photosynthate into grain.**
- ⇒ **Short interval between pollen shed and silk emergence**. This is important because higher planting densities lengthen this interval.
- ⇒ **Ear (cob or spadix)** - shoot prolificacy. In maize, limited sink strength of developing ear is considered as one of the greatest limitations to efficient conversion of photosynthate to grain. Prolificacy would increase sink strength. Further, prolific genotypes are less subject to barrenness at high plant densities

⇒ **Prolificacy** is the ability of plants to produce a second fertile cob, when spaced widely.

⇒ **Small tassel size:** Small tassels would show lower competition for nutrients with the developing ear as well as less shading of the upper leaves.

⇒ **Photoperiod insensitivity:** Adequate genetic variation is available in maize for this trait.

⇒ **Gold tolerance of germinating seeds and developing seedlings.** Adequate genetic variability is reported for this trait in maize populations.

⇒ **Grain-filling period should be as long as practically possible.**

Cotton

Ideotypes for *G. hirsutum* have been proposed for irrigated and rainfed conditions (see. Singh, 1998); their main features are briefly summarized below:

Ideotype for Irrigated cultivation

Singh and coworkers proposed this ideotype in 1974.

⇒ Plants of short stature (90-120 cm)

⇒ Compact and sympodial plant habit making pyramidal shape.

⇒ Determinate fruiting habit with unimodal distribution of bolling

⇒ Short duration (150-165 days). This will reduce the duration and, therefore, cost of crop management and would allow double cropping.

⇒ Responsive to high fertilizer dose

⇒ High degree of interplant competitive ability (is strange unless wide spacing is aimed in the crop. What perhaps is meant is the ability to perform well at closer spacing, i e , to use the environment resources efficiently)

⇒ High degree of resistance to insect pests and diseases

⇒ High physiological efficiency

In addition, boll size in proposed to be between 3.5 and 4 g, and there should be synchrony of bolling in flush of flowering.

Ideotype for Rainfed conditions

Singh and Narayanan proposed this ideotype in 1993.

⇒ Short stature (75-80 cm) and compact plant habit.

⇒ Intermediate growth habit with atleast two monopodia.

⇒ Few smaller and thick leave with sparse hairiness.

⇒ Medium to big boll size (3.5 to 4 g).

⇒ Responsive to nutrients.

⇒ High degree of resistance to insects and diseases.

⇒ Synchronous bolling habit.

Cole crop (*Brassica spp.*)

It has been suggested that yield in *Brassica* spp. equals the product of number of siliqua/m², number of seeds/siliqua and seed weight. Analysis of yield and yield traits has been used to propose the following ideotype by Bhargava *et al.* in 1984 for an optimum production environment.

⇒ Height of 1.0-1.25 m

⇒ Only 5-6 primary branches at an angle of 40-45°

⇒ The mains tem should bear only 40 siliquae, the lower 3 branches only 15 siliqua each and the upper two branches only 20 siliqua each.

⇒ There should be only 7-10 smaller and thicker leaves, with a higher photosynthetic rate, low dark respiration rate and high nitrate reductase activity.

⇒ Each siliqua should have 20 seeds and each seed should weigh 5 mg

⇒ Root system should be deep

This ideotype should be able to perform well in a monoculture population of 40-plants/m² densities. Genetic variation is present in the gene pool for all the traits, except for tolerance of the proposed plant density. The current Indian varieties are grown at densities of 10-25 plants/m². In contrast, rapeseed genotypes in Canada and Europe are grown at densities around 150 plants/m².

Thurling (1991) has proposed an ideotype for *B. napus* for southern Australia (rainfed cultivation). The main features of this ideotype are summarized below.

1. It should be early flowering in order to allow a longer period of reproductive development and to avoid drought stress that occurs at the end of the growing season. Genes for early flowering have been introgressed into leading *B. napus* cultivars from an early flowering *B. napus* line and from early flowering *B. campestris*.
2. It should have apetalous flowers so that at peak flowering developing pods receive greater light. Apetalous lines showed 30% greater light penetration at peak flowering than that in the normal-flowered line. Two major genes govern apetalous condition.

3. It should have longer pods so that a larger number of seeds/pod is produced. Long pod character is governed by two dominant genes showing complementary interaction.
4. The ideotype should show reduced pod shattering. This trait could be introgressed into *B. napus* from related species, such as, *B. campestris* and *B. juncea*.

   ⇒ The plant type should have adequate field resistance to the disease black leg caused by *Leptosphaeria maculans*.

   ⇒ The ideotype must have canola quality, i.e., <0.5% erucic acid in the oil, and <30-μ mol of the four specified glucosinolates per gram of oil and moisture free meal.

IDEOTYPE FOR TREE FRUIT CROPS:

Few example of ideotype breeding in the broad field of horticulture can be found. Many horticulture plants are selected or bred primarily for qualitative, often aberrant, morphological qualities and then propagated asexually; hence, the ideotype concept, which is predicted largely on improvement qualitativeyield, has less relevance. Our aim will focus on perennial fruit tree crops, where both yield and quality are paramount, and where enhancement of yield quality through genetic manipulation is important and ongoing.

Few ideotypes are perennial tree fruits exist in the chapter. Stover (1982) proposed an ideotype for banana (Musa spp.), based on the performance of the variety 'Grand Nain' in Honduras and Panama. This ideotype is a dwarf triploid with small, upright leaves producing a compact crown, and with resistance to disease and nematodes. Tyagi's (1986) ideotype for mango (Mangifera indica) is also dwarf, and includes numerous small leaves producing an open canopy, high leaf area duration, early production of early branches with high tensile strength, and several characters relating to flowering and fruiting. Most of the idetypic charters are present in the current Indian mango cultivars 'Malaviya Bhog'. Both the banana and mango ideotypes are designed for high-density population

Apple is the most widely grown tree fruit and has received the most attention from breeders. Table – I presents a dwarfed apple for culture in a typical high-density orchard in Michigan or in any other region with a similar climate

Although adapted to a wide range of environmental conditions, apple cultivars must have specific traits for a particular cultural regime. Apple cultivars are usually asexually propagated by grafting or budding the scion of a specific genotype onto a rootstock, so that the fruit remains true to type. Rootstocks also offer the potential for wide adaptability. Because they can be changed to fit a particular environment. These toorstock may be of seedling origin or, more commonly, clonally propagated. Both the scion and rootstock influence the growth, morphology, and physiology of the tree. Another approach with some advantage would be to vegetatively propagate apple genotypes by rooting stem cuttings. By not having to go through a grafting procedure, some economic gains might be made, but such cultivars would have to posses the positive attributes of both a conventional scion and rootstock.

Table-1 An ideotype for apple grown in a high-density orchard under Michigan (USA) or similar environmental conditions.

The following traits are considered for developing high yield apple orchard.

- Scion traits.
- Rootstock traits.
- Growth and development.
  - ⇒ Vegetative & Reproductive stages.
- Physiology
  - ⇒ Photosynthesis.
  - ⇒ Water relations.
  - ⇒ Cold hardiness.

→ Scion traits:

- **Fruit quality**
  - Large size, proper flavour (acid-sugar ratio), good texture, red or yellow colour
  - Free from russet
  - Length-to-width ratio greater than 1
  - Multiple use (fresh, processing, juice)
  - Not easily bruised
- Fruit storable up to 12 months under controlled conditions
- High fruit productivity
- Late season ripening
- Chilling requirement
- Spurred habit
- Foliage and fruit resistance to common disease like insects, virus
- Trunk and roots unpalatable to rodents

→ Root stock traits

- Dwarfing tree height no, more than 3-4m(10-12 ft)
- Good anchorage
  - Minimum no of structural branches
  - High no Of fruiting branches
- Graft compatible with most scion cultivars
- Tolerant of environment stress (cold drought anoxia)
- Resistance to disease, insect and virus
- Tolerant to variable soil condition
- It should not produce any phytotoxicity

→ Growth & Development

- Vegetative
  - ⇒ Rapid initial growth for three to four years, followed by slow growth as fruit occurs.
  - ⇒ Responsive to pruning
  - ⇒ Wide branch angles. ($60\text{-}90^0$) With a strong centre leader
  - ⇒ Absence of water sprouts.

- **Reproductive**
  - ⇒ Begins fruiting in the 1st or 2nd year;
  - ⇒ Regular bearing, Uniform anthesis.

→ **Physiology**

- **Photosynthesis**
  - ⇒ High rate of co2 fixation per unit of leaf area
  - ⇒ Co2 fixation rate adjusts for leaf loss or high fruit –to-leaf ratio
  - ⇒ Greater than 50 percent of fixed carbon portioned into fruit (high harvest index)
  - ⇒ High water use efficiency (co2 fixed per unit of H2O transpired)
- **Water relation**

  Capable of withstanding drought through dehydration postponement or dehydration tolerance (Kramer 1983)
- Cold hardiness
  - ⇒ Early to acclimate and drop leaves in the fall, late to deacclimate in the spring
  - ⇒ Maxi. Deep winter hardiness of –40 to –50.

Conclusion

All attributes of the ideotypes are morphological character, but all are based on physiological consideration. Decades of work, have made significant gains in research on theories and methods for breeding. The foundation, development and perfection of these and methods will lay a solid foundation for the future.

DISCUSSION:

1) What is the opinion about success of ideotype is Kerala?
   Ans: Market idetype is suitable for Kerala.

2) How can you justify ideotype with preference of people?
   Ans: Identify the donor parents make a cross and release a new generation ideotype.

3) Is there any other crop than rice?
   Ans: Wheat, Maize, Apple, etc.,
4) In what name is the NPT lines released by IRRI famous among farmers?
   Ans: Super rice
5) Is there any new ideotype in rice released in Kerala?
   Ans: No. The research work is going on this year (2005-2006). It will be done by KAU.

ABSTRACT

Most of the plant breeding programmes aim for defect elimination or selection for yields. A valuable additional approach is available through breeding for crop ideotypes (model / ideal plant types), i.e., plants with model characteristics known to influence photosynthesis, growth and yield potential, in cereals and other horticultural crops. Model characteristics may vary from crop to crop.

The ideotype concept was first proposed by Donald (1968), a cereal breeder, working on wheat crop. According to him it is a "biological model", expected to perform in a predictable manner within predefined environment.Since its postulation by Donald (1968), the use of ideotype concept in plant breeding has expanded. Ideotypes are still applied most frequently in field crops, although examples in horticultural crops and fodder crops can be found.

The crop ideotype consists of several morphological and physiological traits contributing to enhanced genetic yield potential than currently prevalent in crop cultivars. In many cases, progress has been made towards ideotype attainment after several cycles of combination breeding for identified and desirable plant traits (Adam, 1982) with an increase in crop yield.

Successful ideotypes popularly known as New Plant Types or super rice has been developed in rice (Khush, 1994). Model characters will vary for different systems of rice cultivation. In general NPT lines will have only 6-8 sturdy tillers with 90-130 cm height. The plant will be ready to harvest in 100 to 130 days and may yield 13-15 t per ha in the dry season. This is 20 to 30 percent higher yield than improved semi dwarf rice. In 1999 two of the NPT lines have been released in the Yunnan province of China, with a yield of more than 13 t per ha at farmer's field.

In addition ideotypes have also been proposed for wheat (Donald, 1968), maize (Mock and Pearce, 1975), cotton (Coffey and Davis, 1985), apple (Stover, 1982), brinjal (Lieshout, 1993) and napier grass (Wright, 1976).

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Transgenics for crop improvement-Scope and realities

By

Vishnu Vardhan Reddy

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SEMINAR REPORT

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COLLEGE OF HORTICULTURE

KERALA AGRICULTURAL UNIVERSITY

VELLANIKKARA – 680 656

THRISSUR

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DECLARATION

I, Vishnu Vardhan Reddy (2004-11-05) hereby declare that this seminar entitled **"Transgenics for crop improvement-Scope and realities"** has been prepared by me, after going through the various references cited at the end and has not been copied from any of my fellow students

Vellanikkara

Date:

Vishnu Vardhan Reddy

(2004-11-05)

CERTIFICATE

This is to certify that the seminar report entitled **"Transgenics for crop improvement-Scope and realities"** has been solely prepared by Mr. Vishnu Vardhan Reddy (2004-11-05) under my guidance, and has not been copied from any seniors, juniors or fellow students.

Vellanikkara

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1. INTRODUCTION

Transgenic crops are a reality. "Genetic engineering" emerged in the 1970s, at the same time as *in vitro* fertilization techniques (Hindmarsh, 1992). The first "genetically modified" crops were planted in China in 1993; large scale production commenced in 1996 (Huttner, 1997) and, by 1998, almost 28,000,000 hectares of transgenic crops were planted, world-wide.

If positive perceptions gain the ascendancy then transgenic crops may well prove to be the superhighway, which leads World agriculture to a profitable and sustainable future. If, however, negative perceptions hold sway then transgenics could prove to have been an expensively surfaced road to nowhere.

The science is not new. The double helix structure of DNA was discovered more than forty years ago (Watson and Crick, 1953) and recombinant DNA research has taken place since the late 1960s. To many, perhaps most, of those involved in crop improvement, transgenics are merely a further step in an evolution. Which, in the case of a crop such as wheat, extends from the dawn of agriculture. Gene technologies offer a new set of tools to be applied to the on-going task of enhancing crop productivity and profitability by improving the use of scarce resources, including water and nutrients, tolerance to abiotic stresses, including drought and frost, tolerance to biotic stresses, including pests and diseases, quality characteristics, including a diversified product range, and food safety, by reducing dependence on pesticides and herbicides

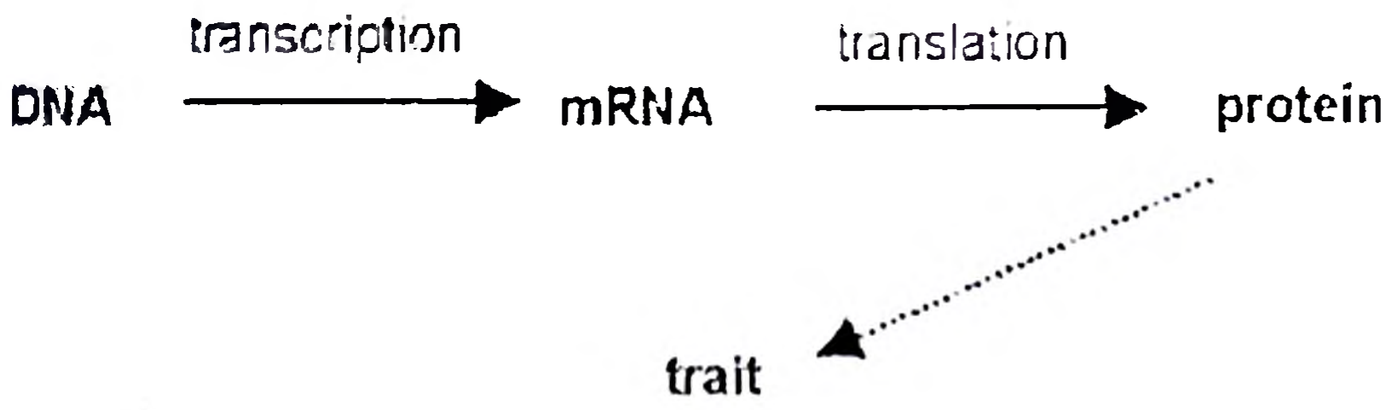
Incremental gains from conventional plant breeding run at 1 to 1.5% per annum. Grain crops will not meet future demand without a step change in yield improvement, such as occurred during the "Green Revolution". While "transitional high technology" plant breeding, perhaps including the delivery of improved traits via hybrids, may help to bridge the food gap, transgenics appear to offer the best prospect of meeting food needs in a sustainable way.

At present, transgenic maize is the only one of the three major cereals in agricultural production (Metcalfe, 1996). While progress is being made with barley, rice and wheat, attaining acceptable levels of transformation, especially with wheat, remains challenging.

2. HOW TO DEVELOP A TRANSGENIC PLANT?

2.1 Introduction to DNA

The underlying reason that transgenic plants can be constructed is the universal presence of DNA (deoxyribonucleic acid) in the cells of all living organisms. This molecule stores the organism's genetic information and orchestrates the metabolic processes of life. Genetic information is specified by the sequence of four chemical bases (adenine, cytosine, guanine, and thymine) along the length of the DNA molecule. Genes are discrete segments of DNA that encode the information necessary for assembly of a specific protein. The proteins then function as enzymes to catalyze biochemical reactions, or as structural or storage units of a cell, to contribute to expression of a plant trait. The general sequence of events by which the information encoded in DNA is expressed in the form of proteins via an mRNA intermediary is shown in the diagram below



The transcription and translation processes are controlled by a complex set of regulatory mechanisms, so that a particular protein is produced only when and where it is needed. Even species that are very different have similar mechanisms for converting the information in DNA into proteins; thus, a DNA segment from bacteria can be interpreted and translated into a functional protein when inserted into a plant.

Among the most important tools in the genetic engineer's tool kit are enzymes that perform specific functions on DNA.

2.2 Locating Genes for Plant Traits

Identifying and locating genes for agriculturally important traits is currently the most limiting step in the transgenic process. We still know relatively little about the specific genes required to enhance yield potential, improve stress tolerance, modify chemical properties of the harvested product, or otherwise affect plant characters. Usually, identifying a single gene involved with a trait is not sufficient; scientists must understand how the gene is regulated, what other effects it might have on the plant, and how it interacts with other genes active in the same biochemical pathway. These efforts should result in identification of a large number of genes potentially useful for producing transgenic varieties.

2.3 Designing Genes for Insertion

Once a gene has been isolated and cloned (amplified in a bacterial vector), it must undergo several modifications before it can be effectively inserted into a plant.

1. A **promoter sequence** must be added for the gene to be correctly expressed (i.e., translated into a protein product). The promoter is the on/off switch that controls when and where in the plant the gene will be expressed.
2. Sometimes, the **cloned gene is modified** to achieve greater expression in a plant. For example, the Bt gene for insect resistance is of bacterial origin and has a higher percentage of A-T nucleotide pairs compared to plants, which prefer G-C nucleotide pairs. In a clever modification, researchers substituted A-T nucleotides with G-C nucleotides in the Bt gene without significantly changing the amino acid sequence. The result was enhanced production of the gene product in plant cells.
3. The **termination sequence** signals to the cellular machinery that the end of the gene sequence has been reached.
4. A **selectable marker gene** is added to the gene "construct" in order to identify plant cells or tissues that have successfully integrated the transgene. This is necessary because achieving incorporation and expression of transgenes in plant cells is a rare event, occurring in just a few percent of the targeted tissues or cells. Selectable marker genes encode proteins that provide resistance to agents that are normally toxic to plants, such as antibiotics or herbicides. As explained below, only plant cells that have integrated the selectable marker gene will survive

when grown on a medium containing the appropriate antibiotic or herbicide. As for other inserted genes, marker genes also require promoter and termination sequences for proper function.

2.4 Transforming Plants

Transformation is the heritable change in a cell or organism brought about by the uptake and establishment of introduced DNA. There are two main methods of transforming plant cells and tissues:

1. The **"Gene Gun" method** (also known as microprojectile bombardment or biolistics).
2. The ***Agrobacterium* method**, which is described below. Transformation via *Agrobacterium* has been successfully practiced in dicots (broadleaf plants like soybeans and tomatoes) for many years, but only recently has it been effective in monocots (grasses and their relatives). In general, the *Agrobacterium* method is considered preferable to the gene gun, because of the greater frequency of single-site insertions of the foreign DNA, making it easier to monitor.

Agrobacterium Method of Plant Transformation

Agrobacterium tumefaciens is a remarkable species of soil-dwelling bacteria that has the ability to infect plant cells with a piece of its DNA. When the bacterial DNA is integrated into a plant chromosome, it effectively hijacks the plant's cellular machinery and uses it to ensure the proliferation of the bacterial population. Many gardeners and orchard owners are unfortunately familiar with A *tumefaciens*, because it causes crown gall diseases in many ornamental and fruit plants.

The DNA in an A *tumefaciens* cell is contained in the bacterial chromosome as well as in another structure known as a Ti (tumor-inducing) plasmid. The Ti plasmid contains

- a stretch of DNA termed T-DNA (~20 kb long) that is transferred to the plant cell in the infection process.
- a series of *vir* (virulence) genes that direct the infection process.

A. tumefaciens can only infect a plant through wounds. When a plant root or stem is wounded it gives off certain chemical signals. In response to those signals, the *vir* genes of *A.*

tumefaciens become activated and direct a series of events necessary for the transfer of the T-DNA from the Ti plasmid to the plant's chromosome. Different *vir* genes

- Copy the T-DNA.
- Attach a product to the copied T-DNA strand to act as a leader.
- Add proteins along the length of the T-DNA, possibly as a protective mechanism.
- Open a channel in the bacterial cell membrane, through which the T-DNA passes.

The T-DNA then enters the plant cell through the wound.

2.5 Selection and Regeneration

Following the gene insertion process, plant tissues are transferred to a selective medium containing an antibiotic or herbicide, depending on which selectable marker was used. Only plants expressing the selectable marker gene will survive, as shown in the figure, and it is assumed that these plants will also possess the transgene of interest. Thus, subsequent steps in the process will only use these surviving plants. When grown on selective media, only plant tissues that have successfully integrated the transgene construct will survive

2.6 Regeneration of whole plants

To obtain whole plants from transgenic tissues such as immature embryos, they are grown under controlled environmental conditions in a series of media containing nutrients and hormones, a process known as tissue culture. Once whole plants are generated and produce seed, evaluation of the progeny begins. This regeneration step has been a stumbling block in producing transgenic plants in many species, but specific varieties of most crops can now be transformed and regenerated.

2.7 Plant Breeding and Testing

Intrinsic to the production of transgenic plants is an extensive evaluation process to verify whether the inserted gene has been stably incorporated without detrimental effects to other

plant functions, product quality, or the intended agroecosystem. Initial evaluation includes attention to:

- Activity of the introduced gene
- Stable inheritance of the gene
- Unintended effects on plant growth, yield, and quality

If a plant passes these tests, most likely it will not be used directly for crop production, but will be crossed with improved varieties of the crop (Metcalfe, 1996). This is because only a few varieties of a given crop can be efficiently transformed, and these generally do not possess all the producer and consumer qualities required of modern cultivars. The initial cross to the improved variety must be followed by several cycles of repeated crosses to the improved parent, a process known as backcrossing. The goal is to recover as much of the improved parent's genome as possible, with the addition of the transgene from the transformed parent.

2.8 The next step in the process is multi-location and multi-year

Evaluation trials in greenhouse and field environments to test the effects of the transgene and overall performance. This phase also includes evaluation of environmental effects and food safety

3. TRANSGENICS CURRENTLY ON THE MARKET

3.1 Biotic stresses - Insect resistance

Although it has the world's largest acreage of 8.9 million hectares under cotton, India is only the third largest global cotton producer, with about 2.86 million tonnes of cotton lint a year. The average productivity of cotton lint at 320 kilograms per hectare is amongst the lowest in the world. The productivity ranges from 200 kg per hectare to 600 kg for hybrid varieties. Since many of the land holdings are characterized by small-scale and resource-poor farming, a sudden and high increase in productivity using present methods is unlikely

Cotton is essentially grown in the kharif, the rainy season and treated as a perennial crop. Nearly 70 per cent of the crop is cultivated under rainfed conditions in the central and southern regions of the country: Gujarat, Maharashtra, Madhya Pradesh, Tamil Nadu, Andhra Pradesh and Karnataka (Gupta *et al.*, 2001). The sowing dates in Southern India differ according to the specific regions. Only in the northern regions of the country, mainly the states of Punjab, Haryana and Rajasthan, is cotton predominantly irrigated. Here, the plantings are homogenous and the emphasis is on planting high yield varieties.

Pests and Pesticides

About 162 species of insects are known to devour cotton at various stages of growth, of which 15 are considered to be key pests. Among these are jassids (*Amarasca biguttulla*), aphids (*Aphis gossypii*), white fly (*Bemesia tabaci*), spotted bollworm (*Earias vitella*), pink bollworm (*Pectinophora gossypiella*) and American bollworm (*Helicoverpa armigera*) (Firoozabady *et al*, 1987). Important diseases are bacterial blight, fusarium wilt, Alternaria leaf spot and grey mildew. Together these pests and diseases result in an estimated loss of 50 to 60 per cent of the potential yield. This is similar to losses in other countries.

Pests appear in quick succession at various stages of the growth of the cotton plant. First to infest are sucking pests like aphids and jassids, followed by white flies. It is then the turn of bollworms and by the time the crop enters the flowering stage, bugs have taken over (Bent and Yu, 1999). Farmers therefore use a combination of expensive chemical pesticides to control pest infestation. Currently, pesticides account for one-third of the total cultivation costs. Increasing reliance on pesticides over the years have replaced traditional methods that included a variety of labour-intensive practices like hand picking to remove pests and cultural practices like intercropping, crop rotation, and the burning or removal of cotton residues from the soil.

Intensive cultivation practices and indiscriminate use of conventional as well as fourth generation pesticides like synthetic *pyrethroids* have created resistance among some of key pests, including the American bollworm. Monocropping and favourable climatic

conditions in certain years have further accentuated the problem. In the early 1990s, the outbreak of leaf curl virus reached epidemic proportions in the northern plains. The reasons for the outbreak of leaf curl virus are not fully known, but based on the experience of other countries, it is reasonable to assume that excessive use and abuse of pesticides is a major contributing factor. Dependence on chemicals has, in some cases, been so heavy that farmers often resort to a mix of several pesticides, so-called pesticide cocktails, and it is not uncommon to spray more than 30 times per season.

If the crop fails because of weather conditions and/or pest resistance, a rising number of farmers have been known to consume the same chemicals to end their lives and escape the humiliation that comes with mounting debts. According to the official records, more than 500 cotton farmers in Andhra Pradesh, Karnataka, Maharashtra and Punjab committed suicide in 1998.

Disease Resistance

Developing disease resistant crop cultivars is one of the main objectives of plant breeders. In the past, breeders have been successful in introgressing natural disease resistance genes into crop plants from wild relatives in order to protect them against specific pathogens. However, for a number of diseases, resistant sources are not available in sexually compatible genotypes. Recently, resistance genes from different sources have been isolated and introduced into susceptible crop cultivars through genetic engineering for making them resistant against viral, bacterial and fungal diseases (Bent and Yu, 1999). Among various diseases, focus has been primarily on developing transgenics for viral resistance using coat protein strategy. By this approach, gene encoding the coat protein, i.e. the outer protective envelop of the virus, is introduced in plants. The presence of this viral gene in transgenic plants hampers virus multiplication and thus imparts resistance against the infecting virus. The coat protein mediated resistance against virus in plants was first introduced and tested in transgenic tobacco plants against tobacco mosaic virus. Since then, this novel strategy has been successfully applied in producing transgenic virus resistant crop plants, notable examples being papaya resistant to papaya ring spot virus, tomato resistant to tomato mosaic virus, potato resistant to both potato

virus X and Y, and squash resistant to zucchini yellow mosaic virus. These virus resistant transgenic plants have been field tested, and a significant increase in yield (upto 90% in case of papaya and squash) has been reported. In the Indian context, it is important to develop transgenics resistance to viruses in crops such as cotton against leaf curl virus, mungbean against yellow mosaic virus, tomato against leaf curl virus and potato against potalo virus Y. Work is in progress in many laboratories in the country to produce such transgenics. As the coat protein approach is specific against a particular strain of virus and does not often provide broad-spectrum protection, efforts are in progress to introduce in plants multiple coat-protein genes from more than one virus to make them resistant against all types of infective viruses.

3.2 Abiotic Stress Tolerance

Research over the past two decades has provided a better understanding of the molecular biology of stress responses in plants. This has led to the identification of several genes and genes products that are induced upon exposure of the plants to various abiotic stresses, *viz* drought, salinity, and low and high temperature. Consequently, genetic engineering has been applied to transfer candidate genes from diverse sources to susceptible crop plants for developing transgenics resistant to abiotic stresses. A few of the notable transgenics of this category are described here. The most recent report is in a model crop tobacco, achieved through overexpression of two genes together of a pathway, viz gly! and gfyll, providing strong evidence of improved tolerance to drought and salinity. Transgenic rice with increased tolerance to drought and salinity is another recent example. These transgenic rice plants overexpressing two genes (otsA and otsB) from *E. coli* produced high amounts of trehalose, a non-reducing disaccharide of glucose. Trehalose is known to play an important role in imparting stress tolerance to a large variely of organisms ranging from bacteria and fungi to invertebrate animals. Another notable example is the biosynthesis of glycine betaine through expression of a baclerial *codA* gene encoding choline oxidase in transgenic rice.

Transgenic wheat transformed with mflD gene of *E.coli* for the biosynthesis of mannitol showed improved growth performance under drought and salinity stresses. It

is also possible to introduce abiotic stress tolerance in transgenic plants by efficient scavenging of reactive oxygen species. This was recently demonstrated by expressing wheat catalase gene in transgenic rice.

3.3 Herbicide Tolerance

The only problem with this seemingly miraculous product is that it kills just about any plant onto which it is directly sprayed. Thus, until recently, this synthetic amino acid, known as glyphosate (N-phosphono-methyl glycine) has had limited utility in agricultural production (Burks and Fuchs, 1995). And then along came genetic engineering. In just the last five years, soybeans, corn, cotton, and rape (canola) are genetically engineered to resist the toxic effects of glyphosate and trademarked these transgenic cultivars as Roundup Ready (RR), in reference to their commercial formulation of glyphosate.

So everyone should be cheering about the decreased numbers of pesticide applications (or at least the potential to decrease them)

3.3.1 Biochemical Basis for Glyphosate Resistance

All Roundup Ready crops contain an enzyme known as EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) that is resistant to the effects of glyphosate. EPSPS is naturally found in all plants, fungi, and bacteria but is absent in animals (18). The enzyme is an important catalyst in the biochemical pathway for synthesis of the aromatic amino acids phenylalanine, tryptophan, and tyrosine. Because animals do not contain EPSPS, they must ingest these aromatic amino acids in their diets (Delannay, 1995).

EPSPS is localized in the chloroplasts of plants, the cell organelle responsible for photosynthesis. Glyphosate latches on to EPSPS, inhibiting its synthetic activity. The inability to produce the aromatic amino acids eventually leads to cell death. The glyphosate-tolerant form of EPSPS has a low affinity for binding glyphosate yet it still helps synthesize the amino acids just as efficiently as the glyphosate-susceptible EPSPS.

3.3.2 Genetic Basis for Glyphosate Resistance

Plant species have long been known to be highly variable in their response to herbicides. For example, grasses are very tolerant to 2,4-D and other growth hormone mimics, but dandelions exposed to it wither and die. Soybeans can tolerate trifluralin, but corn never gets big enough to produce an ear. Furthermore, weed populations can become resistant to herbicides. During the 1980s, agricultural scientists tried in vain to take advantage of plants' natural variability to herbicide toxicity and their penchant to develop resistance. Attempts to conventionally breed glyphosate-tolerant crops failed (Kishore, *et al.*, 1992). Such failure is not surprising, after twenty-five years of glyphosate use, plant resistance in the field has been noted in only two grass species (10). As molecular manipulation technologies developed (i.e., the ability to purposefully transfer specific genetic sequences from one organism to another), the stage was set for engineering plants resistant to glyphosate.

So how does one "make" a plant resistant to glyphosate? Mimic Mother Nature. As with all cases of resistance evolution, two main mechanisms are responsible for herbicide tolerance in plants-an increased ability to detoxify the pesticide and/or an altered biochemical site of interaction with the pesticide. Both mechanisms involve altered protein functioning and/or production. In the case of detoxification, the proteins involved are enzymes that possess an enhanced capacity for breaking down the herbicide. Biochemical sites attacked by a herbicide may also be enzymes or alternatively receptors that trigger a cascade of physiological reactions. Altered enzymes and receptors have less affinity than their "normal" counterparts for binding the herbicide.

Whatever the mechanism of herbicide tolerance, genes ultimately determine the characteristics of the proteins. Researchers either search for the genes of an organism which already possesses a detoxification mechanism (such as GOX from O. anthropi), or they add chemical reagents to plant cells in vitro (i.e., in cell culture) that change the genetic code and produce an "altered" enzyme (i.e., one with less affinity for glyphosate).

Presently, only canola plants have been successfully engineered to contain a functional GOX enzyme (1). However, all the commercial RR crops contain a tolerant EPSPS gene. For soybean, cotton, and canola the glyphosate-resistant EPSPS was obtained from a soil bacterium in the genus Agrobacterium (strain CP4) (Nida, 1996). For corn, the source of EPSPS was its own cloned gene that had been mutagenized in vitro (i.e , in cell culture) This technique involves changing the DNA bases of cultured plant cells by adding mutagenic chemical reagents Resulting changes in DNA bases could slightly affect the amino acid composition of the host (i.e., corn) enzyme. Normally, mutagenesis will produce nonfunctional enzymes, but in some cases a few changes in amino acid sequence can still produce a functional enzyme With the mutagenized corn line, the resulting EPSPS was 99.3% similar to the nonmutagenized EPSPS and still functional (i.e., it produced the aromatic amino acids), but it was resistant to the effects of glyphosate (Sidhu, 2000).

3.3.3 Substantial Equivalence and Ecological Concerns

The principle of substantial nutritional equivalence might be analogously applied to the two remaining concerns about RR crops--glyphosate safety and potential for superweeds. Is the widespread implementation of the technology doing anything to the environment that conventional agriculture has not already wrought? Might there, in fact, be environ-mental benefits from RR technology that surpass conventional crop management? Does more widespread use of glyphosate pose a substantially different risk than the amount currently used for weed control? Is glyphosate perhaps "greener" than other herbicides? Get ready for subsequent issues of this newsletter, I will round up the answers to these burning questions.

3.4 Quality Improvement

In the past, it was possible to increase the quantity of food grains by conventional breeding methods; however, substantial improvement in nutritional quality of foods could not be achieved Another avenue where improvement could not be attained through classical breeding is the post-harvest management of fruits and vegetables (Harrison, 1996). A post-harvest loss of 10 to 30% has been reported to occur in fruits and vegetables due to physical damage, pathological decay and over-ripening. This especially occurs in developing countries including India where storage

conditions are poor and inadequate. Transgenic plants hold a great promise to circumvent such problems.

Numerous studies have demonstrated the power of plant genetic engineering to produce transgenic plams with enhanced nutritional traits and a better keeping quality. The most notable example in this category is the development of 'golden rice' enriched with pro-Vitamin A, in which three different genes, viz, phytoene synthase from daffodil, phytoene desaturase from a bacteria, and lycopene cyclase from daffodil were mobilized into rice. Vitamin A deficiency, which also interferes with the bio-availability of iron, affects 413 million children worldwide. Ferritin rice rich in iron is another example developed by introducing the ferritin gene of Phaseolus into rice. Iron fortification in transgenic rice can provide 30-50% of the daily adult iron requirement

Transgenic tomato and many other fruit crops are also being currently produced with delayed ripening to save the post-harvest losses that occur primarily due to over-ripening. In fact, a transgenic tomato with delayed ripening was the first transgenic crop variety product to be commercialized in the world in 1994. Since then, many public and private institutions have produced transgenic tomatoes fit for processing and possessing and improved post-harvest characteristics. Work is in progress in India to regulate the expression of ripening-related genes for improving texture and delayed ripening in tomato. Production of antigens in transgenic plants is also another important application of this technology, Such transgenic plants, the so called 'edible vaccines', have gone through clinical trials in some countries with encouraging results.

4. BIO-SAFETY

Bio-safety comprises environmentally safe application of modern biotechnology. The term is used to describe the policies and procedures adopted to ensure the environmentally safe application of modern biotechnology. Biosafety is essentially required to protect human health and environment from the possible adverse effects of the products of modern biotechnology (FDA, 1992).

Several countries have formulated safety guidelines and regulations. Most of these occur at or amidst the range of predominantly the two ends viz. substantial equivalence and precautionary principle

In India, genetically modified organisms and r-DNA products are governed by several acts/legislations such as Environment (Protection) Act, 1986 and its Rules, [989, Protection of

Plant Varieties and Farmers' Rights Act, 2001, Drugs & Cosmetics Act, -1940 and its Rules, 1945 etc. The Rules for the Manufacture, Use/Import/ Export and Storage of Hazardous Micro Organisms/Genetically Modified Organisms or Cells formulated under the Environment (Protection) Act provide for the following multi-tiered regulatory framework to assess and ensure bio safety of genetically engineered organisms. It comprises of (i) Recombinant DNA Advisory Committee (RDAC), under the DBT, (ii) Review Committee on Genetic Manipulation (RCGM), under the DBT, (iii) Institutional Biosafety Committee(s) (IBSC), (iv) Genetic Engineering Approval Committee (GEAC), under the Ministry of Environment and Forests (v) State Bio-technology Coordination Committee(s) (SBCC) and (vi) The District Level Committee(s).

The existing system of approval of GM Varieties for cultivation needs rationalization. There is a need for reduction in the levels and number of steps required in evaluation and environmental clearance of GM products/transgenics (FDA, 1992). Once a transgene has been declared bio-safe, its derivatives may not be evaluated for bio-safety to the same extent again as done previously The expression of the transgene in the new background should. however, necessarily be checked which should be in the desirable range so that the performance of the product and delaying of the resistance build-up is ensured. Such derivative crop varieties could be evaluated on the basis of large-scale trials and released after satisfactory performance

5. RISKS AND CONCERNS

The introduction of transgenic crops and foods into the existing food production system has generated a number of questions about possible negative consequences. People with concerns about this technology have reacted in many ways, from participating in letter-writing campaigns to demonstrating in the streets to vandalizing institutions where transgenic research is being conducted

5.1 Concerns about human health

5.1.1 Allergenicity

The possibility that we might see an increase in the number of allergic reactions to food as a result of genetic engineering has a powerful emotional appeal because many of us

experienced this problem before the advent of transgenic crops, or know of someone who did.

However, there is no evidence so far that genetically engineered foods are more likely to cause allergic reactions than are conventional foods. Tests of several dozen transgenic foods for allergenicity have uncovered only a soybean that was never marketed and the now-famous StarLink corn. Although the preliminary finding is that StarLink corn is probably not allergenic, the scientific debate continues. Every year some people discover that they have developed an allergy to a common food such as wheat or eggs, and some people may develop allergies to transgenic foods in the future, but there is no evidence that transgenic foods pose more of a risk than conventional foods do.

5.1.2 Horizontal transfer and antibiotic resistance

The use of antibiotic resistance markers in the development of transgenic crops has raised concerns about whether transgenic foods will play a part in our loss of ability to treat illnesses with antibiotic drugs. At several stages of the laboratory process, developers of transgenic crops use DNA that codes for resistance to certain antibiotics, and this DNA becomes a permanent feature of the final product although it serves no purpose beyond the laboratory stage. Will transgenic foods contribute to the existing problems with antibiotic resistance?

One aspect of this topic is the risk of horizontal gene transfer, that is, transfer of DNA from one organism to another outside of the parent-to-offspring channel. Transfer of a resistance gene from transgenic food to micro-organisms that normally inhabit our stomach and intestines, or to bacteria that we ingest along with food, could help those micro-organisms to survive an oral dose of antibiotic medicine. Although horizontal transfer of DNA does occur under natural circumstances and under laboratory conditions, it is probably quite rare in the acid environment of the human stomach.

Another concern is that the enzyme product of the DNA might be produced at low levels in transgenic plant cells. While high processing temperatures would inactivate the

enzyme in processed foods, ingestion of fresh or raw transgenic foods could result in the stomach containing a small amount of an enzyme that inactivates an orally administered dose of the antibiotic. This issue was raised during the approval processes for Calgene's FlavrSavr tomato and Ciba-Geigy's Bt corn 176. In both cases, tests showed that orally administered antibiotics would remain effective. While the risks from antibiotic resistance genes in transgenic plants appear to be low, steps are being taken to reduce the risk and to phase out their use.

Eating foreign DNA

When scientists make a transgenic plant, they insert pieces of DNA that did not originally occur in that plant. Often these pieces of DNA come from entirely different species, such as viruses and bacteria. Is there any danger from eating this "foreign" DNA?

We eat DNA every time we eat a meal. DNA is the blueprint for life and all living things contain DNA in many of their cells. What happens to this DNA? Most of it is broken down into more basic molecules when we digest a meal. A small amount is not broken down and is either absorbed into the blood stream or excreted in the feces. We suspect that the body's normal defense system eventually destroys this DNA. Further research in this area would help to determine exactly how humans have managed to eat DNA for thousands of years without noticing any effects from the tiny bits that sneak into the bloodstream.

So far there is no evidence that DNA from transgenic crops is more dangerous to us than DNA from the conventional crops, animals, and their attendant micro-organisms that we have been eating all our lives.

5.1.3 CaMV promoter

When scientists use transgenic technology to put a new gene into a plant, they put in additional pieces of DNA to direct the activity of that gene. One of these pieces is the "promoter" that turns the gene on.

The most widely used promoter is the cauliflower mosaic virus 35S promoter, often abbreviated as the CaMV promoter or the 35S promoter. This promoter was obtained from the virus that causes cauliflower mosaic disease in several vegetables, such as cauliflower, broccoli, cabbage, and canola. There are concerns that the CaMV promoter might be harmful if it were to invade our cells and turn on our genes.

A multi-step chain of events would have to occur for the CaMV promoter to escape the normal digestive breakdown process, penetrate a cell of the body, and insert itself into a human chromosome. While there have been no tests to determine whether the CaMV promoter has invaded human tissues, experiments with mice indicate that normal body defenses eliminate stray fragments of foreign DNA that sneak into the blood stream from the digestive tract.

There is some evidence that the CaMV promoter poses little threat to human health. People have been eating it in small quantities for hundreds of years when we eat vegetables that are infected with the disease. Although vegetables heavily infected with CaMV are unappetizing, there have been no documented negative effects on health from eating the virus or its promoter.

5.1.4 Changed nutrient levels

How do genetically engineered foods compare with conventional foods in nutritional quality? This is an important issue, and one for which there will probably be much research in the future, as crops that are engineered specifically for improved nutritional quality are marketed (Hammond, 1996). However, there have been only a few studies to date comparing the nutritional quality of genetically modified foods to their unmodified counterparts.

The central question for GE crops that are currently available is whether plant breeders have accidentally changed the nutritional components that we associate with conventional cultivars of a crop. Because isoflavones are thought to play a role in preventing heart disease, breast cancer, and osteoporosis, the isoflavone content of RoundupReady soybeans has been investigated by several researchers.

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The studies completed so far do not resolve the issue of whether RoundupReady soybeans have isoflavone levels comparable to conventional varieties, but the differences found in experiments appear to be small or moderate in comparison with natural variation in isoflavone levels. Additional evidence may clarify the arguments for and against Roundup applications as a risk factor in soybean cultivation. Industry studies submitted in support of applications for permission to sell transgenic crops indicate that the nutritional components that are commonly tested are similar in transgenic foods and conventional foods.

5.2 Concerns about damage to the environment

5.2.1 Monarch butterfly

The suggestion that Bt corn pollen might kill Monarch butterfly larvae galvanized public interest in the effect of transgenic crops on the environment.

5.2.2 Crop-to-weed gene flow

Hybridization of crops with nearby weeds may enable weeds to acquire traits we wish they didn't have, such as resistance to herbicides. Research results indicate that crop traits may escape from cultivation and persist for many years in wild populations. Genes that provide a competitive edge, such as resistance to viral disease, could benefit weed populations around a crop field.

Many cultivated crops have sexually compatible wild relatives with which they hybridize under favorable circumstances. The likelihood that transgenes will spread can be different for each crop in each area of the world.

For example, there are no wild relatives of corn in the United States or in Europe for transgenic corn to pollinate, but such wild relatives exist in Mexico.

Soybeans and wheat are self-pollinating crops, so the risk of transgenic pollen moving to nearby weeds is small. However, that small risk must balanced against the fact that there are wild relatives of wheat in the United States.

There are no wild relatives of soybean in the United States, but such wild relatives exist in China. Thus each crop must be evaluated individually for the risk of gene flow in the area where it will be grown.

5.2.3 Antibiotic resistance

There is also concern that transgenic plants growing in the field will transfer their antibiotic resistance genes to soil micro-organisms, thus causing a general increase in the level of antibiotic resistance in the environment. However, many soil organisms have naturally occurring resistance as a defense against other organisms that generate antibiotics, so genes contributed occasionally by transgenic plants are unlikely to cause a change in the existing level of antibiotic resistance in the environment.

5.2.4 Leakage of GM proteins into soil

Many plants leak chemical compounds into the soil through their roots. There are concerns that transgenic plants may leak different compounds than conventional plants do, as an unintended consequence of their changed DNA.

Speculation that this may be happening leads to concern about whether the communities of micro-organisms living near transgenic plants may be affected. The interaction between plants and soil micro-organisms is very complex, with the micro-organisms that live around plant roots also leaking chemical compounds into the soil. Much more research must be done before we understand the relationships that occur between micro-organisms and conventional crops. Attempts to discover whether transgenic plants are changing the soil environment, and whether they are changing it in good ways or bad ways, are hindered by our lack of basic scientific knowledge

5.2.5 Reductions in pesticide spraying: are they real?

One of the most appealing arguments in favor of transgenic plants is the potential for reducing the damage we do to our environment with conventional methods of farming. Pest-resistant crops such as Bt corn and Bt cotton have been promoted as a means to reduce the

spraying of pesticides, while herbicide-tolerant crops such as RoundupReady soybeans are said to reduce the application of herbicides. Large reductions in chemical spraying have been claimed to result from the introduction of these transgenic varieties. Are the claims true?

Bt cotton is the only crop for which claims of reduced spraying are clear. Analysts paint a mixed picture on the results of planting RoundupReady soybeans. Bt corn and herbicide-tolerant cotton and corn have not resulted in clear reductions in the spraying of chemicals.

5.3 Concerns about damage to current farming practices

5.3.1 Crop-to-crop gene flow: Hybridization of transgenic crops with nearby conventional crops raises concerns about separation distances to ensure purity of crops and about who must pay if unwanted genes move into a neighbor's crop. As "Identity Preservation" and segregation of GM from non-GM crops become factors in marketing products, it will be important to ensure that hybridization is not occurring in the field.

Many factors influence the potential for gene flow from crop to crop. Some crops are highly out crossing, with pollen carried to other fields by wind and by insects. Other species are highly self-pollinating, with little potential for pollen transfer to neighboring plants. Because of the differences among crops species, every case must be evaluated individually for potential to contribute to gene flow from transgenic to conventional crops.

If GM pollen pollinates plants in a neighboring field, then the issue of genetic trespass may arise. What level of GM presence, if any, should be allowed in products that are sold as organic or conventional? Should GM farmers and companies bear responsibility for preventing gene flow, or should conventional and organic farmers pay to protect their products from gene flow? Should GM versions of outcrossing plants be banned as too risky, while GM versions of self-pollinating plants are permitted? These issues have already prompted several lawsuits and they will continue to be a factor in the development and use of trangenic plants for years to come.

6. CONCLUSION

Transgenic research in the developing countries, including India, has focused largely on traits such as insect pest resistance, disease resistance, improved nutritional quality and adaptation to abiotic stresses. In relation to the priorities and preparedness with respect to transgenic crops in the Indian context, the focus is on the following major aspects: (i) Prioritization of the problems in target crops relevant to the country; ii) Critical components of transgenic development and commercialization that need particular attention: (iii) Building an adequate technology base, appropriate facilities, and human resources; (iii) Ensuring effectiveness of biosafety regulations and capacity to assess risks and benefits; (iv) Developing a strong scientific base in supporting disciplines; and (v) Promoting stakeholders' dialogue and public awareness of the transgenic technology (Lindner, 1999).

The impact of transgenic crops on Indian agriculture would depend largely on the ability of our scientists to identify the most limiting conditions, developing the proper combinations of genes and promoters to overcome those constraints, and transferring effective constructs into proper agronomic backgrounds. Translating the potential of a 'transgenic line' into a successful 'transgenic variety' would also warrant closeness of linkages between biotechnologists and other plant scientists, particularly plant breeders, besides the efforts devoted towards ensuring biosafety (Gebhard and Smalla, 1998). Public funding of the basic sciences that support the development of transgenic research and development is, therefore, critical for the success of transgenics. We also need to ensure that biotechnology is effectively maintained in the public domain while working towards a mix of public and private goods. For successfully achieving this, we require carefully planned investments in research and biosafety regulation, maximized synergy through inter-institutional collaborations, efficient dissemination systems to reach the end users, and the ability to impact farmers with proven and safe technologies. The broadening of technological choices should certainly go hand in hand with a commitment to safety and social responsibility.

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7. DISCUSSION

1. *What is the difference between organic foods and transgenic foods with regard to quality?*

Organic foods are those which are the products of organically grown crops. Transgenic foods are those which are derived from the plants or crops whose genetic background has been changed by introduction of transgene. Organic foods are devoid of pesticides and herbicides residues. Incase of transgenic foods, they have new quality traits because of the completion of metabolic pathways by the synthesis of absent enzymes by introduced transgene.

2. *Is golden rice available for commercial cultivation in India?*

No, Golden rice has not been released in India for commercial cultivation. Trials are conducted in research stations regarding its performance in varieties that are grown in India. It takes 3-4 years for the release of golden rice for commercial cultivation in India.

3 *What is your opinion about Transgenic crops?*

As a plant breeder I appreciate the technology. We cant improve the quality of the trait unless we change the or add genes which control that character from other or related organisms. The disadvantages of conventional breeding can be overcome by transgenics.

4. *What about transgenic soyabean?*

Soyabean's performance is good with regard to the quality that has been improved. People started saying that they develop some allergies by eating transgenic soyabean. We do develop allergies when we eat normal foods. Nobody bothers about this when it is regarding the transgenic crops then everybody raise their voice.

5. *Transgenics revert back to its original crops, true false?*

Transgenics cant revert back to their original forms if the introduced gene is functioning in the way we want it to function. But, if the introduced gene has silencing effect on other genes then we revert back to the original form of the crop for further moidification.

6. *What is gene silencing?*

Inactivation of the expression of the transferred gene in the new genetic background due to several genetic reasons is called gene silencing. Sometimes the introduced gene may silence the already present genes. In this case the transgenics are of no use. But, if we want silencing of some genes in the crop then transgenes can be used.

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7. Are farmers satisfied of the Bt-cotton's performance?

where true Bt-cotton seeds are used for cultivation the results are satisfactory . Problems regarding the Bt-cotton mostly arise due to the spurious seeds sold to farmers. In some pockets of India , there are reports saying increase in the sales of the Bt-cotton seed every year.

8. *How we can check the purity of the Bt-cotton seed sold to us?*

Agriculture officers in the country are supplied with a kit. The kit has few chemicals by which we can test the purity. The seeds are crushed and put in the solution if the solution turns blue then the seeds are pure otherwise not. The problem here is how many seeds are to be crushed in a seed lot.

9. *What are the transgenic crops in India?*

Cotton is the only transgenic crop grown commercially in India With in 2-3 years we will have soyabean and other fruit crops The delay in commercializing the transgenics in India is because of the biosafety and the results shown by Bt-cotton in some areas are of more concern.

8. ABSTRACT

Gene technologies offer new set of tools to be applied to the ongoing task of enhancing productivity and profitability by improving the use of scarce resources including water and nutrients, tolerance to abiotic stresses, including drought and frost, tolerance to biotic stresses, including pests and diseases, quality characteristics, including a diversified product range, and food safety by reducing dependence on pesticides and herbicides Genetic engineering is the basic tool set of biotechnology. Transgene is the genetically engineered gene added to a species and transgenic is an organism containing a trangene introduced by biotechnological methods The importance of transgenics is the transfer of genes without any barriers *i.e* kingdom barrier or species barrier.

Genes are introduced into the organisms mainly by Gene Gun method (also known as microprojectile bombardment or biolistics) or *Agrobacterium* method. Transformation via *Agrobacterium* has been successfully practiced in dicots (broad leaf plants), but recently it has been effective in monocots too In general, the *Agrobacterium* method is considered preferable to the gene gun, because of the greater frequency of single-site insertions of the foreign DNA, making it easier to monitor

Transgenics impart herbicide tolerance, insect resistance, disease resistance, and ability to grow plants under harsh environments and improves nutritional qualities of food. Bt cotton is a classic example of transgenic developed for insect resistance Similarly there are other examples of insect resistant transgenics *viz* , Bt maize, Bt soyabean *etc*.

Recently, resistance genes from different sources have been isolated and introduced into susceptible crop cultivars through genetic engineering for making them resistant against viral, bacterial, and fungal diseases. The notable examples being Papaya resistant to papaya ring spot virus, tomato resistant to tomato mosaic virus *etc*.

Transgenics were developed by identifying candidate genes from diverse sources against abiotic stresses. The tool, genetic engineering is also used in improving the nutritional qualities. The classic example is Golden Rice.

New techniques for producing transgenic plants will improve the efficiency of the process and will help resolve some of the environmental and health concerns. The expected changes are: more efficient transformation, that is, a higher percentage of plant cells will successfully incorporate the transgene. Better marker genes to replace the use of antibiotic resistance genes. Better control of gene expression through more specific promoters, so that the inserted gene will be active only when and where it is needed.

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ORIGIN AND EVOLUTION OF CROP PLANTS

By

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SEMINAR REPORT

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DECLARATION

This is to declare that myself **M. Latha**, Admn. No. 2004-21-08 has prepared this seminar report on going through various references and has not copied from previous student's report in
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ABSTRACT

Evolution means an opening out, an unfolding, a realization potential as in the opening of a flower or in the germination of seeds (Harlan, 1975). Crop plant evolution is a divergent evolution, which increases genetic diversity and leads to change in allelic frequencies in a population. It is a gradual process. Cultural man was in this earth 2,00,000 million years ago and during this 99% period he was a hunter and only during the last 10,000 years he shifted to food production. This shifting from hunting to cultivation is referred to as Neolithic revolution or Agricultural revolution. There are many views regarding the origin of agriculture.

Crop evolution was accelerated when man started domesticating crop plants after the Neolithic revolution. About 100-200 of the thousands of plant species were domesticated and only 15 among them supply human diet. A Russian geneticist, N.I. Vavilov, identified regions where crop species and their wild relatives exist with great diversity. He proposed eight primary centres of origin of crop plants. Among them Hindustan centre and South American centres were divided into two and three sub centres respectively and totally 11 centres were recognized. He also proposed secondary centres of origin. Later, Harlan identified three centres and non-centres. The first step in the development of cultivated plants was domestication. The prehistoric man selected for characteristics that made the plant more suited to his needs. The changes brought out by domestication were enormous (Singh, 2000). Cultivation of vegetatively propagated plants was primitive to seed propagated plants (Donald and Hamblin, 1983).

Both natural and artificial selection are responsible for evolution of crop plants. There are three ways in which genetic variability has arisen in various crop plants (Chrispeeds and Sadava, 1994).

1) Interspecific hybridization, 2) Mendelian variation and 3) Polyploidy.

Polyploidy have played a major role in the evolution of more than 50% of our crop plants. The variations in secondary centres are due to long history of continuous cultivation, ecological diversity and human diversity. The different evolutionary patterns include endemic, semiendemic, noncentric, monocentric and oligocentric. Crop origin can be diffuse in both space and time. It may change radically as it is dispersed from its centre of origin and they differ morphologically from the wild progenitors from which they evolved. There are also regions called micro centres, in primary centres of origin where the variability is maximum.

Another feature of domestication is the land race population that is adapted to traditional agriculture and are highly variable. Their compositions are deliberately manipulated. Landraces forms a good source of genes for modern plant breeding.

Analysis of variation pattern of crops is essential in order to understand its evolution so that it can be effectively used. The old centres of diversity are disappearing and land races are being replaced by the modern cultivar, which reduces the sources of variation for plant breeders. The time will probably come when essentially all the variation available for plant breeding will come from two sources 1. Collection made in gene banks 2. Cultivars in current cultivation. Changes brought out by Genetic engineering in crop plants are also comparable with classical plant breeding.

Origin and Evolution of Crop Plants

Introduction:

In recent years, scientists have made a concerted effort to increase food production around the world by breeding genetically improved crop plants. This effort has led to a renewed interest in the origins of agriculture and crop domestication; that is, in how crop plants evolved from their wild ancestors. Plant geneticists are now searching the world to collect seeds from both the wild relatives and the many cultivated races of some of the important modern crop plants. They hope to incorporate some of the desirable genetic characteristics of these wild relatives into modern crop plants. A better understanding of plant domestication and of the relationship between the crop plants and their wild relatives may help plant breeders produce superior varieties of crop plants.

Another reason for the renewed interest in the origins of agriculture and in various primitive agricultural systems comes from the recognition that agriculture is part of the natural environment and that agricultural systems operate under the constraints of nature. Thus we need to know more about the natural systems in which the crop plants were domesticated, and about the way in which the agricultural systems gradually replaced the natural ones in many parts of the world

"Evolution means an opening out, an unfolding, and a realization of potential as in the opening of a flower or the germination of seed" (Harlan, 1976) Crop plant evolution is a divergent evolution which increases genetic diversity and lead to change in allelic frequencies in population It implies a gradual process rather than sudden catalytic events with each living things being derived genetically from preceding living things. Evolution as a process means change with time and the changes may be relatively slow or rapid, the time relatively long or short. Thus the difference brought out by evolution over time may be small or great. As we shall see, some cultivated plants differ very little from the wild forms while some others differ enormously from progenitors. The same is with

the evolution of agricultural economies and the sociological changes that have occurred in the agricultural and industrial societies from the hunting gathering systems. Crop evolution consists of three phases: the natural evolution of a species to the 'roto-crop' stage, domestication, and further evolution with the domesticated species (Donald and Hamblin, 1983).

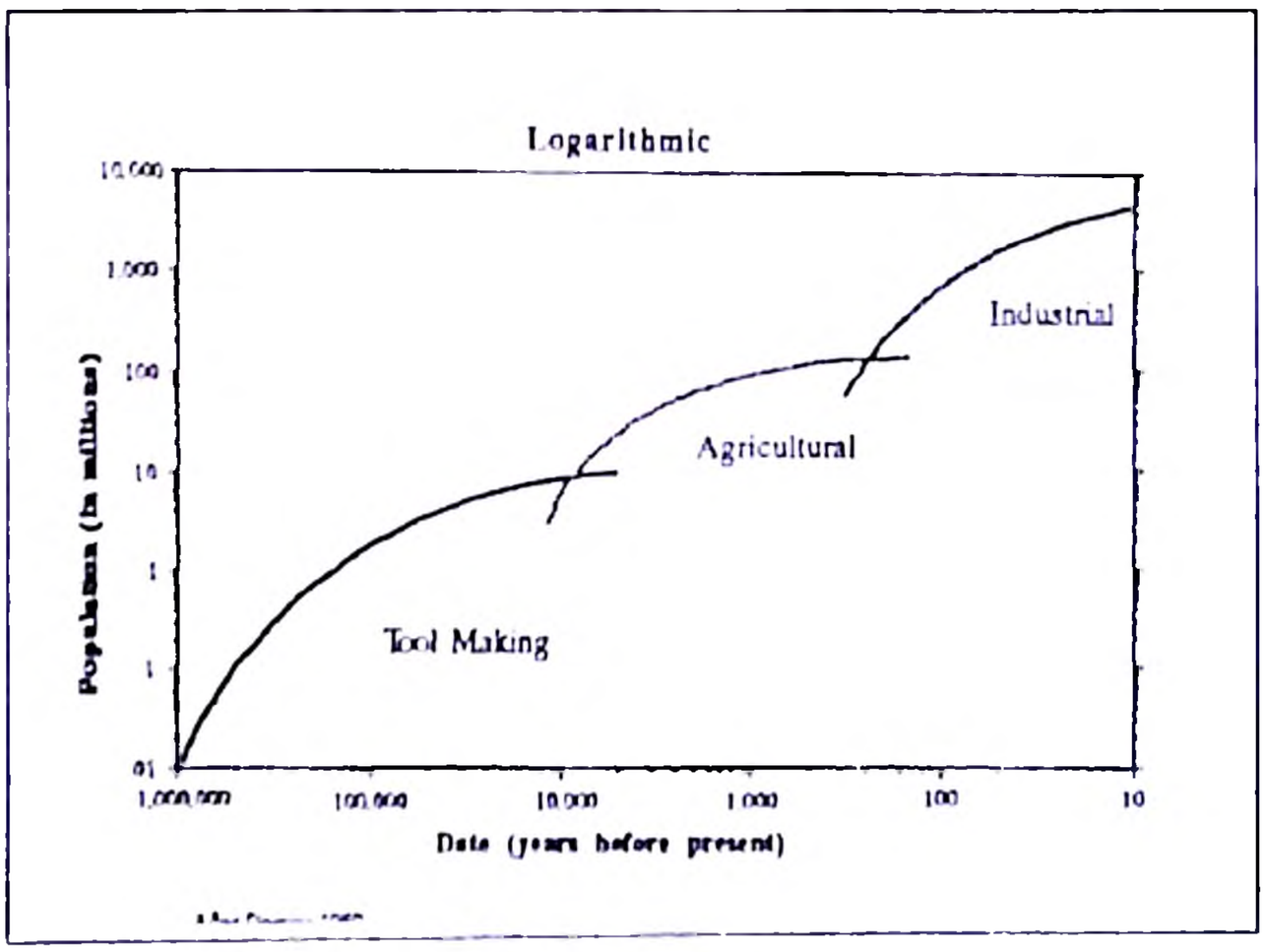
Hunters and Gatherers:

Human beings have been a distinct kind of animals for at least two million years and perhaps much longer. Little is known about the diet of our earlier ancestors, but it is generally believed that our more recent ancestors, were hunters and gathers of food, and had a varied diet that included both plants and animals. Agriculture did not become the usual method of procuring food until 10, 000 years ago, or about 1% of the time humans have existed. Of the estimated population at that time, 90% lived as hunters and 6% as agriculturalist and remaining as industrialists. Traditionally, agricultural people have looked down on hunting people who are described as savage, backward, primitive, ignorant, indolent, lazy, wild and lacking intelligence. Gatherers clear or alter vegetation, plant and sow materials, conduct ceremonies, pray for rain. They do spin fibers, weave clothes, make basket, spears and arrows etc. They paint, play musical instruments. They know poisonous and non-poisonous plants, time of harvest, drug and medicinal plants etc. They understand life cycle of plants. They make by products of seeds they harvest. They harvest plant food resources in greatest abundance with least efforts. The diets of gatherers are better than cultivators and they are less susceptible to disease.

A common misconception is that all hunters-gatherers had primarily a nomadic way of life. Obviously, this may have been true of those who followed large game herds. Their material culture and social organization would have been adapted to the exploitation of their principal food source. The use of wild plants for food may have been ancillary in these highly specialized hunting economies.

Views on origin of Agriculture:

About 10, 000 years ago, a remarkable change occurred in the way people procured food. They domesticated plants and animals, and started practicing agriculture. The gatherer became a farmer. This transition has been called the agricultural revolution or the Neolithic revolution, for it heralded a fundamental change in human beings material wealth, social organization and cultural achievements. The hunters – gatherer was very much a part of nature, competing with other organism for food supply. The farmers started to modify the ecosystem to suit human needs. They interfered with the normal flow of energy in the biosphere and diverted it to products they could eat. They decided which plant grew where, protected useful plants from diseases and even altered the course of plant evolution, by helping to evolve new species that would not have survived without human care



There are several views proposed for the origin of agriculture.

Agriculture as a Divine gift:

In the classical mythologies of all civilization, agriculture is fundamentally of divine origin. It arrived in different ways from different deities and various circumstances. In the Mediterranean region, the source was a goddess; Isis in Egypt, Demeter in Greece and

Ceres in Rome. In China it was the ox headed god Shen-nung; in Mexico, Quetzalcoatl disguised as a plumed serpent or other animal. The appearance of agriculture in mythology was almost always associated with other features of civilization.

Agriculture as Discovery:

The most extensively developed model for agricultural origin is that cultivation was an invention or discovery. Darwin and others was convinced that nomadic people could not develop agriculture. He developed 'Eureka' model of plant domestication. No motive is required only the brilliant revelation that seeds can be sown to produce plants when and where desired. There are several ideas in his view:

1. Man must be sedentary before he can cultivate plants
2. Useful plants are more likely to be discovered in manured reuse heaps
3. Useful plants are likely to be first planted in dump heaps.
4. A wise old savage is required to start the process

Carl O Sauer (1952), a geographer developed 'Agricultural Origins and Dispersals' where he listed six pre supposition as a basis for his search

1. Agriculture did not originate from chronic shortage of food
2. The hearth of domestication is to be sought in areas of marked diversity of plants and animals. This implies well diversified terrain and variety of climate.
3. Primitive cultivars could not establish themselves in large river valleys
4. Agriculture began in wooden lands. Primitive cultivars could readily open spaces for planting by deadening trees
5. The invention of agriculture has previously acquired special skills
6. Founders of agriculture were sedentary folk

With this, he proposed, South East Asia as the oldest hearth of agriculture. From there, system spread northward into China and westward across India and the near east into Africa and the Mediterranean region and finally into northern and western Europe.

In America, he located the original hearth in the northwestern part of South America. From there agriculture spread northward into Mexico, then to eastern North America, southward along to Andean Chain, eastward to the Atlantic coast of Brazil and to the Caribbean island chain.

Edgard Anderson (1954) added some genetic threads to this idea. He saw weeds as potential domesticates. He thought that an increase in hybridization with disturbed habitats could result in increased variation and new genetic combinations from which useful selection could be made. He also felt that vegetative propagation predominated at the beginning.

Agriculture as an extension of Gathering:

Gatherers all they need to know to develop agriculture, so they thought why not farming?, if you are well equipped with all the materials and information to do so. But this alone is not a reason for the gatherers to do cultivation because olden data shows that increasing food supply through cultivation means increase in work load. Another reason the agriculturist and industrialist have less leisure time to elaborate culture than the hunters or gatherers. So, what might have motivated man to domesticate plants? Binford . (1966) and Flannery (1968) model proposed that gatherers are sophisticated, applied botanists who know their material and how to exploit it. They are prepared to cultivate when they think it is worth than the effort to gather. It was also proposed that long before there was a food resource crisis among the fisher folk, groups would move out and migrate into less well endowed regions and ecological zones. The fisher folk population remained stable but the migrants precipitated crisis along the interface between the sedentary peoples and the nomadic hunter – gatherers. It was in response to this crisis that people were willing to go to the effort of cultivation. Changes in climate and slowly changing ecological relationship between human beings and the plants and animals they used for food, also forced them to become farmers.

Geography of plant domestication:

After agricultural revolution, farmers started domesticating plants and animals. They started to modify the ecosystem to suit human needs . They interfered with normal flow of energy in the biosphere and diverted it to products they could eat. They decided which plant grew where, protected useful plants from diseases and even altered the course of plant evolution, by helping to evolve new species that would not have survived without human care. Finding out where, when, why and how this transition occurred are the combined efforts of plant biologist, geographers, archaeologists and many others. People have domesticated about 100-200 of thousands of plant species and of these more than 15 now supply most of the human diet (Chirspeeds and Sadava, 1994).

Cereals – Rice, wheat, maize, sorghum and barley

Roots and stem – Sugar beet, sugarcanes, potatoes, yam, cassava

Legumes – Beans, soybean, peanuts

Fruits – Coconut, bananas

N.I. Vavilov – a Russian geneticist and a plant geographer traveled and collected plants from all over the world, identified regions where crop species and their wild relatives live with their great genetic diversity. He proposed eight primary centres of origin of crop plants (Vavilov, 1926). Later crops moved to other areas due to human activities. These areas generally lack the richness in variation found in the primary centres of origin. But in some areas, certain crop species show considerable diversity of forms although they did not originate there. Such areas are known as secondary centres of origin of that species

Now the concept that centres of diversity represent the centres of origin has been questioned. Plants of a species growing in different environments are likely to be different in diverse. Thus it is likely that a species may show a greater variation in a region with varied climate and other ecological conditions. Further the centres of diversity of many species have shifted with time. This shift in diversity was

brought about by a shift in the area of greatest cultivation and due to introduction of a species into an area with a greater ecological diversity. These processes have given rise to the secondary centres of diversity. Thus several species have two or more centres of diversity and the centres of origin may be appropriately called as centres of diversity. So, the centres of diversity may not be the centres of origin, but they are the areas of maximum diversity of the species.

The eight primary centres of origin of crop plants including the sub centres are given below.

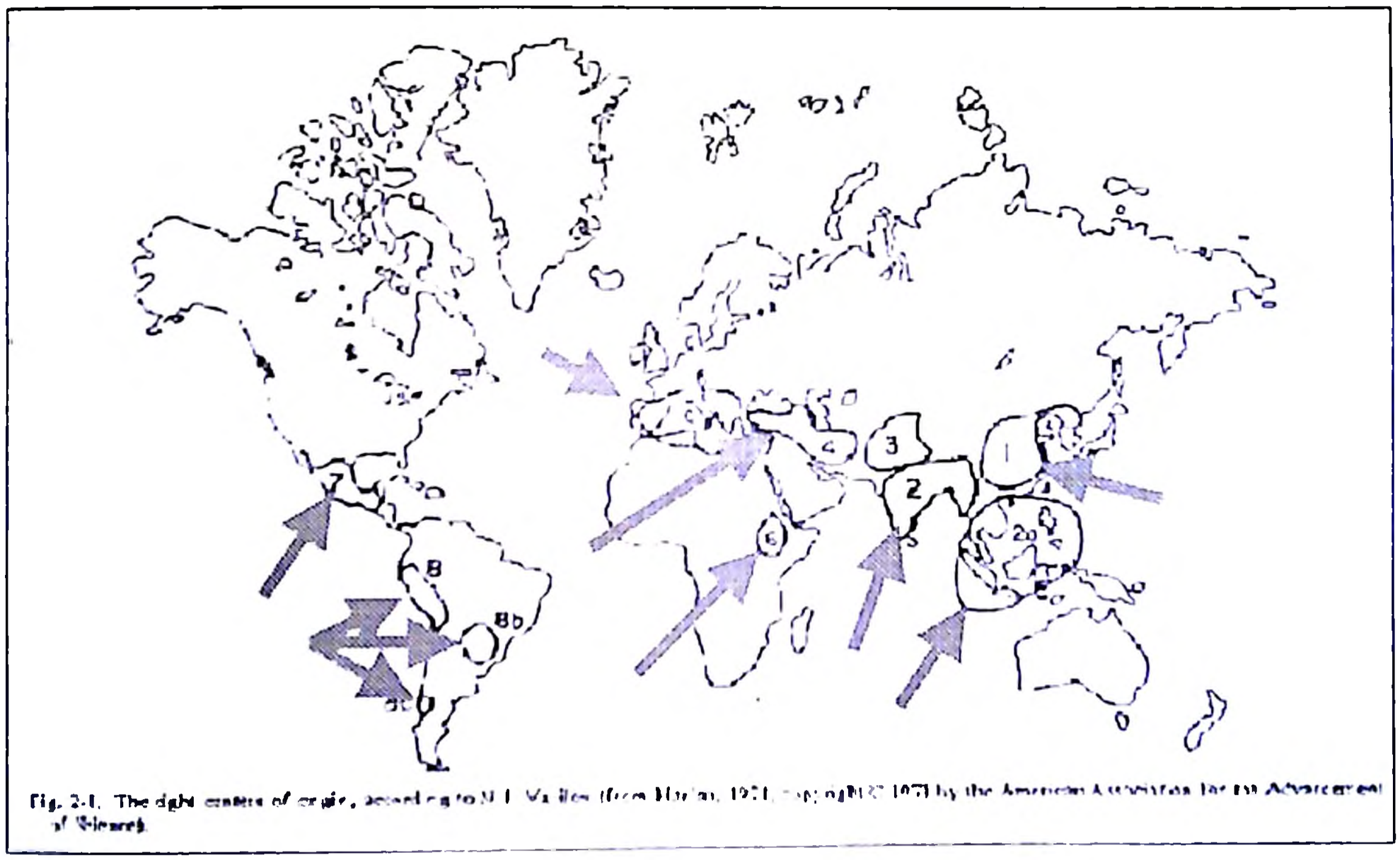


Fig. 2-1. The eight centers of origin, according to N. I. Vavilov (from Stevens, 1951, [illegible] 1951) by the American Association for the Advancement of Science).

1. Chinese centre
2. Hindustan centre
   - 2a. Indo-Burma centre
   - 2b. Siam Malayan centre
3. Central Asiatic centre
4. Asia minor

5. Mediterranean centre
6. Abyssinian centre
7. Central American centre
8. South American
   8a. Brazilian-Paraguay centre
   8b. Peru centre
   8c. Chile centre

Subsequently, a U.S. geographer J. Harlan challenged Vavilov's hypothesis because many cultivated plants did not fit Vavilov's pattern and appeared to have been domesticated over abroad geographical range over along period of time. When actually analysed by crop to crop, it soon becomes apparent that many of them did not originate in Vavilovian centres Some crops do not even have centres of diversity. Activities of plant domestication went on almost everywhere south of the Sahara and north of the equator from the Atlantic to the Indian Ocean Such a vast region could hardly be called as centre, so he called as non-centre of crop origin Thus three major centres and non centres were delineated as given below (Harlan, 1971)

1. Northern China centre (B1) Rice, soybean
2. Southeast Asian non centre (B2) Banana, coconut, sugarcane
3. Near east centre (A1) Barley, wheat
4. African non centre (A2) Sorghum, yam
5. Mesoamerica centre (C1) Bean, corn
6. South America non centre (C2) Cassava, peanut, potato, sugarbeet

Fig. 2–2. Centers and nonconters of agricultural origin: (A1), Near Eastern center, (A2), African nonconter, (B1), North Chinese center, (B2), Southeast Asian and South Pacific nonconter, (C1) Mesoamerican center, and (C2) South American nonconter (from Harlan, 1971; copyright © 1971 by the American Association for the Advancement of Science)

Later J Harlan gave a term 'microcentre' for an area in the primary centre where there is maximum diversity of crop plants The crop evolution appears to proceed at a more rapid rate in these microcenters These are important for plant collection and for studying the evolution of plants

Role of weeds:

Weeds are undesirable or troublesome plants and growing where they are not wanted and they are primary colonisers of disturbed ground Weeds are therefore associated with people The ancestors of modern crop plants may have been weeds that grew naturally in the disturbed and fertile soil surrounding the semi permanent settlements of humans People may have collected the seeds of the weeds in the same manner that they gathered the seeds of other plants At first, they may have gathered them over great distance, but

the abundance of these plants near human settlement may have increased, as seeds were scattered on the grounds. Thus gathering gave way to harvesting. Finally, early agriculturist may have sown seeds in fields or gardens in which the soil had been prepared.

Domestication is an accelerated form of evolution:

Domestication is the process of bringing wild species under human management. The present day cultivated plants have been derived from wild weedy species. Therefore, the first step in the development of cultivated plants was their domestication. Most of the crops were domesticated by the pre historic man. Knowingly or unknowingly he must have selected for the characteristics that made the plants more suited to his needs Under domestication, the crop species have changed considerably as compared to their wild species The changes brought about by selection either by man or nature great that the crops are classified into distinct species. As a result, in many cases the parental wild species are not known The domesticated species were selected for the characters which are different from the natural selection and hence two groups of plants develop in two different directions Domestication of wild species is still being done and is likely to continue for a long time in the future This is because the human needs are likely to change with time Consequently, the wild species of little importance today may assume great significance tomorrow Several members of Euphorbiaceae produce latex. The latex is used in extraction of petroleum products including petrol and diesel Hevea sp., milkweed, *Euphorbia lathyrus* are called as living oil fields Jojoba (*Simmondasia* sp.) which contains oil is comparable to sperm whale oil and is highly suitable as an industrial lubricant.

Selection under domestication:

It is of two types as proposed by Singh, 2000

1. Natural selection 2. Artificial selection

Natural selection: The selection that occurs due to natural forces like climate, soil, biological factors and other factors of environment is called natural selection. It occurs in

natural population *i.e.*, wild forms and wild species and determines the course of their evolution. Generally, all the genotypes of the population reproduce, plants become more adapted to the prevailing environment and population retains considerable genetic variability.

Artificial selection: In contrast, artificial selection is carried out by man. This type of selection is confined to domesticated species. It allows only the selected plants to reproduce, ordinarily makes plants more useful to man and generally leads to marked decline in genetic variability in the selected progenies/population. Usually plants become less adapted to the natural environment and they have to be grown under carefully managed conditions. Our present day products are the products of continued artificial selection. The precise sequence of events during the evolution of crop plants under domestication is not known. Presumably in the initial stages, considerable genetic variability existed in each domesticated species. This variability was acted upon both artificial and natural selection. It may be expected that man always tried to pick out the plant types, which better suited to his needs. He would obviously have selected for larger fruits and seeds. Our planned and systematic evaluation started only in the middle of the nineteenth century. Before this period, selection efforts were obviously unfocussed and primitive. But judging from the results i.e., the differentiation of crops from their wild prototypes, the then completely unscientific man was not a bad plant breeder at all. The domesticated species have undergone several important changes as a consequence of these efforts.

Changes in plant species under domestication:

Almost all the characters of plant species have been affected under domestication. The characters that show more distinct changes are those that have been objects of selection and still plant breeding objectives in many cultivated species. Some of the important changes occurred are:

- **Morphology and Physiology**
  - Non-shattering habit
  - Reduced seed dormancy
  - Reduced plant size, determinate growth habit
  - Shorter life cycles
  - Less branching, fewer flowers
  - Altered photoperiodic or vernalization requirements
  - Reductions in defense mechanisms and defense compounds
  - Changes in flower, seed, and fruit color
  - Reduction in plant height as in rice, wheat
  - Increase in plant height as in sugarcane, jute
  - Decrease in toxins
  - Increase in size of fruits and grains
  - Preference for polyploidy as in potato, wheat, tobacco
  - Multiple uses
  - Variability within a variety has drastically decreased under domestication

Patterns of evolution in crop plants:

It is apparent that selection by nature and man has been responsible for evolution of crop plants. However selection is effective in altering a species only when genetic variability exists in the population of that species. Donald and Hamblin, 1983 were in view that the cultivation of vegetatively propagated plants was primitive to seed propagated plants. There are three major ways in which genetic variability has arisen in various crop species viz., 1. Interspecific hybridization 2. Mendelian variation generated by gene mutation 3. polyploidy (Singh, 2000)

1. Interspecific hybridization:

Interspecific hybridization refers to crossing of two different species of plants. The F1 may be vigorous but F2 segregation result in vast range of genotypes. This is because the

parents differ from each other with large number of genes. Most of them are weak, undesirable and sterile. There are only few evidences for involvement of interspecific hybridization in evolution of crop plants.

Sometimes introgressive hybridization would occur, where in the interspecific hybrids may have repeatedly crossed with on of the parental species and as a result all the genotype of the parent to which the hybrids have been repeatedly backcrossed would have recovered along with one or two genes from the donor parent. Eg. Modern maize is the introgressive hybridization between primitive maize and *Tripsaccum*. Interspecific hybridization has led to the development of many species of strawberry. The F1s between *Fragaria virginiana* and *F. chilonesis* was back crossed to the two parent species to produce many varieties of commercial value. Other examples incude pears, plum, cherries, grapes and ornamentals like *Iris* sp., *Rosa* sp., *Lilium* sp., etc. where vegetative propagation is common. Here one has to the gene pool concept proposed by Harlan and de Wet (1971)

Primary gene pool (GP-1) – This correspond s to the traditional concept of the biological species. Among forms of this gene pool, crossing is easy, hybrids are generally fertile with good chromosome pairing, gene segregation is approximately normal and gene transfer is generally simple. The biological species always includes spontaneous races as we as cultivated races

Secondary gene pool (GP-2) – This includes all biological species that will cross with the crop and approximates an experimentally defined cenospecies. Gene transfer is possible, but one must struggle with those barriers that separate biological species. Hybrids tend to be sterile, chromosomes weakly pair, some hybrids may be weak and difficult to bring to maturity and recovery of desired types in advanced generations may be difficult.

Tertiary gene pool (GP-3) – At this level, crosses can be with the crop, but the hybrids tend to be anomalous, lethal or completely sterile. Gene transfer is possible only with advanced techniques

Now, GP 4, the gene pool of all the organism that cannot be crossed with the plants of GP-1 can only be introduced via technique of genetic engineering.

2. Mendelian variation or Mutation:

Many crops have evolved through variation generated by gene mutations and by hybridization between different genotypes within that same species followed by recombination. Ultimately, all the variability in any species originates from gene mutations. Most of the gene mutation are harmful and are eventually eliminated. But some mutations are beneficial and are retained in the population. The mutation may be grouped into two categories. 1. macro mutation 2. micro mutation. A macro mutation produces a large and distinct morphological effect and often affects several characters of the plants. A single macro mutation is believed to have led to the differentiation of the modern maize plant from the grassy pod corn. This mutation has affected the position of male and female inflorescence, the habit of the pant and several other characters. Similarly, cabbage, cauliflower, broccoli and Brussels sprout have originated from a common wild species and they differ from each other in few major genes.

The greater part of variation however has resulted from mutations with small and less drastic effects *i.e.*, micro mutations. Since micro mutations have only small effects they tend to be accumulated in a population. Natural selection would accumulate and select for more favourable gene combinations. Man would have selected from the populations desirable plant types leading to the differentiation of domesticated species from the wild ones. Several important crops have evolved through mendelian variation *e.g.*, barley, rice bean, peas, tomatoes, linseed, jowar, bajra etc.

3. Polyploidy

Polyploidy have played a major role in the evolution of modern crop plants, plant strains, which early farmers selected for their vigorous growth or higher yield, often turned out to be polyploids. In some cases an increase in the number of chromosome set is correlated

with increase in size of the leaves, stems, fruits and flowers. This is especially true for ornamentals such as petunias where series of both wild and cultivated varieties show both additional sets of chromosome and large flowers. Autopolyploidy leads to increased vigour, larger flowers and fruits as in triploid apple, water melon, sugar beet. Potato is an autotetraploid. Other autopolyploid includes oat, alfalfa. Autoplolyploid has played a limited role in evolution. Polyploids can arise from chromosome complements of two different species called as allopolyploid. Plum tree is a diploid with 2n=24 of which 8 of these chromosomes derived from cherry plum and 16 from black thorn. Thus plum, yield large fruit and higher yield. Tobacco is other example derived from a cross followed by doubling of chromosomes between *Nicotiana sylvestris* and *N. tomentosa*. The other example is bread wheat which is hexaploid derived from chromosomes of three different species as depicted below:

Triticum monococcum X unknown
(AA) (BB)
(2n=14) (2n=14)

↓

AB
(2n=14)

‖

↓ (chromosome doubling)

T. turgidum X *T. tauschii*
(AABB) (DD)
(2n=28) (2n=14)

↓

ABD
2n=21

‖

↓ (Chromosome doubling)

T. aestivum
(AABBDD)
(2n=42)

The two wild grasses that contributed the A and D chromosomes to this evolutionary scheme have been identified as goat grass identified as goat grass endemic to turkey and region of the Fertile Crescent, where wheat was domesticated.

Biological Species Concept: Species is a population whose members have the potential to interbreed and produce fertile offspring. Species are reproductively isolated from each other.

Speciation in Angiosperms:

Allopatric:

-a parent species is separated into two or more smaller groups by a geographical barrier. Differences accumulate in each group until the point that even if the barrier was removed, interbreeding between the groups would be unsuccessful

Sympatric:

-intrinsic factors, such as chromosomal changes result in a population of the same species becoming reproductively isolated from the parent species. Crop plants evolved by sympatric mode of speciation

Mechanisms of Reproductive isolation

Prezygotic barriers:

Temporal isolation: Mating occurs at different times

Habitat isolation: Mating occurs in different places

Behavioral isolation: Mates recognize species specific sexual signals

Structural isolation: Mating is physically impossible

Gametic isolation: gametes fail to unite

Postzygotic barriers:

Hybrid inviability: hybrids never reach sexual maturity

Hybrid sterility: hybrids can't produce functional gametes

Hybrid breakdown: offspring of hybrids are inviable

Kinds of patterns of variation:

Origin of a crop could be identified by the simple procedure of analyzing variation patterns and plotting regions where diversity was concentrated. Centres of diversity are not same as centres of origin, yet many crops do exhibit centres of diversity. The variations in secondary centres are due to the following reasons proposed by Vavilov, 1949:

1. long history of continuous cultivation
2. ecological diversity where many habitats accommodate many races
3. human diversity- different tribes are attracted to different races of crop
4. introgression with wild or weedy relatives or between different races of crop

There are many other causes but the reasons for secondary centres are human, environmental and internal biological dynamics of hybridization, segregation and selection. Crop by crop analysis shows that many crops did not originate in Vavilov's centres of origin, some do not have centres of diversity, several can be traced to very limited and specific origins and others would have originated all over the geographical range of species. If the crop have not spread to any other area, the centres of diversity an origin will be same. Different crops have different evolutionary patterns, the main patterns can be classified as,

a. **Endemic**. Crops that originated in a limited area and did not spread appreciably. Eg. *Brachiaria deflexa* in Guinea, *Eusele verticosa* in Ethiopia, *Digitaria iburcia* in West Africa

b. **Semi endemic**: Crops that originated in definable centre and with limited dispersal Eg. *Oryza glaberimma*

c. **Mono centric**. Crops with definable centre of origin and wide dispersal without secondary centres of diversity. Eg. Arabica coffee and Hevea rubber. Crops of this group are plantation or industrial crops.

d. **Oligocentric**: Crops with a definable centre of origin, wide dispersal and one or more secondary centres of diversity. Eg. Near east complex of barley, emmer wheat, flax, pea, lentil, oats, chickpea, *Brassica* sp., all have secondary centres in Ethiopia and some also have centres in India and China.

e. **Non centric**: Crops whose pattern of variation suggest domestication over a wide area. Centres are either not apparent or anomalous. Eg. Sorghum, common bean, banana and *Brassica campestris*

Diffuse origins:

Crop origin can be diffuse in both space and time. Even if a crop enters the domestic fold in a limited area, it may change radically as it is dispersed from its centre of origination. As it spreads, it may receive infusions of germplasm from its wild relatives and people in different regions may apply very different selection pressures. The most highly derived end products may be far removed geographically and morphologically from the wild progenitors fro which they evolved

Maize was domesticated first in Southern Mexico and spread slowly in all directions fro its centre of origin At the time of European contact, it was being cultivated from Southern Canada to Southern Argentina and Chile and throughout the Caribbean islands Each region has its own characteristics array of races Some larger areas had fewer races and smaller areas with larger variations These areas of diversity occurred in Southern Mexico, Guatemala, parts of Colombia and Peru is noted for its extreme diversity in Maize Many unique races have not found in centres of origin Similarly Barley was first domesticated in Near East It is being cultivated from above the Artic circle to Southern Argentina and Chile as well as in tropical latitudes The progenitor is wild two rowed *Hordeum spontaneum* and the earliest barley from archeological sitesare two rowed. Variations are not seen in centres of origin but seen in other geographical and ecological region with specific characters Barley of Ethiopian plateau is favorable for development of leaf disease, In Tibet it has naked seeds and some are hooded and peculiar characters are seen in barleys of Chinese and Japan. All these originated in the

respective area and they differ considerably from the primitive two rowed barley first cultivated by man in the near east. Wheat is another example where the hexaploid wheat has originated outside the nuclear area where einkorn and emmer wheat have originated.

Variation in a crop may be increased considerably if the crop is used for different purpose by different people.

Commom bean – used for green and dry beans

Garden pea – green and dry peas, edible pods

Mung bean – used as flour in India, sprouted in China

Jute – fibre in India, vegetable in Africa

Flax – fibre, edible seeds, industrial oil

Hemp – fibre, edible seeds, narcotics

Cereals – multiple uses in different areas

The variation pattern of crops are largely artifacts resulting from human activity; therefore, the larger the number of people who grow a crop and the greater their diversity, the more variable the crop is likely to be

Microcentres:

Within the centres of diversity, a very small region show enormous diversity and this region is called microcenter. Microcentres are relatively small regions, 100-500 km across in which may be packed an astonishing variation from one to several crops. The source of variability involves introgression between contrasting populations. Microcentres are scattered across the near east Turkish Thrace, Transcaucasia with parts of Turkey, parts of Iran and Afghanistan (Harlan, 1951)

Landrace populations:

With the advent of modern agriculture it seems necessary to describe landrace populations. It is only within the last century or less landraces have been replaced by uniform breeding cultivars or hybrids of controlled parentage. Traditionally, field crops consisted of landrace populations rather than cultivars of modern sense. Landraces are

still grown in areas where traditional agriculture is practiced. Land race populations are highly variable in appearance and are easily identifiable and have local names. A land race has particular properties or characteristics. Some are considered early maturing and some late. Each has reputation for adaptation to particular soil type. They may be classified according to their usage also.

Genetic variation within and races may be considerable they are offsprings of lines having under gone local selection for many generations. Landraces are adapted to conditions of traditional agriculture, they are adapted to low soil fertility, low plant population and low yield. On the other hand, genetic variability provides some built in insurance against hazards. Devasting diseases epidemics are unlikely because the population contains such an array of resistance genes and no single race of pathogen can built upto epidemic proportion. Some would be affected each year and not all of them. Land race can emerge from a wide range of planting depth, they can withstand many biotic and abiotic stresses. In traditional agriculture yield was never been necessary.

The composition of landraces is frequently deliberately manipulated by cultivators. For example in maize and sorghum a farmer will select an ear head or cob from the field just before harvest as seeds for next season sowing. Sometimes the heads are similar but sometimes remarkable array of ear heads are assembled. Farmers select a really variable range and the reason given is that a mixture of type is more nutritious than uniform strains. Whether the selection of land race is uniform or variable, all compounds are from adapted materials and the compounds are selected, reassorted, recombined and rearranged, but the local materials are constantly adjusted to local conditions. The great sources of variability of land races makes them good sources of genes for modern plant breeding.

Implications of evolution and patterns of variability in plant breeding:

Analysis of variation patterns of crops are essential in order to understand the germplasm that went into their evolution and to make efficient use of the available variability in plant breeding. Typical variation patterns include:

1. wild population that are often highly variable especially when they cover a considerable geographic range and / or ecological amplitude
2. land race populations which are balanced, integrated mixtures of genotypes adapted to region and to cultural practices in vogue.
3. weed population frequently evolved from genetic interaction between wild and cultivated races
4. microcenters in which enormous diversity is found in a restricted geographical area, usually due to genetic interaction between cultivated races and/or spontaneous races
5. secondary centres in which great variation has accumulated in certain special geographic regions, usually with considerable isolation from other regions for long periods of time.

Geographical patterns of variation help direct pant exploration and germplasm assembly for breeding programs. Collecting is more rewarding in centres of diversity and microcenters when they can be found. Not al the useful genes are present in centres of diversity. Cultivars being grown near the climatic or ecological limit, of a crop may have special attributes

The old centres of diversity are disappearing and some have already gone. New, modern cultivars are replacing the land races. Due to modernization of agriculture, the wild populations are being ploughed or grazed out in many parts of the world. Genetic erosion is far advanced in many regions. The sources of variation for plant breeders are reducing. But many steps are being taken up to conserve the same. The time will probably come when essentially all the variation available for plant breeding will come from two sources: 1. collections maintained in gene banks and their satellite working collections maintained by pant breeders. 2. cultivars in current production.

Role of genetic engineering in evolution:

Several claims have been made about genetic engineering (GE) in comparison with crop domestication and classical plant breeding, including the similarity of genetic changes between those taking place during domestication and by GE, the increased speed and accuracy of GE over classical plant breeding, and the higher level of knowledge about the actual genes being transferred by GE compared with classical breeding. In reviewing evidence pertaining to these claims, I suggest that (i) it is unlikely that changes introduced by GE will make crops weedier, although exceptions have been noted, (ii) changes brought about by GE currently often involve gain-of-function mutations, whereas changes selected during domestication generally involve loss-of-function mutations, (iii) adoption of GE cultivars has been much faster than any previous introduction and spread of agriculture that occurred earlier but has occurred at about the same rate as the spread of cultivars obtained by plant breeding, (iv) introduction of agriculture reduced the health of agriculturists compared with that of hunter–gatherers, suggesting that introduction of innovations do not automatically improve well being, (v) although GE is not a substitute for plant breeding, it can significantly contribute to plant breeding by generating additional genetic diversity, (vi) uncertainties associated with the site of insertion of transgenes in the genome and the expression of transgenes following insertion, makes GE less rapid and precise than originally claimed, and (vii) a potential advantage of GE over classical breeding is the knowledge of the actual gene(s) being inserted, although few cases of unwanted gene introductions through classical plant breeding have been documented. Further advances in GE will increase the precision of the technique, its relevance to consumers, and its environmental friendliness. What is most needed are even-handed, case-by-case assessments of the benefits and potential pitfalls of GE in comparison with other crop improvement techniques. Classical plant breeding may, in the end, also be regulated in the same way as GE (Gepts, 2002).

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Discussion:

1. *Most of the crops originated in South East Asia according to your presentation, but reports shows that most of the crops are from Near eastern centres?.*

Ans: The region of South east Asia was proposed by Carl O'Sauer based on some pre suppositions.

2. *What is the evolutionary process of Oryza malampuzhensis?*

Ans: It must have been derived from the introgression from weedy species.

3. *What is the secondary centres of origin?.*

Ans: These are the regions where a crop have spread from its primary centre of origin and more variability exist in the secondary centres of origin due to prolonged cultivation and crossing between other weedy and wild relatives existing in that region

4. *How are primary centres different from the secondary centres of origin?*

Ans Primary centres are enriched with dominant characters and wild relatives of a crop plants are seen only in primary centers of origin

5. How many species of rice are cultivated?

Ans Two are cultivated i e , *Oryza sativa* and *O. glaberrimma*

PHAGE DISPLAY:
BIOPANNING AND ANTIBODY LIBRARAY

SEMINAR REPORT
(PRESENTED On 26-11-2005)
(SUBMITTED IN PARTIAL FULFILLMENT OF THE COURSE NO.PBT.651)

By

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(2004-11-49)
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DECLARATION

I, Premjith gopinath (2004-11-49) hereby declare that the seminar entitled "Phage display: biopanning and antibody library" have been prepared by me, after going through various references cited at the end and has not been copied from any of my fellow students

Vellanikkara

29-11-2005

Premjith Gopinath

2004-11-49

CERTIFICATE

This is to certify that the seminar report titled "PHAGE DISPLAY: BIOPANNING AND ANTIBODY LIBRARY" has been solely prepared by Mr.Premjith Gopinath(2004-11-49), under my guidance, and has not been copied from any seniors, juniors or fellow students seminar reports.

Vellanikkara
Date: 22-11-2005

Dr. P.C. Rajendran
Major advisor
Associate professor
Centre for Plant Biotechnology and Molecular Biology

1. INTRODUCTION

Antibodies are well recognized as indispensable tools for recognizing and tracking target molecules. However, traditional methods for preparing antibodies are cumbersome and labor intensive. As a result, researchers are working to develop faster and easier ways to capitalize on the target-recognition qualities of antibodies. Molecular antibodies have been generated either by the hybridoma technology or more recently, from antibody libraries. The development of antibody libraries has been greatly influenced by the development of display technologies and vice versa. The physical connection of antibody phenotype (protein) and genotype (cDNA) effectively allows selection rather than screening of antibody libraries. The integration of antibody libraries and phage display technology approximately a decade ago was a key event in this respect. More recently, display technologies other than phage display have been applied to antibody libraries, including ribosome, yeast and bacterial display.

Typically, antibody libraries are selected for mAbs that bind with high affinity and specificity to a given target antigen. However, mAbs exhibit selectable phenotypes other than affinity and specificity. For example, mAbs that mediate endocytosis of phage by mammalian cells can be selected from antibody libraries, which has potential application in gene therapy. Enzymatic activity is another selectable phenotype of mAbs. Whereas the hybridoma technology is practically confined to rodents, antibody libraries allow the generation of mAbs from virtually any species whose immunoglobulin genes are known. It is conceivable that the ability to generate mAbs from a variety of species will be important for the identification of highly conserved human antigens or highly conserved epitopes of human antigens. The epitope repertoire of a given human antigen recognized by nonhuman antibodies is different for each species. As a result, epitopes that are not immunogenic in one species might be immunogenic in a different species. Highly epitopes often display functional binding sites. The generation of mAbs against functional binding sites, that is, the generation of mAbs that agonize or antagonize functional interactions, is relevant for therapeutic applications.

2. PHAGE DISPLAY

Phage display, first introduced by G. Smith (Smith, 1985) is a very effective way for producing large numbers up to 10^{10} of diverse peptides and proteins and isolating molecules that perform specific functions. This technique can also be used to study protein-ligand interactions, receptor and antibody-binding sites, and to improve or modify the affinity of proteins for their binding partners (Cesareni, G. 1992). Phage display involves the expression of proteins, including antibodies, or peptides on the surface of filamentous phage. DNA sequences of interest are inserted into a location in the genome of filamentous bacteriophage such that the encoded protein is expressed or "displayed" on the surface of filamentous phage as a fusion product to one of the phage coat proteins. This technology thus couples the displayed antibody's phenotype to its genotype, allowing the DNA that codes for the selected antibody to be retrieved easily for future use. Collection of these antibody-covered phage are called a library.

Phage libraries each typically contain a billion different antibodies, a number comparable to that in human immune systems. To select the phage with the desired antibody from a library, the phage are allowed to bind to the target molecule bind tightly, and the remaining (unbinding) phage are simply washed away. Phage display even permits researchers to select antibodies with different binding characteristics for a given target. The DNA contained within the desired phage then can be used to produce more of the selected antibody for use in research or medical diagnostics. Therefore, instead of having to genetically engineer proteins or peptide variants one-by-one and then express, purify, and analyze each variant, phage display libraries containing several billion variants can be constructed simultaneously specific phage particles bearing sequences with desired binding specificities from the nonbinding variants.

Antibody phage display

DNA encoding millions of variants of certain ligands (e.g. peptides, proteins or fragments thereof) is batch-cloned into the phage genome as a fusion to the gene encoding one of the phage coat proteins (pIII, pVI or pVIII) (fig. 1).

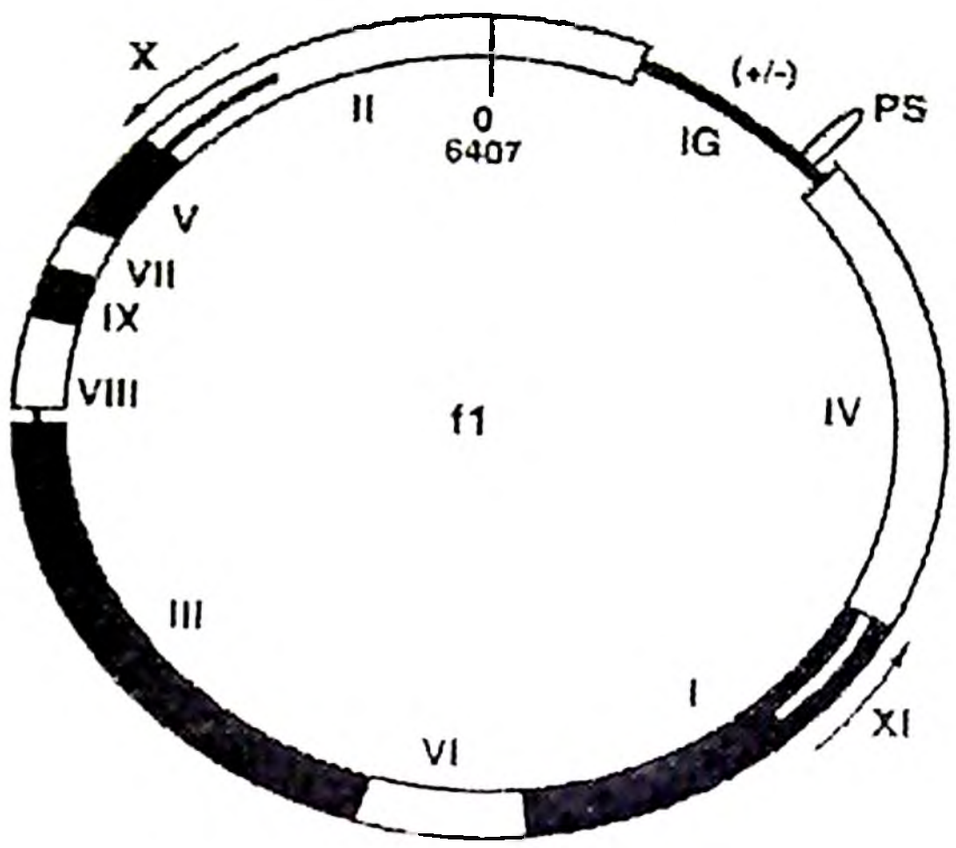

Figure.1

Upon expression, the coat protein fusion will be incorporated into new phage particles that are assembled in the bacterium. Expression of the fusion product and its subsequent incorporation into the mature phage coat results in the ligand being presented on the phage surface, while its genetic material resides within the phage particle (fig.2). This connection between ligand genotype and phenotype allows the enrichment of specific phage, e.g. using selection on immobilized target. Phage that displays a relevant ligand will be retained, while nonadherent phage will be washed away. Bound phage can be recovered from the surface, reinfected into bacteria and re-grown for further enrichment, and eventually for analysis of binding.

GENE TRANSFER PROCEDURE

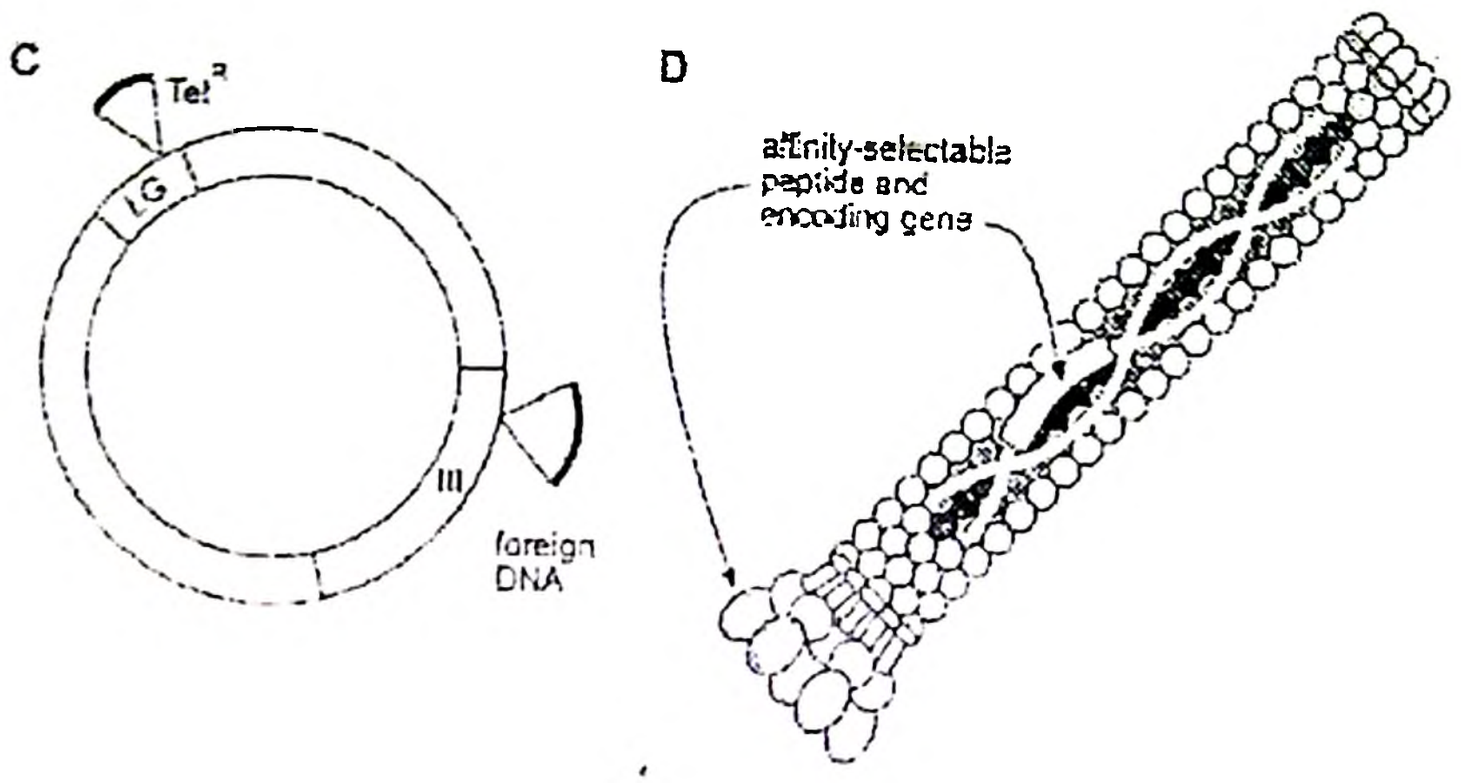

Figure.2

The success of ligand phage display hinges on the combination of this display and enrichment method, with the synthesis of large combinatorial repertoires on phage. The phage display cycle. DNA encoding for millions of variants of certain ligands (e.g. peptides, proteins or fragments thereof) is batch-cloned into the phage genome as part of one of the phage coat proteins (pIII, pVI or pVIII). Large libraries containing millions of different ligands can be obtained by force cloning in *E. coli*. From these repertoires, phage carrying specific binding ligands can be isolated by a series of recursive cycles of selection on antigen, each of which involves binding, washing, elution and amplification

3. FILAMENTOUS PHAGE BIOLOGY

The most popular phage that has been used for display is the filamentous bacteriophage. The non-lytic filamentous phage fd or M13 infect strains of *E. coli* containing the F conjugative plasmid. Phage particles attach to the tip of the F pilus that is encoded by genes on this plasmid, and the phage genome, a circular single-stranded DNA molecule, surrounded by a cylinder of coat proteins and are about 1 μm in length, 7 nm in diameter, and have a molecular mass of ≈1.6 x 10^7 Da(fig.3).

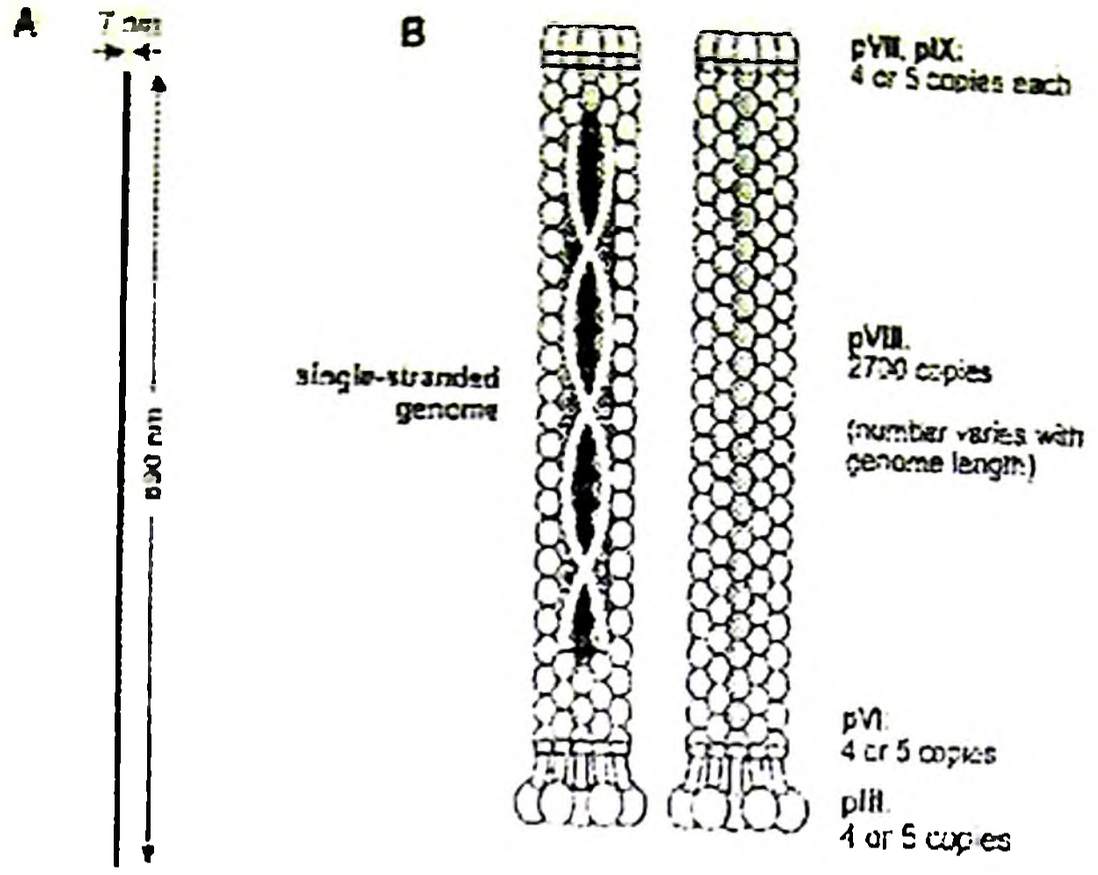

Figure.3

Most of the viral capsid consists of the major protein pVIII, of which there are ≈2700 copies per phage. At one end of the phage particle, there are five copies each of pIII and pVI that are involved in host cell binding and in the termination of assembly process. The other end contains five copies each of pVII and pIX. The genome is replicated involved both phage and host derived proteins, and packaged by the infected cell into a rod-shaped particle which is released into the media. All virion proteins will undergo transport to the cell periplasm prior to assembly and extrusion

Life cycle

Filamentous phage does not produce a lytic infection in *E. coli*, but rather induces a state in which the infected bacteria produce and secrete phage particles without undergoing lysis. Infection is initiated by the attachment of the phage g3p to the f pilus of a male *E. coli* (e.g., *E. coli* TG1). Only the circular phage ssDNA enters the bacterium where it is converted by the host DNA replication machinery into the double-stranded plasmid like replicative form (RF). The RF undergoes rolling circle replication to make ssDNA and also serves as a template for expression of the phage proteins g3p and g8p. Phage progeny are assembled by packaging of ssDNA into protein coats and extruded through the bacterial membrane into the medium. Recombinant antibodies, and folded proteins,

are typically expressed as g3p fusion proteins and are displayed at the tip of the M13 phage.

4. PHAGEMID CLONING VECTORS

With the M13 phage, there are two forms of the phage DNA: ssDNA templates that can be easily prepared from phage media and used for sequencing and dsDNA (plasmid-like RF) that can be isolated from the infected bacterial host and used for cloning of a target fragment. Phagemids, a more popular vector for display, are hybrids of phage and plasmid vectors. Phagemids are designed to contain the origins of replications for both the M13 phage and *E. coli* in addition to gene III, appropriate multiple cloning sites, and an antibiotic-resistance gene. However, they lack all other structural and nonstructural gene products required for generating a complete phage. Phagemids can be grown as plasmids or alternatively packaged as recombinant M13 phage with the aid of a helper phage that contains a slightly defective origin of replication (such as M13KO7 or VCSM13) and supplies, *in trans*, all the structural proteins required for generating a complete phage. This process if termed "phage rescue"(fig.4). The resulting phage particles may incorporate either pIII derived from the helper phage or the polypeptide-pIII fusion protein encoded by the phagemid. The ratios of polypeptide-pIII fusion protein will type pIII may range between 1:9 and 1:1000 depending on the type of phagemid, growth conditions, the nature of the polypeptide fused to pIII, and proteolytic cleavage of antibody-pIII fusions.

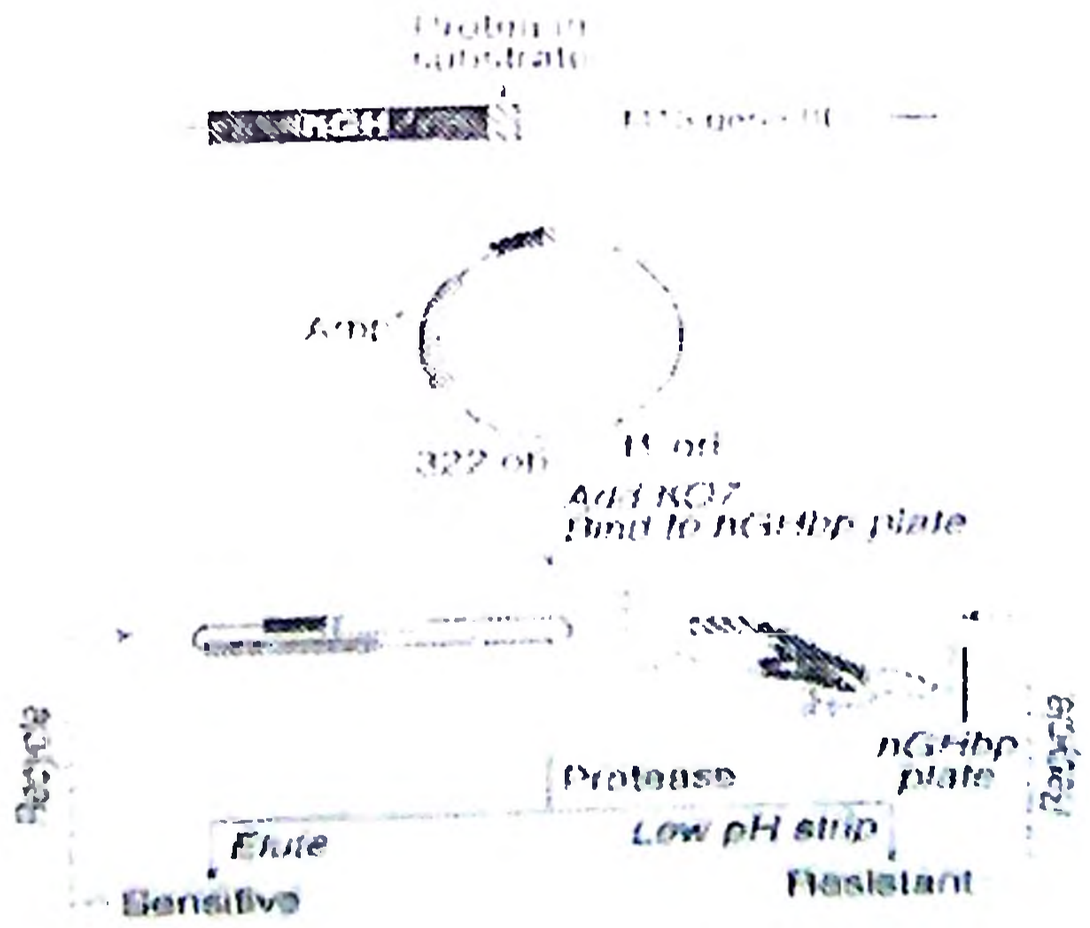

Figure.4

5. ANTIBODY STRUCTURE

All antibodies have a basic structure consisting of an identical pair of heavy chain polypeptides and a pair of identical light chain polypeptides held together by disulfide bridges and noncovalent bonds. Each of the heavy chains is encoded for by: variable (VH), diversity (D), joining (JH), and constant (CH) genetic segments: while each of the light chains is encoded for by VL, JL, and CL segments. The DNA and the amino acid sequences of the C region are relatively conserved within a given species while those of the V region are antigen-dependent. Pairing of the heavy chain V-D-J regions and light chain V-J regions creates an antigen-dependent. Pairing of the heavy chain V-D-J regions and light chain V-J regions creates an antigen-binding site (paratope), which recognizes a single antigenic determinant (epitope). Each V region consists of an alternating framework (FW), which is more conserved, and three hyper-variable or complementarity-determining regions (CDRs) with greatest sequence diversity. The CDRs, and to a lesser extent the FW regions, interact with the antigen to form the core of an antigen-binding site. The V segment encodes the first 2 CDRs, while the third CDR is the product of the junction of V-D-J for the heavy chain or V-J for the light chain

Antibody fragments

Antibody molecules contain discrete protein domains that can be separated by protease digestion or produced by recombinant technology. Two antigen-binding fragments designated Fab and Fv have been cloned and displayed on phage. The larger Fab (fragment antibody) consists of VH-CH and VL-CL segments linked by disulfide bonds. The smaller Fv (fragment variable) is composed of the VL and VH regions only. The recombinant version of the Fv is termed the single-chain variable fragment (scFv). The two variable regions in the scFv are artificially joined with a flexible peptide linker, usually a 15 aa linker is used with the sequence $(Gly_4Ser)_3$, and expressed as a single polypeptide chain. The linker allows the association of the VH and VL to form the antigen-binding site.

6. CONSTRUCTION OF SCFV PHAGE DISPLAY LIBRARIES

To construct an scFv library using phage display, genes of variable heavy (VH) and variable light (VL) chains of antibodies are prepared by reverse transcription of mRNA obtained from B-lymphocytes. Then mRNA is affinity purified by affinity chromatography on oligo(dT)-cellulose. mRNA is reverse transcribed into cDNA using radom hexamers. The heavy and light chain antibody genes are amplified in two separate reactions using two sets of primers designed to hybridize to opposite ends of the variable region of each chain. The purified heavy and light chain DNA products are assembled into a single gene using a DNA linker fragment constructed to hybridize to the 3'end of the heavy chain and the 5'end of the light chain. The assembled scFv DNA fragment is amplified by PCR using a set of primers designed to introduce restriction sites for cloning into a phagemid vector. Following restriction digestion and ligation of the scFv DNA to the phagemid, the ligated vector is introduced into competent *E. coli* by $CaCl_2$ transformation or electroporation. Phagemid-containing bacterial cells are grown and then infected with a helper phage (M13VCS or KO7), a process known as phage rescue, to yield recombinant phages which display scFv antibody fragments as fusion to one of the coat proteins.

7. ANTIBODY LIBRARIES

Immune library

Libraries are created from the IgG genes of spleen B-cells of mice immunized with antigen or from immune donors. An immune phage antibody library will be enriched in antigen specific antibodies, some of which will have been affinity matured by the immune system. Repertoires may be created from the IgG genes of spleen B-cells of mice immunized with antigen or from immune donors. An immune antibody library has two main characteristics: (i) it will be enriched in antigen-specific antibodies, and (ii) some of these antibodies will have undergone affinity maturation by the immune system (Clackson *et al.*, 1991). Immune libraries were used to produce antibodies against carcino-embryonic antigen (CEA) (Chester *et al.*, 1994), and major histocompatibility complex/peptide complexes. High-affinity antibodies were reportedly derived from mice (Andersen *et al.*, 1996), chickens (Yamanaka *et al.*, 1996), and rabbits (Lang

et al., 1996). Antibody libraries were also derived from sheep (Chariton *et al.*, 2001), cows and nonhuman primates (Tordsson *et al.*, 2000).

Applications

Cloning of high-affinity antibodies present after viral infections or cancer, and antibodies to self-antigens present in patients with autoimmune diseases is made possible. Analysis of such antibodies could aid in the identification of antigenic epitopes involved in the humoral immune response. A second application of immune libraries involves the removal of irrelevant antibodies: this can be used to generate antibodies against minor or poorly immunogenic antigens.

Disadvantages

(i) long time required for animal immunization, (ii) lack of immune response to self or toxic antigens, (iii) the unpredictability of the immune response to the antigen of interest, (iv) a new antibody library must be constructed for each antigen this increases the total time of the procedure by 1-3 months, and (v) restrictions in generating human antibodies.

Synthetic library

It has become obvious from structural studies that five of the six CDRs have limited structural variation (Chothia, 1987). The CDR3 of the heavy chain (VH-CDR3) is the most diverse loop in composition and length (estimated potential diversity of 10^{18} sequences) and is most central to the antigen-binding site of all CDRs (Sanz, 1991). Therefore, synthetic libraries can be made by randomizing the VH-CDR3 region synthetic repertoires were made using different strategies including the use of randomized light and heavy chain CDR3s (Akamatsu *et al.*, 1993) and diversifying all three CDR loops in one V-gene segment (Garrard and Henner, 1993). In the first synthetic antibody library constructed according to these principles (Siegel *et al.*, 1997), a set of 49 human VH-segments was assembled via PCR with a short CDR3 region (encoding either five or eight amino acids) and a J-region, and cloned for display as a scFv with a human lambda light chain. From this library, many antibodies to haptens and one against a protein antigen were isolated. Subsequently, the CDR3-regions were enlarged (ranging from 4 to 12 residues) to supply more length diversity in this loop (Bruin *et al.*, 1999). Antibodies with nanomolar affinity were eventually isolated from a synthetic antibody library which combined a novel synthesis

method to construct combinatorial libraries, *in vivo* recombination, with a strategy to maximally mimic natural antibody diversity.

A major advantage of synthetic library over naïve ones is the potential to control and define the contents, local variability and overall diversity of synthetic libraries. The choice of V-gene segments for the construction of synthetic antibody repertoires may be guided by factors that will increase the overall performance of the library, such as good expression and folding and low toxicity in *E. coli*: this will increase the functional library size.

Naïve library: "single pot libraries"

V-genes from the IgM mRNA of B-cells of unimmunized human donors are isolated from peripheral blood lymphocytes, bone marrow, spleen cells, or from animal sources. From small sized human single-pot libraries (3×10^7 antibody clones), antibodies have been isolated against "foreign" antigens (e.g., bovine serum albumin and lysozyme), haptens (2-phenyloxazol-5-one), or "self" antigens (thyroglobulin, tumor necrosis factor α) (Nissim *et al.*, 1994). The affinity of these antibodies was similar to that seen in naive primary immune response and was sufficiently reactive in Western blot, ELISA, and FACS analysis. A large sized naive library was constructed using lymphocytes from over 40 nonimmunized human donors and contained 1.4×10^{10} clones (Vaughan *et al.*, 1996). Antibody libraries from non-immunized donors clone high-affinity antibodies present after viral infections or cancer, and antibodies to self-antigens present in patients with autoimmune diseases. Analysis of such antibodies could aid in the identification of antigenic epitopes involved in the humoral immune response. A second application of immune libraries involves the removal of irrelevant antibodies: this can be used to generate antibodies against minor or poorly immunogenic antigens.

Advantages

(i) isolation of human antibodies to self, nonimmunogenic or toxic antigens; (ii) a single library can be used for all antigens; (iii) short time needed for antibody generation (2-4 rounds of selection in two weeks) and (iv) direct isolation of high affinity antibodies when very large repertoires are used.

Disadvantages

(i) low affinity of antibodies isolated from small sized libraries: (ii) the time needed to construct large libraries, and (iii) content and quality of the library are influenced by the unequal expression of the V-genes repertoire, unknown history of the B-cell donor, and potential limited diversity of the IgM repertoire.

8. SELECTION OF ANTIBODY LIBRARIES: "BIO-PANNING"

Antibody libraries are screened and enriched for antigenspecific clones by a technique known as bio-panning in which phages displaying scFv are incubated with an immobilized antigen of interest (Nissim *et al.*, 1994). Unbound phages are removed by washing whereas phages displaying scFv that specifically bind the antigen are eluted, by changing the binding conditions, and amplified in *E. coli*.

Selection using immobilized antigens

Phage libraries are selected by flowing through an affinity column with the immobilized antigen of interest. Following washing of the column to remove nonspecific clones, specific binders are eluted and amplified in *E. coli*. It should be noted that selection of the immobilization method must take into consideration the conformational integrity of the immobilized antigen. Some phage antibodies selected against an adsorbed antigen may not be able to recognize the native form of the antigen. One way to circumvent such problem is to employ indirect antigen coating through the use of antigen-specific capture antibodies. One way to circumvent such problem is to employ indirect antigen coating through the use of antigen-specific capture antibodies.

Selection using antigens in solution

This technique allows solution binding and overcomes issues with conformational changes that are encountered upon coating antigens on solid surfaces. The use of labeled soluble antigens also allows a more accurate quantification of the antigen used during selection (Hawkins *et al.*, 1992) and consequently enhances the ability to use lower concentrations of the antigen to favor selection of high-affinity phage antibodies. Phage bound to the labeled antigen are recovered with avidin or streptavidin-coated paramagnetic beads following incubation of phage-antibodies with biotinylated antigen.

Selection of cells

Direct selection of antibodies against markers on cell surfaces may be carried out on either monolayers of adherent cells or on cells in suspension. Unbound phage can be washed away by rinsing tissue culture flasks (monolayers) or centrifugation (cell suspension). To optimize the isolation of antigen-specific binders and minimize the binding of irrelevant binders, a simultaneous positive and negative selection may be applied (Kruif *et al.*, 1995). In this approach, a competition is set up between a small number of antigen-positive target cells and an excess of antigen-negative "absorber" cells to bind antibodies of phage library: the absorber cells serve as a sink for the nonspecific adherence of irrelevant binders. A fluorescent labeled antibody against an irrelevant antigen present only on the target cells is added and FACS is used to isolate the target cells binding the specific phage antibodies

***In vivo* selection**

In this method phage repertoires are directly injected into animals and then tissues are collected and examined for phage bound to tissue-specific endothelial cell markers as was demonstrated for peptide phage (Pasqualini and Ruoslahti, 1996)

Advantages

(i) The isolated phage-displayed peptides home selectively to "intact" targets of interest (ii) an inherent blocking step is included where most of the phage-displayed peptides that recognize ubiquitous plasma and cell surface proteins are eliminated (iii) these peptides may be useful for the functional analysis of new receptors and potential identification of novel drug target candidates because some of the isolated peptides have been found to bind to endothelial receptors expressed in the vasculature of specific tissues

Pathogens provide research tools, diagnostics and potential pharmaceutical reagents for the prophylaxis and treatment of viral infections (Zwick *et al.*, 2001). Random phage peptides selected against antibodies from HIV-infected individuals uncovered immunogenic epitopes that behave as antigenic mimics of gp120 and gp41 HIV proteins and, therefore, have the potential for use as broadly protective HIV-1 vaccine candidates (Chen *et al.*, 2001).

9. CATALYTIC ANTIBODIES

Native immune system has already been exploited to obtain catalytic antibodies (Lerner *et al.*, 1999). Antibody phage libraries should also prove a useful source of catalytic antibodies. A potential added advantage for searching antibody libraries for catalytic antibodies is the ability to select for actual catalysis rather than for binding activity only (Janda *et al.*, 1991).

10. PHAGE DISPLAY IN PLANT SCIENCE

Production of diagnostic reagents for single-step detection of target antigens: genetic fusions of antibody fragments to reporter molecules. Two aspects of phage antibody use in relation to plant science are their use as molecular probes, and for the *in vivo* immunomodulation.

Phage antibodies as immunocytochemical probes

Phage antibody binding can be detected by the use of secondary antibodies with specificity for phage coat proteins. For example PAM1 phage can be detected using secondary antibodies with specifity for the M13 pVIII coat protein. The large size of M13 is a disadvantage for immunocytochemical localization studies because of the diffuse signal resulting from secondary antibody binding to the multiple copies of pVIII distributed along the phage particle in order to overcome this it is necessary to use soluble (non-phage bound) scFvs. When used for immunocytochemical labeling the small size of PAM1scFv provides much superior resolution compared to PAM1 phage. Recently, the immunocytochemical applications of phage display antibodies have been extended by the production of scFv/GFP fusions. Functional analysis has established that in many cases such fusions can be made in which both the scFv and GFP moieties retain their original activities (Morino *et al.*, 2001, Casey *et al.*, 2000).

Immunomodulation

If the genetic pathways controlling a particular process or molecule are not characterized an alternative strategy is to directly disrupt gene products in order to elucidate their functions. One such direct approach is immunomodulation - the disruption of antigen function by the action of antibody binding (Smith and Glick, 2000). This can be achieved either by micro-injection of antibodies into cells incorporation of antibodies into plant or plant cell growth media, or by the expression of antibodies in plants.

One problem associated with the expression of whole antibodies or Fab fragments in plants is that the intracellular environment is not conducive to correct antibody assembly. In this regard scFvs, with their relatively undemanding folding requirements, are particularly well suited for this role and have been successfully used to immunomodulate a variety of plant antigens (De Jaeger *et al.*, 2000). Immunomodulation of the activity of the plant hormones abscisic acid (Straub *et al.*, 2001) and gibberellin (Shimada *et al.*, 1999) and the receptor protein phytochrome (Owen *et al.*, 1992) has been demonstrated.

11. CONCLUSIONS

Antibody phage display, system, has yielded hundreds of antibodies for therapy, research and diagnostics. Several antibody (synthetic, immune, scFv, Fab, etc.) and peptide (linear, cyclic, etc.) libraries have been constructed and characterized. Screening of these libraries can yield a set of recombinant antibodies or peptides and their coding genes within 2 to 3 weeks. However, given the current patent status of phage display, none of these libraries is for sale but they may be obtained under license agreements from either academic research laboratories or biotechnology companies. Biotechnology companies such as Cambridge Antibody Technology (CAT), Dyax, and MorphoSys are now driving the new developments and clinical applications of phage display. Exploitation of the phage display technology to identify new diagnostic targets has the potential to direct the discovery of new classes of diagnostic reagents. Antibodies are central to mastering the next main task of 'postgenomic' research: that is, to understand gene function by analyzing the proteome. As we enter the era of proteomics, recombinant antibodies represent a possible approach to close the gap of information that currently exists between genomics and proteomics.

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13. DISCUSSIONS

1) What are the application in plant science and works in India?

Ans.) There has been reports of use of phage display in detection of deesterified Pectic Polysaccharide Rhamnogalacturonan II in Plant Cells and some work in identifying novel genes for control and deterrence of sucking insect pests. There are works going on cholera, HIV and many other diseases in Delhi University, ICMR, IISc, TIFR and other premiere institutes in India.

2) What is the meaning of phage?

Ans.) Phages are the common term used for 'Bacteriophages'. They are the viruses, which affect the bacteria and cause death to the organism.

3). Can this technique be used against bacteria?

Ans.) Yes, this technique was used against bacteria, *Streptococcus mutans* using a phage display chain shuffling approach and there are reports of its use to detect pathogenic bacteria in foods

4) What were the earlier techniques in identification of antibodies?

Ans) The earlier techniques involved hybridoma technology, enzyme-linked immunosorbent assay (ELISA) and testing each antibody against the host in the hope of the perfect drug

5) What is biopanning?

Ans.) Antibody libraries are screened and enriched for antigen specific clones by a technique known as biopanning in which phages displaying scFv are incubated with an immobilized antigen of interest. Unbound phages are removed by washing whereas phages displaying scFv that specifically bind the antigen are eluted, by changing the binding conditions, and amplified in *E. coli*.

6) Is phage display used only for antibody production?

Ans.) Phage display is a complex tool, which can be used for wide range of application one of which is antibody production in novel drug discovery. The other functions include the use of phages for transporting cell or tissue specific drugs or ligands, eg.in case of tumor cells we can use tumor specific antibodies to transport the drugs.

Abstract

Antibodies are well recognized as indispensable tools for recognizing and tracking target molecules. However, traditional methods for preparing antibodies are cumbersome and labor intensive. As a result, researchers are working to develop faster and easier ways to capitalize on the target recognizing qualities of antibodies. Molecular antibodies have been generated either by the hybridoma technology or more recently from antibody libraries. The development of antibody libraries have been greatly influenced by the development of display technologies and vice versa.

Phage display, first introduced by Smith, P.G. (1985) is a very effective way for producing large numbers up to 10^{10} of diverse peptides and proteins and isolating molecules that perform specific functions. This technique can also be used to study protein-ligand interactions, receptor and antibody binding sites, to improve or modify the affinity of proteins for their binding partners (Cesareni , 1992). Antibody libraries were created from mice (Andersen *et al.*, 1996), chickens (Yamanaka *et al.*, 1996), rabbits (Lang *et al* , 1996), cows and nonhuman primates (Tordsson *et al.*, 2000).

Biopanning is the process by which antigen specific clones are screened and enriched from antibody libraries in which, phage displaying Single chain variable Fragment (scFv) are incubated with an immobilized antigen of interest (Nissim *et al* , 1994). Unbound phages are removed by washing whereas phages displaying scFv that specifically bind the antigens are eluted, by changing the binding conditions, and amplified in *E.coli*.

In this way pathogen specific research tools, diagnostics and potential pharmaceutical reagents for the prophylaxis and treatment of viral infections have been developed (Zwick *et al.*, 2001). Such as HIV-1 vaccine candidates (Chen *et al.*, 2001), Hepatitis C virus antibody (Zhong *et al* , 2002) and malarial phage libraries (Lauterbach *et al.*, 2003).

DECLARATION

T.Eliza Lincy (2004-11-37) hereby declare that the seminar entitled "Molecular farming" have been prepared by me, after going through various references cited at the end and has not been copied from any of my fellow students.

Vellanikkara,
Date 29-11-2005

T.Eliza Lincy,
2004-11-37

CERTIFICATE

This is to certify that the seminar report titled "Molecular Farming" has been solely prepared by Ms.T.Eliza Lincy (2004-11-37), under my guidance, and has not been copied from any seniors, juniors or fellow students seminar reports.

Vellanikkara

Date 30 - 11 - 05

Dr.A.Augustin.
Major advisor.
Associate professor.
CPBMB.

CONTENTS

MOLECULAR FARMING

Introduction:

Plants are the most efficient producers of protein on the planet, with simple nutritional requirements and the potential to be grown on an agricultural scale.

Plant molecular farming is a new and promising industry involving plant biology. "Molecular farming is a term coined to describe the application of molecular biological techniques to the synthesis of commercial products that are completely novel to plants". The products identified as targets for molecular farming include variety of carbohydrates, proteins, fats and secondary products are produced in plants as biofactories / bioreactors (Horn *et al*, 2004)

Many of the proteins being farmed in plants are antibodies, vaccines or biopharmaceuticals aimed at improving human and animal health. Molecular farming is the area where many of the most exciting and beneficial developments in plant biotechnology are taking place. Through molecular farming very few products have reached the market and some are expected to come to market within the next 5 years. Similar to the evolution of new biological organisms, new technical breakthroughs have been achieved through molecular farming

History:

Molecular farming has existed since the first higher plant was successfully transformed (Fraley *et al*, 1983), because any protein has the potential of being a protein product one of the earliest market genes the scientists have used in developing transformation systems in plants, uids (Jefferson *et al* 1987), is now a molecular farming product (kusnadi *et al*, 1998,Witcher *et al* 1998) The first report of human antibodies produced in plants was by during 1988 and was expanded to include secretary antibodies by (Hiatt *et al* 1989). The first report of a protein being produced in plants for the specific purpose of extraction, purification and sale of that protein (Hood *et al*1997),which detailed the production of avidin, an egg protein with several important properties, Aprotein, one of the first molecularly farmed pharmaceutical proteins to be produced in plants(Zhong *et al* 1999), may soon be used on medical

patients for wound closure and to suppress the systematic inflammatory response during surgery.

What Is Molecular Farming?

Biotechnology in agriculture has two categories:

(1) Improvements to existing livestock and crops,

(2) Development of entirely new uses for both animals and plants (Bio pharming)

Types of Molecular Farming:

1. Medical
2. Non-Medical.

Molecular Farming is the term for new use plants only [not animals] and is different in that this does not affect and has nothing to do with food directly.

DEFINITION:

Growing of plants in agriculture to produce pharmaceutical or industrial compounds instead of food, feed, or fiber. The possibilities range from the manufacture of medical products, such as pharmaceuticals (drugs) and vaccines, to the production of products like biodegradable plastics and industrial chemicals. (Canadian Food Inspection Industry)

Why plants are selected for molecular farming?

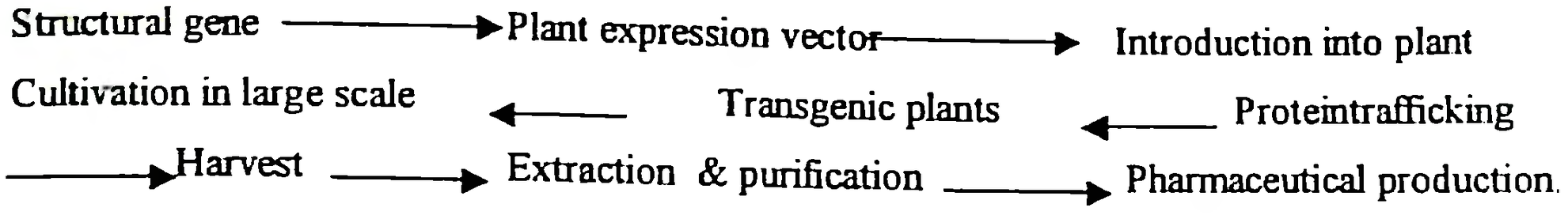
1. Mammalian cell systems are expensive and having pathogens and animal viruses cause contamination.
2. Bacterial or fungal cell system can be scaled up but often-recombinant proteins are not properly processed. (They are not properly folded and disulphide bridges are not formed) So intracellular precipitation of non-functional protein can occur.
3. Large growing vessels of high cost required for growing of bacteria and fungi.

4. Plant material can be scaled up so that large quantity of material can be harvested and processed, allowing industrial scale amounts of protein to be purified.
5. In some cases it may be possible to omit purification and the plant material containing recombinant enzyme can be added directly to animal feed or industry process.
6. There are some difference between plant and animal posttranslational modification systems and the difference in glycosylation patterns has caused some concerns, particularly when expressing antibodies in plants.
7. Plants are able to fold, cross-link and translationally modified i.e. functional proteins are obtained.
8. The combination of protein products with toxins or pathogens of animal and humans very much reduced.

Lot of work was first done with tobacco, but this is not a suitable plant for feeding to animals. For work in which edible products are being developed in plants such as potatoes, tomato, maize and lettuce. For bulk production this leaves of plants such as tobacco and alfalfa remains a favorite, because they can be harvested several times per year. However, plants such as rice, wheat, maize & soybean have also been used although their biomass yields are lower.

Crop	Annual biomass (t/ha)
Tobacco	>1000
Alfalfa	25
Maize	2
Rice	6
Wheat	-3

Steps in molecular farming:



MOLECULAR FARMING SYSTEM

Methods Of Protein Production From Plants:

1. **Stable nuclear transformation of a crop species grown in field / greenhouse.**
2. **Stable plastid transformation.**
3. **Transient transformation of a crop species using recombinant viruses.**
4. **Transient transformation of a plant species that is grown hydroponically.**

I. STABLE NUCLEAR TRANSFORMATION:

The most common methods is, nuclear transformation of a crop species, which produced all products available in the market place today. This requires a method of transferring the foreign genes into the plant cells, usually using *Agrobacterium tumefaciens* or particle bombardment in which the genes are taken up and incorporated into the host nuclear genes in a stable manner.

1.AGROBACTERIUM MEDICATED TRANFORMATION:

1 Isolation of desired gene.

2 Insertion of gene into a vector.

3 Introducing the vector into host i.e., transformation.

4 Selection of transformed cells Agrobacteria.

Infecting plants using transformed Agrobacteria

Induces tumor or hairy root by the Ri plasmids T-DNA from Agrobacteria onto plant chromosome (Mary *et al*, 2004)

EXPLANT

(Decapitated seedlings, cell, protoplast, leaf discs)

Addition of acetosyringone

Wounding

Agrobacteria

Co cultivation to allow infection

↓

Antibiotics to kill bacteria

(Carbenillicin, cefotaxime)

Transformed & non-transformed tissues

↓

Selective media to kill non Transformed tissue

(kanamycin, hygromycin)

Transformed tissue/callus

↓

Transformed shoots

↓

Rooted shoots

↓

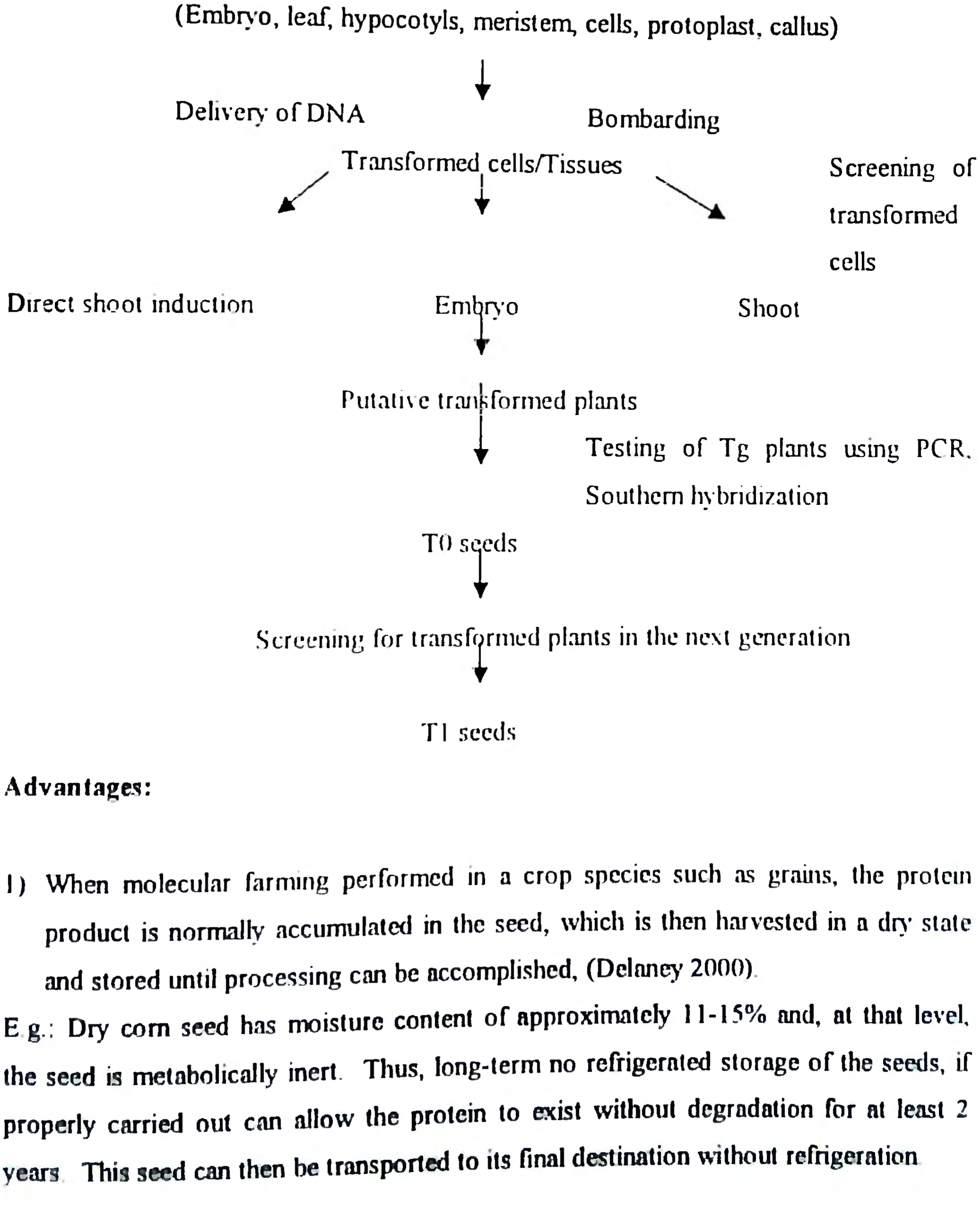
Adult plants

↓

Seed

2. PARTICLE BOMBARDMENT:

Foreign DNA is coated with golden particles and bombarded against the target tissue.



Advantages:

1) When molecular farming performed in a crop species such as grains, the protein product is normally accumulated in the seed, which is then harvested in a dry state and stored until processing can be accomplished, (Delaney 2000).

E.g.: Dry corn seed has moisture content of approximately 11-15% and, at that level, the seed is metabolically inert. Thus, long-term no refrigerated storage of the seeds, if properly carried out can allow the protein to exist without degradation for at least 2 years. This seed can then be transported to its final destination without refrigeration.

2) This system we can utilize large acreage with the lowest possible cost. Since crop such as rice and corn are grown throughout the world, the products have the potential to be produced near the target markets.

Disadvantages:

Some grains, such as corn, have the potential to cross with native species or food crops. There are technologies that will prevent out crossing eg: mechanical detasseling or genetics-based male sterility. Such technology generally reduces the cost advantage of the system due to higher manual labour requirements, lower yields, and less effective genetics.

2.PLASTID TRANSFORMATION:

The laboratory of Hentry Daniell at the university of Central Florida has put emphasis on plastid transformation for the purpose of molecular farming. Svab first described a system for plastid transformation etal 1990,using tobacco. Tobacco appears to be the only species in which plastid transformation has been established as routine (Svab and Maliga 1993, Daniell *et al* 2002).

Advantages:

1. There is no risk of transgene being transmitted through pollen to plants.
2. Transgenes integrated into the chloroplast genome show very high level of expression (up to 40% of the soluble protein in all may be the recombinant protein)
3. The transgene expression is not affected by gene silencing.
4. Chloroplasts are better able to express bacterial genes than are nuclei.
5. The very high level of transgene expression ensures a high level of mortality of the pest organism.
6. Introduction of foreign gene into chloroplast giving an estimated 10,000 copies per cell. So recombinant protein level reached maximum. High gene copies and

no gene silencing i.e. low expression. Chloroplast can assemble and fold complex foreign protein, so protein-processing problem get reduced. They are not maternally inherited.

Disadvantages:

1. Transformation frequencies are much lower than those for nuclear genes.
2. Prolonged selection procedures under high selection pressure are required for the recovery of transformants (Singh, 2004).
3. The method of transgene transfer into chloroplasts are limited, and they are either expensive or require regeneration from protoplasts.
4. These transformation systems are far more successful with tobacco than with other plant species.
5. Products of transgenes ordinarily accumulate in green parts only. As with any fresh tissue molecular farming system protein stability overtime will change even with refrigeration.
6. Extraction and purification must be performed at very specific times following harvest. Tobacco is currently a highly regulated crop and is not edible. Large volume products and edible vaccines would not appear too feasible using this system.

3.Transient transformation of crop species using recombinant plant viruses:

This system depends on the ability of recombinant plant viruses, which transiently express a target protein in the plant tissue. The protein will accumulate in the interstitial spaces. The interstitial fluid can than be collected by centrifugation under vacuum.

Recombinant viruses:

1.DNA viruses:

i. Caulimo viruses.

ii. Gemini viruses.

2. RNA viruses

i. Monopartite viruses Eg. TMV

ii. Multipartite viruses Eg. BMV

E.g. TMV vector TB2 containing foreign gene at 3^1 end that was the first plant RNA viral vector able to spread systemically in whole plants (Donson *et al* 1991)

Advantages:

TMV can be readily manipulated genetically and the infection process is rapid. Small quantities of the target protein can be obtained within several weeks.

It's an ideal system for a large number of custom proteins that are needed in relatively small amounts.

Disadvantages:

1.Not suitable for any protein regulated in large (kg) quantities.

2. Product must be processed immediately as storage will cause degradation of the plant tissue. Same, tobacco is a highly regulated crop.

4. STABLE TRANSFORMATION OF PLANT SPECIES THAT GROW HYDROPONICALLY:

In this system, transgenic plants containing a gene coding for the target protein are grown hydroponically in a way that allows release of the desired product as a part of the root exudates into the hydroponics medium (Raskin2000)

Advantages:

Plants grown hydroponically in a green house setting have reduced fears of unintentional environmental release Purification of the desired product is considerably easier, since no tissue disruption is needed and the quantity of contaminating proteins is low.

Disadvantages:

1.Purification not amenable to produce large (kg) quantities of any protein product.

2. Green house or hydroponics facilities are relatively expensive to operate.

Products produced

CARBOHYDRATES

Starch:

Starch is one of the components of plant cells. Tailor made starches with reduced level of amylase or increased amount of starch have been developed in potato. Starch precursors have been rerouted into the biosynthetic pathways of other storage carbohydrates.

1 Nonfructan storing tobacco and potato plants have been transformed with fructosyl transferase gene from *Bacillus subtilis* to accumulate fructan.

2 Tobacco plants have been transformed with the mannitol-1- phosphate dehydrogenase gene (mtl D) from *E.coli*. These transgenic synthesized more mannitol and were also tolerant to high salinity (Tarczynki *et al*, 1993)

3 Cyclodexrins from starch:

One of the high value products that could be made from starch is the cyclodextrins These compounds are typically 6-7 or 8 membered ring comprising glucopyranose will effectively solubilise hydrophonic pharmaceuticals such as steroids. In 1991 only one report of an attempt to produce cyclodextrins has been published

A bacterial cyclodexrins glycosyl transferase gene from *Klebsiella pneumoniae* was fused to a targeting sequence and placed under the control of the promoter from the patatin gene Patatin is a protein that accumulates in potato tubers and the promoter directs high level of expression in the tubers of Tg potatoes However, transformation of potatoes with this construct resulted in very little conversion (0.001-0.01%) of starch to cyclodexrins

It was concluded that the insoluble starch granules may have been inaccessible to the bacterial enzyme or that enzyme became trapped in the growing granule Whatever the reason, no subsequent attempts to produce this have appeared in this literature.

4. Polyfructans:

These compounds are soluble polymers of fructose that are synthesized and stored in the vacuole.

5.Trehalose:

It's an additive in food processing, dehydration and flavour retention. Genes for trehalose synthesis from yeast and *E.coli* have already been introduced in to transgenic tobacco but the purpose of these experiments was to manipulate drought tolerance rather than to manufacture bulk quantities of sugar.

6. Sucrose:

It is a disaccharide of linked glucose and fructose, is the most preferred sweetener, the key enzyme is sucrose synthesis is sucrose phosphatase (SPS). Over expression of SPS holds promise for achieving the goal of increased sucrose synthesis in plants. Maize SPS has been over expressed in tomato. In transgenic plant sucrose level increased with a concomitant reduction in starch.

LIPIDS

Monounsaturated fatty acid level in plants is desirable so that nutritional value of the oil is enhanced.

1.Nutritional value of oil:

Rat desaturase gene introduced in to tobacco plants, the level of palmitoleic acid (16:1) & oleic acid (18:1) levels get increased. (Grayburn *et al*, 1992)

2.Manufacture of detergents: medium chain fatty acids:

Thioesterase gene from California bay tree was incorporated into Arabidopsis for the accumulation of medium chain 12-carbon fatty acid as storage lipids. (Voelker *et al*, 1992)

3.Production of first non-food product:

Lauric acid is the raw material for the production of soaps, cosmetics, and detergents. Genetically engineered rapeseed variety (Laurical) modified to produce lauric acid (12:0) used to make soaps, cosmetics, detergent. The gene was from Californian bay tree (seed)

This gene shut off fatty acid synthesis after 12 carbons rather allow the acid to grow 18 C.

4.Modification of the degree of saturation:

γ-linolenic acid (GLA) is the first intermediate in the bioconversion of linoleic acid to arachidonic acid. GLA alleviates hyper cholesteromia and coronary heart disease, which is not produced in oil seed crops. This conversion is catalyzed by enzyme Δ6 desaturase and this enzyme cloned from *Cyanobacterium synechocystis*. Constitutive expressions of this gene in transgenic tobacco produce GLA. Recently this gene cloned from filamentous fungi (*Mortierlla alpina)* and transferred to canola & tobacco. (Knutzen *et al*, 1998 Sayanova *et al*, 1997)

5.Production of Industrial products:

Hydroxy fatty acid: Ricinoleic acid used in the manufacturing of nylon, paints, varnishes, resins, lubricants & cosmetics.

- ✓ Tg arabidopsis expressing castor oleate hydroxylase gene accumulate ricinoleic acid upto 17% of total lipid.
- ✓ Simularly oleate 12 dehydrogenase desaturase gene transfer from *Lasqurella fendleri* to *Arabidopsis* for the production of hydroxylated fatty acid (Broun *et al*1998)

BIODEGRABLE PLASTICS

One of the most imaginative examples of molecular farming has been the attempt to produce biodegradable plastics Polyhydroxybutyrate (PHB) is the best-characterized polyhydroxy alkanoate (PHA) and is found in intracellular inclusions of bacteria In *Alcaligenes eutrophus*, phb accumulates as a high molecular weight polymer upto 80% of bacterial dry weight Pathways of PHB synthesis, the genes for three enzymes involved in the pathway (phaA, phaB, phaC genes) have been cloned from bacteria

1. In initial experiment it was recognized that the first step of pathway, production of aceto acetyl – CoA, occurs in cytoplasm of plant at the start of the pathway to isoprenoids. Thus, only phaB and phaC encoding acetoacetyl-coA reductase and phb synthase respectively were transformed into *Arabidopsis* without targeting sequences. Microscopic observation of the *Arabidopsis* leaves

indicated the formation of micro bodies of bioplastics accumulating in the cytosol, nucleus and vacuole. However, the amount of bioplastic was relatively low (20-100µg g-1 fresh weight) and the plants were severely stunted in growth.

2. Subsequently, all three genes were transformed into *Arbidopsis* and targeted to chloroplast. In the first generation experiment each gene was separately fused to a sequence encoding the transit peptide plus N-terminal fragment of the Rubisco small subunit protein, and expression of each gene construct was directed by the CaMv35s promoter, each construct was transformed into separate *Arabodopsis* plant and brought together by series of sexual crosses between the individual transformants. In this case, the bioplastics accumulated as 0.2-0.7µm granules in the plastids to level upto14% of plants dry weight and there was no observable effect on growth and fertility.

3 Increased phb production in *Arabidopsis* has been achieved using triple construct. So that three genes are transformed into the plant in a single transformation event. Rapid gas chromatography, mass spectrometry procedures used to screen a large number of Tg plants, permitting the selection of plants accumulating phb in the leaf chloroplasts to more than 4% of their fresh weight (40%dryweight) How ever, the high producing lines showed stunted growth and loss of fertility. Interestingly, the production of phb did not affect fatty acid accumulation or composition, but there was a significant impact on the levels of various organic acids, amino acids, sugars and sugar alcohols

4. Some research has been focused on the potential for the commercial production plastics in oil crops, effectively diverting the pool of acetyl-coA away from fatty acid biosynthesis towards bioplastic production. A team from Monsanto used three genes from phb synthesis pathway from bacteria *Ralstonia cutropha* and fused each one to a seed – specific promoter. These were transferred in to a single, multigene vector, which was used to transform oil seed rape. Phb was found to accumulate in mature oil seeds leucoplasts to level upto 7.7% of fresh seed weight.

5. How ever, apart from in *Arabidopsis*, it has proved difficult to obtain very high levels of PHB in transgenic plants. Recent work indicates that constitutive expression of the β-ketothiolase gene is detrimental to the efficient production

of PHB in some species and the use of inducible or developmentally regulated promoters to drive the PHB A gene permitted some production of PHBs in tobacco & potato.

6. Another interesting direction taken by this research has been the production of bioplastics in cotton fibers. At the start of the work it was recognized that cotton fibers contain β-ketothiolase activity. Therefore, *A.eutophus* PHA B and PHB C genes were transferred into cotton by particle bombardment of seed axis meristems. PhaB gene was driven by cotton fiber specific gene promoter, whilst PHA C was fused to 35s promoter. Clustering of small phb granules were found in the cytoplasm of the fiber cells. The thermal properties of fibers were found to be altered, indicative of enhanced insulation characteristics.

Limitations:

1. Phb is a highly crystalline polymer, which produces stiff and brittle plastics.
2. Pha copolymer made from long monomers has better physical properties like less crystalline and more flexible. Medium chain phaC1 gene from *Pseudomonas aeruginosa* with a peroxisome targeting sequence from an oilseed rape isocitrate lyase The enzyme was targeted to leaf-type peroxisomes in light-grown plants and glyoxisomes in dark-grown plants. These accumulate phas in the glyoxisomes & peroxisomes and in vacuole to a level of 4mg g-1 dry weight

Vitamins & minerals:

Vitamin – A deficiency cause colour blindness. Rice is the staple food, which lacks Provit-A in the endosperm Immature endosperm capable of synthesizing the early intermediate Geranyl geranyl diphosphate (GGDP) which can produce uncoloured carotene 'Phytoene'by the expression of enzyme phytoene synthase. Synthesis of β-carotene requires 3 additional enzymes: Phytoene desaturase (phy), ξ - Carotene

desaturase (crt1), lycopene β-cyclase (lcy). *Agrobacterium* mediated transformation in rice to induce entire β – carotene biosynthetic pathway (Philomina, 2004).

Genes of lycopene synthesis added are,

1. Plant phytoene synthase gene – Daffodil (*Narcissus pseudonaricissus*)
2. Bacterial Phytoene synthase – *Erwinia uredovora*.

They were placed under the control of endosperm specific glutelin (Gt1) and the constitutive promoter CaMV 35s. These 2 genes directed the formation of lycopene in plastids, the site of GGDP formation.

3. Lycopene β –cyclase - Daffodil (*Narcissus pseudonaricissus*) controlled by rice glutelin promoter resulted rice produce sufficient β-carotene converted to Vit – A

Phytoene synthase gene from *Brassica napus* resulted in high carotenoids. Monsanto conducted this work to enhance carotenoid level of oil seeds i.e. of β-carotene in seed of Canola.

GGDP

↓ Phytoene synthase (phy)

Daffodil

PHYTOENE

Erwinia uredovora ↓

Carotene desaturase(Crt1)

LYCOPENE

Daffodil ↓ lycopene β- cyclase(lyc)

PROVIT – A

↓

VIT – A

↓

GOLDEN RICE

Micronutrient:

Major micronutrient deficiency worldwide, is Fe deficiency cause anemia (24% of world population). Potrykus *et al* aimed to,

- Increase Fe content with ferritin transgene from *Phaseolus vulgaris*
- Reduce Phytase in cooked rice with a transgene for heat – stable phytase from *Aspergillus fumigatus*.
- Increase resorption-enhancing effect from a transgene sulfur – rich metallothionin like protein from rice.
- Resulted in two-fold increase in Fe content& cystein – 25%.

Other protein pharmaceuticals:

1.Trichosanthus:

It is a component of tuber of the plant *Trichosanthus kirilowii*, which is used in Chinese medicine. A ribosome inactivating protein TCS inhibits tumor growth and immune response may be useful as a treatment for HIV/AIDS. High levels of TCS accumulation (2% of total soluble protein 2 weeks after inoculation) have been achieved in *Nicotiana bethamiana* using viral RNA- based transfection system.

2.Glucocerebrosidase:

Gauchers disease is an inherited disorder in which glucocerebroside (remove old and damaged red blood cells) accumulates in lysosomes, due to a deficiency in the enzyme glucocerebrosidase. This disease cause swelling of spleen and liver and severe bone damage. Currently drug developed from glucocerebrosidase purified from human placentas. Its one of the worlds expensive drug. A process to produce glucocerebrosidase in Tobacco has recently been patented which will result in cheaper glucocerebrosidase production.

3.Human serum albumin:

HAS used to treat burn and in liver cirrhosis. HAS has been expressed in tobacco and potato under the control of 35s promoter which was indistinguishable from the human protein.

4.Arginin vasopression:

Attempts to express AVP in tobacco culture have proved unsuccessful due to protein insolubility and retarded cell growth.

Although active combinant proteins have been produced through molecular farming one problem associated with production in plant systems is that these often give a relatively low product yield and recovery.

PROTEIN

Major strategies for the production of protein in plants:

1. The stable integration
2. Use of plant viruses as transient vectors.

Stable Expession:

Transgene is regulated by strong, constitutive promoter (35s) most suitable for the bulk production of soluble proteins in leaves, although yields can be low using this approach. Target gene expression and protein production to specific tissue leads to higher yields of recombinant protein. Vaccines are produced only to the parts of plants that are normally eaten. Storage organs such as tubers, seeds are designed to maintain their biological integrity over long periods (weeks - years). Seeds are also great interest because of easy protein purification. In tobacco seeds contain a simpler mixture of protein and lipids with fewer phenolic compounds than do green leaves. Tissue and intracellular targeting are brought together in the use of oleosins as vehicles for protein purification.

Protein quality improvement:

1. **Improvement in the nutritional quality of legumes storage protein:**

Gene responsible for a methionine – rich protein was from Brazil nut (*Bertholletia excelsa*) seed protein Brazil nut 2S albumin, under the promoter of Phaseolin gene was introduced into tobacco, resulted in 30% increase in metheonine in Tg tobacco seed. (Altenbach *et* al, 1989).

Expression and accumulation of some protein tested in Tg *Arabidopsis*, Rape seed, Soybean, French bean, potato.

Unfortunately this gene was found allergenic. (Nordlee *et al*, 1996). So, this is replaced by sunflower seed albumin under the control of the Pea vicilin gene promoter has been transferred to lupin and methionine content increased two fold.

a. Seed storage protein gene (Ama 1) isolated from Amaranthus. This gene has well – balanced amino acid composition. Tg potato contain 0.3% Ama 1 of total soluble protein (Chakraborty *et al*, 1998).

b. Soybean Glycinine gene introduced in to rice to increase protein and make it more digestible. Important amino acids lysine, lacking in quantity of rice have been replenished.

c. Human milk encoding β – casein gene expressed in Tg potato under the control of an auxin – inducible promoter (Chong *et al*, 1997). This finding opens a way for reconstruction of human milk proteins in plant foods.

Proteins, Peptides, Vaccines:

Genetically engineered plants and plant viruses used to produce vaccines against human diseases ranging from tooth decay to life threatening infections such as bacterial diarrhea, cholera & AIDS

1. The neuropeptide (leu) enkephalin was produced in *Brassica napus* as part of the seed storage protein 2s albumin by -

1. Using Viral vectors to produce chimeric coat protein in Tg plants (Turpen *et al*, 1995) have described a method for engineering the capsid protein of TMV as either internal or C – terminal fusion with peptides carrying epitopes derived from malarial sporozites. Both types fusion constructs yielded high titers of genetically stable recombinant virus produced appropriate monoclonal anti-malarial antibodies in infected tobacco plants.

2. Fusing the Zonapellucida ZP3 protein with TMV capsid protein. ZP3 protein of mammalian oocyte has been targeted. Mice immunized with the recombinant TMV particles developed antibodies against ZP3 (Beachy *et al*, 1996). Theoretically, vaccines

with the modified viral coat protein might work as a form of birth control, because antibodies to Zonapellucida could prevent fertilization of egg.

3.Deriving the epitope from HIV which are expressed in alfalfa mosaic virus coat protein fusion products (Yusibov *et al*, 1997)

4.Engineer cowpea mosaic virus (CMV) as an expression system for the production of foreign peptides such as epitope derived from human rhinovirus14 and HIV.

The modified plant viruses elicit the production of mouse antibodies that neutralize virus in Test tube experiments. It indicates the possibility of producing a preventive HIV vaccine. Fusion coat protein may represent a cost effective way of generating vaccines.

Classes Of Proteins in Molecular Farming:

1. Parental therapeutics & pharmaceutical intermediates.
2. Industrial proteins (eg Enzymes)
3. Monoclonal antibodies (Mabs)
4. Antigens for edible vaccines

1.Parental therapeutics and Pharmaceutical intermediates:

It includes all proteins used directly as pharmaceuticals along with those proteins used in making of pharmaceuticals List of proteins include, thrombin, collagen (therapeutics) trypsin, aprotinin (intermediates)

E.g. Hirudin.

It is a small anticoagulant peptide found in salivary glands of medicinal leech *Hirudo midicinalis*. Hirudin is the most potent inhibitor of thrombin (serine protease catalyze blood clotting). It has high therapeutic value in the treatment of thrombosis. Recombinant hirudin was fused with 3^1 end of a *Arabidopsis* gene with its own promoter transferred to *Brassica napus*.

2. Industrial proteins:

The global industrial enzyme market has been estimated to be worth something approaching $US 2 billion per annum.

1. Avidin (from chicken) and 2. β-glucuronidase (from *E.coli*) both of which were produced in maize.

Prodi Gene Inc is the company is leading the race to large-scale production of plant derived Tg enzymes for commercial use.

3. Trypsin: Proteolytic enzyme largely produced by Prodi Gene Inc and aimed to produce Kg quantities of protein from plant sources.

4. Phytase: Useful enzyme that release phosphate

When Tg plants expressing phytase E.g. Seeds added to pigs & poultry diet increased the production of phosphate and phosphate excretion is reduced.

3. Antibodies/ plantibodies:

It includes all antibody forms (Ig) and antibody fragment (Fv). They can be produced in plants in both glycosylated & non-glycosylated forms. Plant derived MAbs have the potential of alleviating the serious production bottleneck that currently exist.

Eg. α – caries for prevention of dental decay, α -herpes transmission.

Immunoglobulins are composed of two heavy chain and two light chains. H chain termed – α, δ, ε, γ, & μ. L chain termed – κ or λ. But not mix of two.

Each antibody has two antigen binding sites formed from the variable regions of the light and heavy chains.

The variable regions of the light and heavy chains are solely responsible for antigen binding can be produced. Single chain variable fragment antibodies are produced from synthetic genes made by fusing the sequence.

Eg. scFv antibodies are most commonly used and its successful.

First example of a functional antibody (IgG1) being produced in plant was reported in 1989.

The construction of this antibody was a two-step process.

1step: Two separate tobacco lines were produced. One contained the gene for γ-chain and other contained the gene for the κ - light chain.

2step: Cross these plants to generate progeny that expressed both these genes.

The F1 progeny expressing both chains 1.3% of the total leaf protein. Produce the various antibody subunits in different plant lines that are subsequently crossed to produce a functional antibody.

E.g. A secretory IgA that protects against dental caries produced by *Streptococcus mutants* has been expressed in plants. The antibody recognizes the native streptococcal antigen (SA) cell surface adhesion molecule, by there preventing colonization up to 4 months.

4. Edible Vaccines

Both stable and transient expression systems have been used to produce vaccines in plants. One of the first attempts in the area used a transient expression system (Chawla. Recombinant cowpea mosaic virus (cpmv) was used as a vector for a linear epitope from the VP2 capsid protein of mink enteritis virus (MEV). These virus replicated in *Vigna ungiculata* isolated and used for subcutaneous injection of mink. This induces resistance to clinical infection by MEV

The ideal production system for edible vaccines in plants that could be eaten raw, such as Banana, Tomato For animals fodder crops and other food crops is possible

One of the drawbacks of edible vaccine is that very difficult to control the dose administered if consumption as a part of foodstuff is the mean of delivery. Charles Arntzen,1998 of Texas A&M University one of the main light in this field, believes that they should be considered vaccines that come from a plant source. His group produce vaccines in Tomato, but they are partially processed so that food based tablets containing a known dose of vaccines can be produced.

Phytoremediation

All commercial and research activity to date in phytoremediation has used naturally occurring plant species. However, many of these are species that can be genetically engineered, including *Brassica juncea*, which is being investigated for phytoremediation of heavy metals from soils (Dushenkov et al., 1995), sunflower-*Helianthus annuus*, being tested for rhizofiltration of uranium (Dushenkov et al., 1995) and poplar trees (*Populus deltoides nigra*), being investigated for the accumulation of nitrates and other organic chemicals from soil (Schnoor et al., 1995). In general, any dicotyledonous plant species can be genetically engineered using the Agrobacterium vector system, while most monocotyledonous plants can be transformed using particle gun or electroporation techniques.

Genetic engineering might be used to improve heavy metal phytoremediation by introducing biochemical traits that enhance hyper accumulation. Examples might include genes controlling the synthesis of peptides that sequester metals, like phytochelatins (e g , the Arabidopsis cad1 gene of Howden et al. 1995), genes encoding transport proteins, such as the Arabidopsis IRT1 gene that encodes a protein that regulates the uptake of iron and other metals (Eide et al. 1996) or genes encoding enzymes that change the oxidation state of heavy metals, like the bacterial merA gene encoding mercuric oxide reductase (Rugh et al. 1996).

PRODUCTS ON MARKET

1. **Avidin:** Glycoprotein found in avian, reptiles and amphibian egg white. It's used primarily as a diagnostic reagent. The avidin in egg is relatively expensive due to the cost of maintaining live animals. Production of Tg corn with transformed avidin gene resulted avidin had properties almost identical to those of avidin from chicken egg white (pI, KI, antigenic properties – identical both glycosylated.) The size of corn-derived avidin was slightly less due to less complex glycosylated composition. Corn derived avidin expressed levels over 20% of total soluble protein have been observed. Processing methods were

developed to extract and purify this novel compound from Tg corn. (Kusnadi et al, 1998). This product is currently being sold by Sigma – Aldrich.

2. **β – Glucuronidase: (GUS)**

GUS is widely used as a visual marker in Tg research. It was first reported to be produced in Tg corn (Kusnadi et al, 1998). Properties were compared with GUS extracted from *E.coli*. The molecular weight, KI, pI, KM – identical V max- similar in both cases. The sequences of the two genes were similar, with the exception of the most N – terminal amino acid. Corn derived GUS has been marketed by Sigma – Aldrich.

3. **Trypsin:** First large scale protein produced from Tg plant technology has a significant market potential than Avidin & GUS.

   Trypsin is a protease used in the processing and some biopharmaceuticals. Availability of maize derived bovine trypsin helps to supply the growing market for animal free reagent

Maize derived trypsin specific activity – 175 U/ mg of protein

Bovine – Pancreas derived trypsin – 1660/ mg of protein

PRODUCTS CLOSE TO MARKET

Besides the 3 products described above, there are at least 9 products are to be close to reaching commercial market within next 5 years.

1. Aprotinin: Protein that inhibits serine protease, which suppress the systemic inflammatory response during surgery. Recombinant bovine aprotinin from Tg corn

seed was first repoted by (Zhong et al, 1999) using particle bombardment – 0.068%TSP Through Agrobacterium – 8.9% TSP.

2. Collagen: It is a structural protein currently rendered from hooves and connective tissues of animals. Vast quantities of collagen are consumed in the form of 'gelatin'. Collagen used in areas of arterial sealants, drug discovery. Gelatin used in the stabilization and delivery of vaccines and drugs in capsules, soft gels, and plasma expanders. The first report of human collagen produced in plants was by Ruggiero et al 2000 through agrobacteria mediated transformation in tobacco.
3. Human gastric lipase:

   It's a protein involved in the condition known as exocrine pancreatic insufficiency (EPI). Absence of this resulted in the inability to digest food lipids. The production of canine gastric lipase produced in Tg tobacco has been reported.
4. Human lactoferin: Is a natural defense iron – binding milk protein that purportedly possesses antibacterial, anti fungal, antiviral, anti-inflammatory properties. Lactoferin pharmaceutical produced in Aspergillus sp was first reported in tobacco suspension cells by (Mitra & Zhang 1994).

Companies involved in molecular farming:

Company	Location	Crop
Meristem therapeutics	Clermont-Ferrand	Maize
Croptech	Charleston,S C	Tobacco
Plant Genix	Philadelphia,Pa	Various
Large scale biology	Vacaville, Calif	Tobacco
Monsanto Pr Tech	St Louis Mo	Maize
SemBio sys	Calgary,Canada	Safflower
Medicago	Quebec city,Canada	Alfalfa
Ventria	Sacramento,Calif	Rice
Epicyte Pharmaceutical	San Diego,Calif	Maize
Planet Biotechnology	Hayward,Calif	Tobacco

Success stories

Human epidermal growth hormone used for wound repair is produced @ 0.01% of plants total soluble protein, giving a 1Ac crop of tobacco estimated value of $175000. Human protein Anticoagulant is produced @ 0.01% TSP with the estimated value of $2,000,000 per Ac. Human serum albumin used as a intravenous protein @0.02% could provide $2,000,000 worth product.

Disadvantages:

•Require keen skill

•Expression of proteins quality is low

•Non preference

Safeguards

Make them different looking:

•Bioluminescence – Firefly luciferase & Green fluorescent protein.

•No destruction of plant tissues & no use of substrate.

•Biopharmaceutical companies using purple, blue, black maize breeds.

•Cross contamination seen easily

Terminator gene technology: Monsanto

•Beneficial in stopping contamination of food crops in open field.

•Stop gene transfers because hybridization relies on fertile gametes.

•Production is suppressed by this gene

•Reversible nuclear genetic system

•Male sterility

Reporter genes

•Easy to test contamination.

•Lack of easily visible signs.

•DNA barcoding.

Human health & environmental impacts:

Compounds from plants developed for molecular farming may have physiological effects on humans other organisms. Workers or bystanders accidentally eat or contact with plants during production. Livestock and wild life accidentally eating plants during field production. Pollen movement to the same or related species (Bhatia, 2003). Unintentional introduction of the plant materials into food or livestock feed supply chains.

Constrains in molecular farming:

Larger isolation distance than required for other PNTs. Additionally toxicity & allergenicity in some cases. Careful disposal and destruction of all residual plant material. Just one mistake by a biotech company and we will be eating other peoples prescription drugs in our food or corn flakes.

Future research:

One of the keys to success in the future will undoubtedly be the level of expression of the recombinant protein in plants. This is one of the most important aspects with regard to economics. The expression level affects the cost of growing, processing, extraction, purification and waste disposal. It is clear that there will be a drive towards higher level of expression and there is much more room for improvement compared to other establishing systems (Horn *et al*, 2004)

Expression of protein action is also a regulatory concern. Whether or not the protein is in specific tissue will enable or nullify exposure to the environment. There has already been work to show that expression can be limited to specific tissues, thus reducing the regulating concerns. As an example, keeping the protein out of pollen can reduce inadvertent exposure to the environment.

Conclusion

There is no question that plant molecular farming is starting to come into its own as a viable new industry. Important questions concerning the glycosylation, immunogenicity, accumulation, and stability of the transproteins are being answered. Academic laboratories have been instrumental in elucidating much of the science behind the potential products and will continue to do so. However, the marketing and delivery of commercial products will necessarily fall to industry. As with any new industry, there have been hurdles to overcome, both technical and regulatory. However, the experience to date has taught us much and the industry is now poised for rapid growth and profitability.

Discussion

1. What is the difference between normal farming and molecular farming?

Here crops are genetically engineered for a specific purpose and cultivated in a large scale in molecular farming. The use of normal plant products is not being altered. The isolation distance required for molecular farmed crops are more. It requires >800m, in UK government put strict rules to keep isolation distance 7km. No contamination happens >3.5 km by insect, bird, wind.

2. What does protein trafficking mean?

The protein produced move through the endoplasmic reticulum and Golgi apparatus for processing, folding and glycosylation.

3. Why gene silencing is low in chloroplast engineering?

a. In transplastomics transgene integration is normally through homologous recombination.

b Chloroplasts contain prokaryotic genome.

c. High copy number is integrated i.e. 10,000 copies /cell.

4. Any toxic effects by using molecular farmed products in humans?

For the improvement in the nutritional quality of legumes storage protein,

Gene responsible for a methionine – rich protein was from Brazil nut seed protein Brazil nut 2S albumin under the promoter of Phaseolin gene was introduced into tobacco Resulted in 30% increase in metheonine in Tg tobacco seed. Unfortunately this gene was found allergenic. So, this is replaced by sunflower seed albumin under the control of the Pea vicilin gene promoter has been transferred to lupin and methionine content increased two fold.

5. Which area you prefer for molecular farming?

Industrial area, Plantibodies.

6. Any special labels for the molecular farmed products?

In the product bottles it is to be written as expressed in corn. For eg. - Maize derived trypsin.

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ABSTRACT

Plant molecular farming is a new and promising industry involving plant biotechnology. The plant systems are used as bioreactors or biofactories for the production of proteins, pharmaceuticals and industrial compounds.

The estimated global area of transgenic crops in 2001 is 52.6 million hectares (James, 2003). A scant 15 years after first report of stable transformed plant, the use of transgenic plants to produce foreign protein with economic value was being realized. Although transgenic animals, bacteria and fungi are also utilized for the production of protein, plants are giving the highest economic benefit (Horn *et.al.*, 2004).

There are currently four methods of protein production from plants. In *Agrobacterium* mediated transformation the product can be stored with out refrigeration for long-term. Plastid transformation is the fresh tissue molecular farming system. The protein stability over time will change even with refrigeration.

The transient transformation of crops by viruses and stable transformation of plant species that are grown hydroponically are also practiced. The three protein products Avidin, β- glucuronidase, Trypsin are already in market and few are expected to come within five years. Vitamins and minerals, which alleviate vitamin A and iron deficiency, were incorporated in golden rice successfully (Philomina, 2004). Many of the industrial enzyme products are made through molecular farming. Plants also serve as a source for the production of biodegradable plastics, edible vaccines and phytoremediation purposes

The possibilities of genetically engineered crops out crossing with other crop cultivars of modern agriculture, traditional land races, wild, weedy, related species and consequent gene flow have emerged as a reality based on scientific data (Bhatia and Mitra, 2003). We would also like to see the companies which use purple maize as an extra precaution, as this would serve as an easy visual marker for any cross contamination.

It has been estimated that the cost of producing immunoglobulin in alfalfa, in a 250 m^2 green house, is $US 500- 600/g compared with the figure of $US 5000/g for a hybridoma produced antibody. Tobacco is the highest exploited crop in molecular farming because it possess little risk to the environment and can be grown in some of

the poorest countries of the world. Banana, tomato and potato are mostly utilized for the production of edible vaccines.

One of the keys to success in future will undoubtedly be the level of expression of the recombinant protein in plants. This is the one of the most important aspects with regard to economics. The expression level affects the cost of growing, processing, extraction, purification and waste disposal. Clearly there will be a drive towards higher levels of expression and there is much more room for improvement compared to other established systems.

PHARMACOGENOMICS

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(2004-11-36)
M.Sc. Agrl (Biotechnology)

SEMINAR REPORT

(PRESENTED On 19-11-2005 FOR THE PARTIAL REQUIREMENT OF THE COURSE. PBT 651)

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AND MOLECULAR BIOLOGY
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CERTIFICATE

This is to certify that the seminar report titled "Pharmacogenomics" has been solely prepared by Siny C.V. (2004-11-36), under my guidance, and has not been copied from any seniors, juniors or fellow students seminar reports

Vellanikkara
Date: 29/11/07

Dr.P A.Nazeem
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DECLARATION

I, Siny.C.V. (2004-11-36) hereby declare that the seminar entitled "Pharmacogenomics" have been prepared by me, after going through various references cited at the end and has not been copied from any of my fellow students.

Vellanikkara

Date 21-11-2005

Siny C.V.

2004-11-36

CONTENTS

ABSTRACT

The complete sequencing of the entire human genome by Human Genome Project has created a new level of opportunity in medical research. Pharmacogenomics is a science that examines the inherited variations in genes, that dictate drug response and explores the way these variations can be used to predict whether a patient will have a good response to a drug, a bad response to a drug or no response at all. The term pharmacogenomics originated from the two words pharmacology and genomics and is thus the intersection of pharmaceuticals and genetics.

Adverse drug reactions have been ranked as the fifth leading cause of death in the United States. It is estimated that 1,00,000 Americans die annually from adverse drug reactions (Adithan, 2002). In cancer therapy of acute lymphocytic leukemia, administration of drugs such as 6-mercaptopurines and 6-thioguanine can cause severe haematological toxicity or even death in patients possessing non functional variants of thiopurine methyl transferase There are more than thirty families of drug metabolizing enzymes in human. All have genetic variants, many of which translate in to functional changes in the protein encoded. An inter individual difference in drug response is due to sequence variants in genes encoding these proteins (Evans and Relling, 1999). The most common variations in the human genome are called single nucleotide polymorphism(SNP). It is estimated to be approximately 1.4 million SNPs in the human population, with an average of one in every 13,000 bases (Sachidanandan *et al* , 2001).

The vision of pharmacogenomics encompasses genetic profile for each individual containing sufficient information to select which drug are most likely to be safe and effective in that person. Genetic testing is commonly done by PCR, RFLP and Southern blotting. It is based on experimenting on a single gene. If one has to analyze a gene having 60 alleles then he has to do 60 separate experiments, which is time consuming. Newer technology like DNA microarray promises to monitor the whole genome on a single chip so that thousands of genes can be analyzed simultaneously (Joseph *et al* , 2002).

Instead of standard trial and error method of matching patients with the right drugs, doctors will be able to analyze a patient's genetic profile and prescribe the best available drug from the beginning. It is anticipated that an increased application of pharmacogenomics shall significantly improve the quality of future drugs in terms of their efficacy and safety profile. This will reduce the extent of their adverse drug reactions with subsequent reduction in the overall cost. Besides maximizing the useful effects of drugs, it will also lead to better patient compliance and accurate monitoring of the appropriate drug dosages.

Introduction

The 20th century has brought us a broad arsenal of therapies against all major diseases: infections, cardiovascular disease, neurological disorders, and mental disorders. However, drug therapy often fails to be curative and may in fact cause substantial adverse effects. Moreover, worldwide use of these drugs has revealed substantial interindividual differences in therapeutic response. Any given drug can be therapeutic in some individuals but ineffective in others, and some individuals experience adverse drug effects whereas others are unaffected.

Recognition of interindividual differences in drug response is an essential step towards optimizing therapy. Over the past decades, much evidence has emerged indicating that a substantial portion of variability in drug response is genetically determined, with age, nutrition, health status, environmental exposure, and concurrent therapy playing important contributory roles. To achieve individual drug therapy with a reasonably predictive outcome, one must further account for different patterns of drug response among geographically and ethnically distinct populations

The observations of highly variable drug response, which began in the early 1950s, led to the birth of a new scientific discipline arising from the confluence of genetics, biochemistry, and pharmacology called pharmacogenetics, it focuses on drug response as a function of genetic differences among individuals Applied to nontherapeutic foreign substances (xenobiotics), the equivalent term "toxicogenetics" is used

Human Genome Project

Two groups, the human genome consortium and Celera genomics announced successful completion of the sequencing of the human genome by human genome project jointly. This was the first draft of human

genome sequence. Human genome contains 3.5 billion base pairs. It contains 23 pairs of chromosome with 44 autosomes and two sex chromosomes. The total number of gene estimated is 30,000. The average size of gene consists of 3000 bases, but size varies greatly. Largest known human gene is dystrophin. It has 2.4 million bases. Almost 99.9 percent nucleotide bases are exactly same in all people. Function are unknown for 50 percent of discovered gene. Less than 2 percent of the genome codes for protein. Junk DNA make up 50 percent of the human genome. Repetitive sequences are thought to have no direct function. These repeats reshape the genome by rearranging it.

The complete sequencing of the entire human genome by human genome project has created a new level of opportunity in medical research. Pharmacogenomics is the study of how an individual's genetic inheritance affects the body's response to a drug. The term comes from the words pharmacology and genomics and is thus the intersection of pharmaceuticals and genetics.

Genomics is the study of how genes and genetic information's are organized within genome, and how this organization determines their functions (Singh, 2005). Cell is the basic structural unit of living things. Each cell typically contains an entire copy of organism's genome. Chromosomes are structures in which DNA and hence genes are housed. Chromosome is a long strand of the DNA double helix where bases are paired.

Mutations in Genome

One in every 1200 bases may be different in any human (David *et al.*, 2005). A change in single Base pair will alter amino acid sequence of the corresponding protein. This polymorphism is responsible for difference between how human respond to drug.

Mutations associated with Variations.

1. Missense Mutation.

In Missense mutation a nucleotide substitution changes a codon so that it codes for a different amino acid in the protein. The change may be harmful or beneficial to the protein.

2. Silent mutation

In silent mutation there is a substitution in the third location of mRNA codon. The resulting new codon may still code for the same amino acid because the genetic code is degenerate.

3. Nonsense mutation

A nonsense mutation is same as missense mutation except the resulting codon codes for a stop signal. The result is premature termination of translation & production of non functional protein.

4. Frame shift mutation

Frame shift mutation is caused by insertion or deletion of a base pair. This will shift the reading frame so that all nucleotide down stream from the point of mutation will be improperly grouped

Drugs

Drug is any substance that can be used to treat an illness (Mohammed, 1993). The word drug is derived from the German word droog which means dry since in the

past most of the drugs were dried plant parts. It is a lead compound that can modulate the target effectively.

Requirements of Drugs.

1. **Potency**-Drug should be able to modulate target effectively
2. **solubility**- Drug should be easily soluble in water for quicker Action
3. **Lipophilicity**- milder lipophilicity is required to penetrate plasma membrane.
4. **Bioavailability**- the term bioavailability is used to refer to the proportion of administered drug that has reached the circulation and that is available to have an effect.
5. **Protein Binding** – The specific protein binding is important. Non specific binding is undesirable.
6. **Toxicity** – should be less toxic
7. **Metabolic stability** – should not get destroyed quickly inside the body. A long shelf life is also desirable

ADMET Properties of Drug.

- Absorption
- Distribution
- Metabolism
- Excretion
- Toxicity

The tern is used in pharmaceutical research where the ADMET properties of new potential drugs describes how the drug is taken up, distributed within and eventually excreted from the body i.e. the pharmacokinetics, (Dandiya and Kulkarni, 1995). Toxicity refers to possible damage a drug or chemical compound may cause a person taking or being exposed to the substance.

Drug Absorption

Drug absorption is the absorption of drug from the site of administration to the circulatory system. Four major type of drug absorption are there.

1. Passive diffusion

Passive diffusion is most important and most common. If the drug present in the gastrointestinal tract in a greater concentration than that is in blood stream, then a concentration gradient is said to exist. Presence of concentration gradient will carry the drug through cell membrane into circulation.

2. Fascilated diffusion

Drug is transported across the cell membrane by combining with a carrier molecule

3 Active transport

This process work against the concentration gradient and energy to be expanded

4. Pinocytosis

Pinocytosis involves the cell membrane invagination and engulfing a fluid filled vesicle or sac

Factors affecting drug absorption

1. Surface area

The rate of dug absorption is greatest in the small intestine due to the large surface area provided by the villi

2. Gut pH

The pH of the gastrointestinal tract varies along its length. The change in environmental pH may have different effects on different drugs. Optimal absorption of a drug may be dependent on a specific pH

3. Blood flow

The small intestine has a very good blood supply which is the one reason why most of absorption occurs in this part of the gut.

4. Presence of food and fluid in the gastrointestinal tract.

The presence of food in the gut may selectively increase or decrease drug absorption For *e.g* Food increases the absorption of dicoumarol, while tetracycline absorption is reduced by the presence of dairy food.

5 Drug composition

Various factors pertaining to the composition of the drug may affect the rate at which it is absorbed for example Liquid preparations are more rapidly absorbed than solid ones, the presence of enteric coating may rapidly absorbed

Distribution

Drug is distributed through blood and organs such as heart, liver, kidney and brain receive most of the drug during the first minutes after absorption. These organs are highly vascular and acquire the drug very quickly. Level of drug in the bone, muscle, skin and adipose may take sometime to rise due to reduced vascularity. The patient's level of activity and local tissue temperature may also affect drug distribution to the skin and muscles. Binding to plasma membrane influences a drug's distribution because only unbound drug may passively diffuse from plasma into tissue. Drugs may accumulate in muscle, adipose, bone, stomach, intestine, etc. these tissues may represent sizable drug reservoirs, if drug binding to tissue is reversible. This has effect on drug availability. Fat tissue act as storage site for lipid soluble drugs.

Metabolism

Drug metabolism or biotransformation refers to the process of modifying or altering the chemical composition of the drug. Most of the drug metabolism occurs in the liver, where hepatic enzymes catalyze various biochemical reactions. Metabolic products are often less active than parent drug and may even be inactive. However some biotransformation products have enhanced activity or toxic properties including mutagenicity, (induce heritable alteration of DNA) teratogenecity, (production of birth defects) and carcinogenicity (cause cellular transformations).

Excretion

Kidney - Because the drugs are small particles, they are filtered into the kidney and then reabsorbed into blood stream. The process of hepatic biotransformation results in conversion of fat-soluble into water-soluble metabolites that are poorly reabsorbed once they filtered into renal tubule. Then it passed out through urine.

Lungs - highly volatile agents such as anesthetics will be excreted through lungs.

Liver - some drug and drug metabolite is excreted into bile and it enters the duodenum via bile duct and move through small intestine. If not reabsorbed by intestine, are excreted from the body through the body through feces.

Historical perspective of pharmacogenomics.

Pharmacogenomics is a science that examines the inherited variations in genes that dictate drug response and explores the ways this variation can be used to predict whether a patient will have a good response to a drug, a bad response to a drug or no response at all.

Biologists have long accepted that the capacity of organism to respond differently to their environment is genetically determined. Investigations into human physiology and chemistry during the mid 19th centaury accelerated by the emergence of organic chemistry, established that ingested chemicals are excreted in a different form. In 1902 two separate investigators Hucien Cuenot and Archibald Garrod discovered the connection between enzymes and genes. Garrod's work on Alcaptonuria constituted the first proof of Mendelian genetics in human. As a result of these studies, he advanced the hypothesis that genetically determined differences in biochemical process could be the cause of adverse reactions after ingestion of drugs (Smith *et al*., 2001).

More than 2500 years ago Pythagorus observed that ingestion of faba bean caused disease in some, but not all people who ate it. It look many years of study to pinpoint the cause of the adverse reaction that Pythagorus had observed haemocyclic anemia in those individuals who were deficient in glucose 6-phosphate dehydrogenare (Majer *et al*., 1965). This is probably one of the earliest recorded instances of metabolic genetic polymorphism.

Adverse drug reactions have been ranked as the fifth reading cause of death in the United Stated (Adithan, 2002). It is estimated that 1,00,000 American die annually from adverse drug reactions (ADRs). Any given drug can be therapeutic in some individuals, but ineffective in others and some individuals experience adverse drug effects where as others are unaffected. Each drug after it enters the body interacts with many proteins such as carrier proteins, transporters, metabolizing enzymes and multiple types of receptors. An inter individual difference in drug response are due to sequence variations in genes encoding these proteins (Evans and Relling, 1999). The most common variations in the human genome are called single nucleotide polymorphism (SNP). It is estimated to be approximately 1.4 million SNPs in the human population, with an average of one in every 13000 bases (Sachidanandan *et al.*, 2001).

Drug response also depends upon cause, severity, patient's age, drug interaction, sex, organ function, life style (smoking, alcohol consumption), environmental factors *etc*

Case studies

In the chemotherapy treatment of a common childhood leukemia mercaptopurines are used as medication. Thiopurine methyl transference converts mercaptopurines into an inactive metabolite called methylmercaptopurines. A small percentage of persons have genetic variants that prevent them from producing an active form of this protein. As a result thiopurines elevate to toxic levels in the patient because the inactive form of TPMT, is unable to break down the drug. Today , doctors ca use a genetic test to screen patients genetic profile for this deficiency and the TPMT activity is monitored to determine appropriate thiopurine dosage level (Pistoi, 2002)

During 2nd world war Primaquine haemolysis was much more common among African-American soldiers in the United States army who were taken antimalarial Primaquine. Subsequent study in the postwar period revealed that the cause of this drug induced haemolysis was a genetic deficiency of glucose 6 phosphate dehydrogenase (Carson *et al.*, 1956).

In 1950 doctors noticed that some patients suffered prolonged respiratory apnea (difficulty in breathing) after being treated with succinylcholine. This syndrome was found to be caused by mutations in gene for the enzyme butrylcholine estrase. Normally butrylcholine estrase in the blood degrades succinylcholine and the anesthetic effects of the drug wear off with time. But in patients with mutations that inactive or weaken butrylcholine estrase, the anesthetics persist in the body causing the dangerous side effects.

Isoniazid is an antituberculosis drug. N acetyl transferase is an enzyme metabolizing this drug and the gene coding for this enzyme is (nat2). These are many polymorphic alleles of the nat2 gene with reduced or accelerated ability to inactivate the drug isoniazid. Some individuals developed peripheral neuropathy in reaction this drug (Jones *et al*., 1954). Some alleles of the nat2 gene are also associated with susceptibility to various forms of cancer.

Drug metabolism

There are more than thirty families of drug metabolizing enzymes in humans (Oscarson *et al*., 1999). The cytochrome P450 (CYP) family of liver enzymes is responsible for breaking down more than 30 different classes of drugs. DNA variations in genes that code for these enzymes can influence their ability to metabolize certain drugs. Less active or inactive forms of CYP enzymes that are unable to breakdown and efficiently eliminate drugs from the body can cause drug overdoses in patients. Today, researchers use genetic tests for

variations in cytochrome p450 genes to screen and monitor patients. In addition, many pharmaceutical companies screen their chemical compounds to see how well they are broken down by variant forms of CYP enzymes (Hodgson and Marshall, 1998). Genes coding for CYP 450 system of enzymes are CYP2D6,CYP3A4,CYP3A5,CYP1A2,CYP2E1, CYP2C9,CYP2C19 etc. The field of pharmacogenomics began with a focus on drug metabolism, but it has been extended to encompass the full spectrum of drug disposition, including a growing list of transporters that influence drug absorption, distribution and excretion (McLeod and Evans, 2001).

CYP 2D6

Patients who are homozygous for the CYP 2D6 null alleles exhibit poor metabolizer phenotype with impaired degradation and excretion of many drugs. The enzyme coded by this gene metabolizes more than 40 drugs used in areas of cardiovascular disease and psychiatric disorder. Poor metabolizers are likely to exhibit adverse drug reactions. Frequency of this recessive trait ranges from 1-2 percent in Asians and 4-5 percent in Americans (Macinelli *et al*.,2000). Determination of patients CYP 2D6 phenotype/genotype may prove useful in treatment with antipsychotic drugs.

CYP 2C9 and CYP 2C19

CYP 2C9 constitute 18.2 percent of liver CYP 450 content. CYP 2C9 and CYP 2C19 enzymes are concerned with the metabolism of many currently used drugs. Substrate of CYP 2C9 include phynitoin, oral anticoagulants like warfarin, ancenocoumarol, oralhypoglycemic drugs like tobutamide, glipizide etc. CYP 2C9 and CYP 2C19 genes are polymorphically expressed. Amino acid substitution at codons 144 and 359 in the coding region of CYP 2C9 result in a 5 fold decline in metabolic activity. This may leads to drug toxicity.

A study was conducted at karnataka based on the family history. DNA was extracted from peripheral leukocytes and amplified by the standard PCR protocol . PCR products were digesteded with restriction endonucleases SmaI and Bam HI. The size of digestedd products was determined by 8% PAGE. Bands were visualized by staining with ethidium bromide. The genotype was identified based on the size of DNA fragments. Samples were sequenced, the frequencies of CYP 2C9*1 and CYP 2C19 *2 and CYP 2C19*3 were identified to be 60.25, 39.3 and 0.5 percentages respectively (Rosemarry *et al.*, 2004).

DRUG RECEPTORS

Receptors are structures that are located either on the surface of cell membrane or within cells themselves. One drug molecule occupies each receptor site and binding is reversible. Combination of drug with a receptor produces a specific response. Drug receptor interactions are analogous to enzyme substrate interaction. When a drug interacts with receptor, several chemical attractive forces are responsible for the initial interaction.

Genetic variations in drug receptors can have a profound effect on drug efficiency. Genetic polymorphism of the β2 adreno receptor can alter process of signal transduction. Three SNPs in ADRB2 have been associated with altered expression, down regulation, or coupling of the receptor in response to β2adreno receptor agonist. Single nucleotide polymorphism resulting in an arginine to glycine amino acid change at codon 16 and at glutamine to glutamic acid change at codon 27 are relatively common

A recent study of agonist mediated vasodilation and desensitization revealed that patients who where homozygous for arginine at ADRB2 codon 16 had nearly complete desensitization after continuous infusion of isoproterenol, with vasodilation decreasing from 44 percent base line to 8 percent after 90

minutes of infusion. In contrast patients homozygous for glycine at codon16 had no significant change in vasodilations regardless of their codon 27 status.

Genetic polymorphism in the apolipoprotein (APOE) gene appears to have a role in predicting response to therapy for Alzheimer's disease and to lipid lowering drugs. There are numerous allelic variants of the human APOE gene(e.g. APOEε3, APOEε4, APOEε5 etc) which contain one or more single nucleotide polymorphism that alter amino acid sequence of the encoded protein. In a study of treatment of Alzheimer's disease with tacrine, 83 patients without APOEε4 allel slowed improvement (Poirier *et al.*, 1995). Patients having APOEe4 genotype cannot overcome therapy.

There are two types of drugs, pro drugs and active drugs. Pro drug needs metabolization to work. Slow metaboliser have poor efficiency which leads to possible accumulation of drugs. Fast metabolizers have good efficiency with rapid effect

eg. Codein is metabolised by CYP 2D6 to Morphine

Active drugs are inactivated by metabolisation. Slow metabolizers have good efficiency,which leads to accumulation of drugs and produce adverse drug reactions. So such persons may need lower dose. Fast metabolisers have poor efficiency, and they need greater dose or slow release formulation.

eg. Omeprazole

Metabolisers

A. PM poor metabolizer - absent or greatly reduced ability to clear or activate drugs

B. IM intermediate metabolizer. Heterozygotes for normal and reduced activity

C. EM extensive metabolizer (normal)

D. UM ultra Metabolizer. Greatly increased activity (accelerated)

MICROARRAY

Genetic testing is commonly done by PCR, RFLP and southern blotting. It is based on experimenting on a single gene. If one has to analyze a gene having 16 allels then he has to do 60 separate experiments, which is time consuming. Newer technology like DNA microarray promises to monitor the whole genome on a single chip so that thousands of genes can be analyzed simultaneously (Joseph *et al.*, 2001). Micro arrays can be used in identifying mechanisms of toxicity in response to action of drugs and chemicals(Bulera, *et al.*, 2001). Micro array is a microscopic array of hundred to thousands of sDNA sequences from plants, animals and human tissues immobilized in a solid surface like glass or silicon.

Micro arrays are of two types.

1. Oligonucleotide array

Oligonucleotide arrays are generated by synthesizing oligonucleotide in a predetermined spatial orientation on a glass surface by a technique known as photolithography.

2. cDNA array

cDNA arrays are preparations of PCR amplified CDNA clones and spotted on a microscopic glass slide.

Steps

1. preparation of cDNA probes.

a. Isolation of RNA

b. Conversion of mRNA to cDNA

c. Labeling cDNA with fluorescent dye

2. Hybridization

If the target DNA has a region complimentary to the probe they will hybridize.

3. Slide scanning and image analysis

We can calculate fluorescent intensity of each spot. Based on that we can analyze the levels of expression of a gene.

Application in medical field

1. Micro arrays can be used in the grading of tumours, thereby improving the accuracy of progonosis (Alizadeh *et al.*, 2000).
2. micro arrays can be used in the detection of mutations.
3. cDNA microarrays can be used to identify amplified genes and monitor their expression levels in cancer(Heiskanen *et al.*,2000).

What are the anticipated benefits of pharmacogenomics?

- **More powerful medicine**

Pharmaceutical companies will be able to create drugs based on the proteins, enzymes, and RNA molecules associated with genes and diseases. This will facilitate drug discovery and allow drug makers to produce a therapy more targeted to specific diseases. This accuracy not only will maximize therapeutic effects but also decrease damage to nearby healthy cells.

- **Better, Safer Drugs the First Time**

Instead of the standard trial-and-error method of matching patients with the right drugs, doctors will be able to analyze a patient's genetic profile and prescribe the best available drug therapy from the beginning. Not only will this take the

guesswork out of finding the right drug, it will speed recovery time and increase safety as the likelihood of adverse reactions is eliminated.

More Accurate Methods of Determining Appropriate Drug Dosages
Current methods of basing dosages on weight and age will be replaced with dosages based on a person's genetics, how well the body processes the medicine and the time it takes to metabolize it. This will maximize the therapy's value and decrease the likelihood of overdose.

- **Advanced Screening for Disease**

Knowing one's genetic code will allow a person to make adequate lifestyle and environmental changes at an early age so as to avoid or lessen the severity of a genetic disease. Likewise, advanced knowledge of a particular disease susceptibility will allow careful monitoring, and treatments can be introduced at the most appropriate stage to maximize their therapy.

- **Better Vaccines**

Vaccines made of genetic material, either DNA or RNA; promise all the benefits of existing vaccines without all the risks. They will activate the immune system but will be unable to cause infections. They will be inexpensive, stable, easy to store, and capable of being engineered to carry several strains of a pathogen at once

- **Improvements in the Drug Discovery and Approval Process**

Pharmaceutical companies will be able to discover potential therapies more easily using genome targets. Previously failed drug candidates may be revived as they are matched with the niche population they serve. The drug approval process should be facilitated as trials are targeted for specific genetic population groups providing greater degrees of success. The cost and risk of clinical trials will be reduced by targeting only those persons capable of responding to a drug.

- **Decrease in the Overall Cost of Health Care**

Decreases in the number of adverse drug reactions, the number of failed drug trials, the time it takes to get a drug approved, the length of time patients are on medication, the number of medications patients must take to find an effective

therapy, the effects of a disease on the body (through early detection), and an increase in the range of possible drug targets will promote a net decrease in the cost of health care.

What are some of the barriers to pharmacogenomics progress?

Pharmacogenomics is a developing research field that is still in its infancy. Several of the following barriers will have to be overcome before many pharmacogenomics benefits can be realized.

- **Complexity of finding gene variations that affect drug response** - Single nucleotide polymorphisms (SNPs) are DNA sequence variations that occur when a single nucleotide (A,T,C,or G) in the genome sequence is altered. SNPs occur every 100 to 300 bases along the 3-billion-base human genome, therefore millions of SNPs must be identified and analyzed to determine their involvement (if any) in drug response. Further complicating the process is our limited knowledge of which genes are involved with each drug response. Since many genes are likely to influence responses, obtaining the big picture on the impact of gene variations is highly time-consuming and complicated.
- **Limited drug alternatives** - Only one or two approved drugs may be available for treatment of a particular condition. If patients have gene variations that prevent them using these drugs, they may be left without any alternatives for treatment
- **Disincentives for drug companies to make multiple pharmacogenomic products** - Most pharmaceutical companies have been successful with their "one size fits all" approach to drug development. Since it costs hundreds of millions of dollars to bring a drug to market, will these companies be willing to develop alternative drugs that serve only a small portion of the population?
- **Educating healthcare providers** - Introducing multiple pharmacogenomic products to treat the same condition for different population subsets undoubtedly will complicate the process of prescribing and dispensing drugs. Physicians must execute an extra diagnostic step to determine which drug

is best suited to each patient. To interpret the diagnostic accurately and recommend the best course of treatment for each patient, all prescribing physicians, regardless of specialty, will need a better understanding of genetics.

Challenges

- **Genome analysis for all individuals**

Rapid, automated methods must be developed to identify SNPs in the 3.5 billion-base-pair genome that influence susceptibility to disease and individual drug response.

- **Studying the biology of genes involved in disease and drug reactions**

It can take decades to study a gene's product, function and association to drug response

- **New techniques need to prove their worth**

SNP analysis and expression profiling are in their infancy, and few success stories used

- **Complex diseases really are complex!**

In reality, disease and drug response can involve hundreds of genes. Environmental factors such as age, nutrition and lifestyle can influence disease and drug response as well

Issues

- **Adopting new practices in healthcare**

Health care providers and pharmacists will have to become educated about new diagnostic tests and how to use them when treating and advising patients.

- **Who will pay for it?**

Today's methods for SNP analysis and expression profiling are expensive. Health insurance companies may not want to pay for extra diagnostic tests and economic issues might influence which drugs pharmaceutical companies choose to develop.

- **Ethical and privacy issues**

Identified genetic susceptibility to disease may have implications for employers and insurance companies. Who will have access to genetic information and databases?

CONCLUSION

Advances in genomic and proteomic sciences along with system biology have greatly influenced the design and approaches to the discovery and development of new drugs. It is anticipated that an increased application of pharmacogenomics shall significantly improve the quality of future drugs in terms of their efficacy and safety profiles. It will also reduce extend of their adverse drug reactions with subsequent reduction in the overall cost of their development. Besides maximizing the useful effects of drugs, it will also lead to better patient compliance and accurate monitoring of the appropriate drug dosages. Application of pharmacogenomics in new drug discovery shall have high impact on the overall cost and time duration of the development offering high competitive advantages to the companies that successfully incorporate these technologies.

Discussion

1. what about faba been causing favism?

Ans: *Viccia faba*- It is a cool season vegetable grown in hilly areas

2. Common name of faba been?

Ans: broad been

3. protein which act as hormone?

Ans: insulin

4. Scientist behind the human genome project?

Ans: Craig ventor

5. When did the human genome sequencing started?

Ans: The project officially began on october 1- 1990

6. What do you meant by teratogenicity?

ans Teratogenicity means production of birth defects

7. How mutation will effect the drug absorption?

Ans Presence of food in the gut may selectively increase or decrease drug absorption For *eg* food increases the absorption of dicoumarol while tetracycline absorption reduced by the presence of dairy food

8. What is the function of dystrophine?

Ans It actually known as DMD gene . and its product is known as dystrophine It is a muscle protein

9. What are the diseases applied to pharmacogenomics?

Ans: cancer and cardiovascular diseases

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PROTEIN TRAFFICKING

CINU SEBASTIAN

(2004-11-32)

M.Sc. Agri. Plant biotechnology

SEMINAR REPORT

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COLLEGE OF HORTICULTURE

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DECLARATION

I, Cinu Sebastian (2004-11-32) hereby declare that the seminar entitled "Protein Trafficking" has been prepared by me, after going through various references cited at the end and has not been copied from any of my fellow students.

Cinu Sebastian
2004-11-32.

Vellanikkara
Date: 23-11-2005

CERTIFICATE

This is to certify that the seminar report titled "PROTEIN TRAFFICKING " has been solely prepared by Ms. Cinu Sebastian (2004-11-32), under my guidance, and has not been copied from any seniors, juniors or fellow students seminar reports.

Vellanikkara

Date: 29-11-05

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PROTEIN TRAFFICKING

INTRODUCTION

The eukaryotic cell has evolved highly elaborate subcellular organelles or structures that specialize in distinct biochemical and biophysical processes. The biogenesis of organelles, progression of localized biochemical reactions and cellular integration of these processes involve extensive subcellular traffic and localization of a wide variety of molecules. Within an organelle, spatially clustered biochemical reactants also require traffic and precise localization of all molecules involved. Thus organized traffic and localization are hallmarks of a living cell. Elucidating the underlying pathways and mechanisms of protein trafficking has vast importance in understanding how biological processes at the individual level are integrated at the whole plant level. This forms the basis of how a plant grows, develops and deals with biotic and abiotic stress.

PROTEINS

The term 'proteins' was derived from the Greek word 'proteios' meaning first. Proteins are the most prominent macromolecules in the cell making up over fifty percent of the dry weight of cells. They are found in every part and every cell of the organism and are fundamental aspects of cell structure and function. Proteins are the direct products and effectors of gene action. Proteins are informational macromolecules and each protein molecule contains a specific information rich sequence of just 20 amino acids (Lehninger, 2002).

Proteins have diversified functions in the living organisms. They are known as the building blocks of the organism.

- Some act as catalysts for biological functions (enzymes)
- Defend body against invasion of foreign organisms i.e., antibodies like gamma globulins.
- Form food reserves e.g. Ovalbumin of egg, casein of milk.
- Act as hormones e.g. Insulin maintains sugar level in blood.

Thus proteins constitute an important group of vital molecules with highly divergent activities. But these wonderful molecules have to be at the right place at the right time. This is where protein trafficking comes to play.

Protein Trafficking

Protein trafficking is the mechanism by which the biological cell transports proteins to its proper destination to have the desired effect. It is more complex in eukaryotes because of the presence of many intracellular compartments like nucleus, mitochondria, peroxisomes, chloroplast, endoplasmic reticulum, golgi bodies etc.

Need for Protein Trafficking

The trial and error process, nature has used for improving the metabolic machinery has led not only to great versatility but also to an intricate control system. This is aimed at producing codes that prevent wasteful overproduction of metabolites. The bioengineer, in contrast, wants to convert a substrate into some product as efficiently as possible with a minimum waste of any protoplasm that is non functional (Heden, 1997). Some enzymes can inhibit their own synthesis, upon accumulation. This is known as feed back mechanism. High concentrations of biosynthetic enzymes and products can be obtained by blocking the build up of an end product or it's derivatives, which is possible by the knowledge about protein trafficking.

Molecular farming is the application of molecular biology techniques for the synthesis of commercial products in plants. The interest of such proteins comes in part from the problems associated with existing fermentation and bioreactor systems for protein production. Mammalian cell systems are expensive and cannot easily be scaled up. Bacterial systems can be scaled up, but often the recombinant proteins not properly processed i.e., they are not properly folded and disulphide bridges are not formed, so intracellular precipitation of non-functional proteins can occur. Plant systems can be easily scaled up and are able to fold, crosslink and post translationally modify even non-plant proteins sufficiently well to ensure that functional proteins are obtained. The production of recombinant

proteins in plants also benefits from the ability to traffic proteins to sub cellular components where processing occurs, or where the proteins may be more stable (Slater *et al*., 2003). Targeting proteins to seeds and tubers increases stability of recombinant protein from weeks to years.

Production of herbicide resistant plants often require transgenic products to be deliberately targeted to chloroplast as most of the potential pathways of herbicide resistance occurs in chloroplast. The knowledge on protein trafficking also assists in sophisticated drug design. Knowing the mechanisms and pathway can help in the regulation of diseases like cancer.

To know where the proteins are before trafficking the different steps in proteins synthesis like transcription and translation have to be discussed.

Transcription

Genetic information is stored in the DNA (deoxyribonucleic acid), the informational macromolecule of the chromosomes. This information instructs each cell to produce a characteristic set of proteins. The genes remain in the chromosomes and the message is first enzymatically transcribed to message RNA (mRNA) whose nucleotide sequence is complementary to that of DNA of the gene. The different steps involved are initiation , elongation and termination.

Initiation occurs with the binding of RNA polymerase to a DNA molecule at a specific position at attachment sites called the promoters. During elongation stage of transcription, the RNA polymerase moves along the DNA, reading the template in the 5'- 3' direction. A RNA-DNA hybrid is formed in the unwound regions. As the RNA polymerase moves further along the DNA template, the newly synthesized RNA strand is displaced and the DNA duplex reforms behind the transcriptional complex. Termination signals are usually complementary palindromes. They exert their influence by enabling a stem loop to form in the growing RNA molecule. Possibly one of the transcription factors detaches from the complex at the end of the gene, destabilizing the complex and leading to the fall off the template at some later point (Chawla, 2002)

Translation

Translation is the process by which the information in the mRNA is decoded into a pdypeptide chain. This process is carried out by ribosomes and t RNAs (transfer RNA). The coding triplets or codons on mRNA, which are complementary to those in DNA, serve as immediate template and provide genetic information specifying the sequence of amino acids during protein biosynthesis on ribosomes. The tRNAs bring the amino acids which are linked by peptide bonds, in association with ribosome, forming the polypeptide. There are three major steps in the synthesis of the polypeptide are chain initiation, elongation and termination.

The first step is the attachment of the small 40S subunit of ribosome to the mRNA molecule. In eukaryotes, the small subunit of the ribosome recognizes the cap structure as its binding site and therefore attaches to the extreme 5' end of the mRNA. The translation starts when an aminoacylated tRNA base pairs with an initiation codon that has been located by the small subunit. Elongation begins when the correct aminoacylated tRNA enters the A site and base pairs with second codon. A peptide bond is formed between the two amino acids which are in close contact in the two sites of thee ribosome. Ribosome shifts one codon in the 3'direction expelling the uncharged tRNA and making the A site vacant again. The third aminoacylated tRNA enters the A site and the elongation cycle is repeated. Termination of translation occurs when a terminator codon (UAA,UAG,UGA)enters the A site. There are no tRNA molecules with anticodons to base pair with any of these terminator codons.

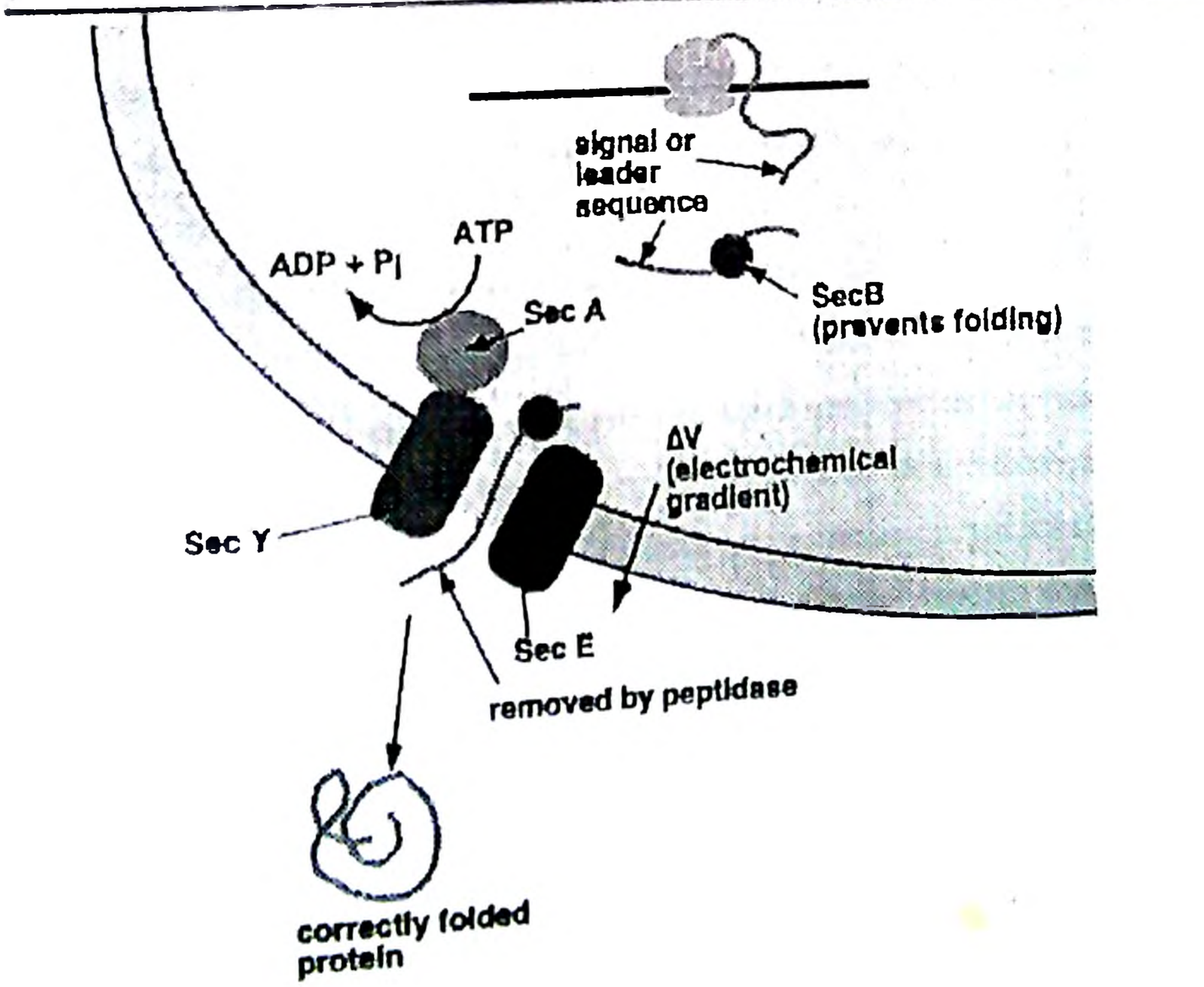
Free cytoplasmic ribosomes are employed in biosynthesis of proteins intended for internal use whereas ribosomes that are attached to outer surface of endoplasmic reticulum are used to make proteins to be secreted outside the cell. Trafficking is essential for proteins destined to work outside the cytoplasm and reach specific sites.

Trafficking In Prokaryotes

The chaperone protein **SecB** binds to the nascent polypeptide chain to *prevent premature folding* which would make transport across the plasma membrane impossible. **SecE** and **SecY** are transmembrane components which form a pore in the membrane through which the still unfolded polypeptide is threaded. The translocation process is energy-dependent (ATP) and is driven by **SecA**. Once the protein has passed through the pore, the signal sequence is cleaved off by an extracellular, membrane-bound ***protease***.

N-terminal signal sequences of representative secreted prokaryotic proteins.

Protein	-20 -10 -1 +1
Leucine-binding protein	MKANAKTIIAGMIALAISHTAMA EE...
Pre-alkaline phosphatase	MKQSTIALALLPLLFTPVTKA RT...
Pre-lipoprotein	MKATKLVLGAVILGSTLLAG CS...



Protein trafficking in kinetoplastid protozoa

The kinetoplastid protozoa infect hosts ranging from invertebrates to plants and mammals, causing diseases of medical and economic importance. They are the earliest-branching organisms in eucaryotic evolution to have either mitochondria or peroxisome-like microbodies. Investigation of their protein trafficking enables us to identify characteristics that have been conserved throughout eucaryotic evolution and also reveals how far variations, or alternative mechanisms, are possible. Protein trafficking in kinetoplastids is in many respects similar to that in higher eucaryotes, including mammals and yeasts. Differences in signal sequence specificities exist, however, for all subcellular locations so far examined in detail--microbodies, mitochondria, and endoplasmic reticulum--with signals being more degenerate, or shorter, than those of their higher eucaryotic counterparts (Clayton *et al.*, 1995).

Proteins Trafficking in Eukaryotes.

Signals direct proteins into a targeting pathway or redirect them once they have reached the initial target (Gillham, 1994). Protein trafficking is complex in eukaryotes due to the presence of subcellular components. Secretory proteins and integral membrane proteins travel through the secretory pathway to a variety of destinations. Mutations that affect the trafficking of proteins are even associated with some human genetic diseases (Amara *et al.*,1992).

Many of the nascent proteins have to be modified after translation in order to become functional. Proteins can undergo a great variety of possible post translational modifications. Knowledge of signal peptides, which direct the proteins to different organelles, is of immense importance for understanding the sorting process that underlies the trafficking of proteins. Specific residues may be modified by phosphorylation, glycolysation or acetylation to regulate the activity and location of large number of proteins.

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Post-TranslationalModification

Release of a completed polypeptide chain from a ribosome is often not the last chemical step in the formation of a protein. Various covalent modifications often occur, either during or after assembly of the polypeptide chain. Most proteins undergo co- and /or post-translational modifications. Knowledge of these modifications is extremely important because they may alter physical and chemical properties, folding, conformation distribution, stability, activity, and consequently, function of the proteins. Moreover, the modification itself can act as an added functional group. Examples of the biological effects of protein modifications include phosphorylation for signal transduction, ubiquitination for proteolysis, attachment of fatty acids for membrane anchoring and association, glycosylation for protein half-life, targeting, cell:cell and cell:matrix interactions. Consequently, the analysis of proteins and their post-translational modifications is particularly important for the study of heart disease, cancer, neurodegenerative diseases and diabetes.

Glycosylation

Protein glycosylation is acknowledged as one of the major post-translational modifications with significant effects on protein folding, conformation distribution, stability and activity. Carbohydrates in the form of aspargine-linked (N-linked) or serine/threonine (O-linked) oligosaccharides are major structural components of many cell surface and secreted proteins

Phosphorylation

Reversible protein phosphorylation, principally on serine, threonine or tyrosine residues, is one of the most important and well-studied post-translational modifications. Phosphorylation plays critical roles in the regulation of many cellular processes including cell cycle, growth, apoptosis and signal transduction pathways.

Without a signal the protein will remain in the cytoplasm. Gunter Blobel gave the signal hypothesis for which he was honoured with the Nobel Prize in 1999.

Following is a partial time line of Günter Blobel's original, groundbreaking accomplishments:

1971 - Proposed, with David Sabatini, that information for translocation of secretory proteins across the endoplasmic reticulum membrane resides in the NH2 terminal sequence.

1975 - Developed the first cell-free system that faithfully reproduces protein translocation. This system became the paradigm for all other subsequently developed cell-free translocation systems (bacteria, mitochondria, chloroplasts, peroxisomes). More importantly, it provided the opportunity for extensive biochemical analysis of protein translocation. Expanded and shaped the proposal he and Sabatini made in 1971 into the "signal hypothesis."Determined the partial primary structure of signal sequences of several presecretory proteins by Edman degradation.

1977 - First demonstrated that a nucleus-encoded, cytosol-synthesized protein of the chloroplast stroma is synthesized as a precursor, consistent with the idea that it contains a transient, chloroplast-targeted signal sequence.

1978 - Provided the first example of an integral membrane protein shown to contain an NH2 terminal sequence extension that is the structural and functional equivalent of the signal sequence of pre secretory proteins. Established for the first time a cell-free protein translocation system that mimicked the integration of a bacterial integral membrane protein into the bacterial plasma membrane. First demonstrated a membrane-associated bacterial signal peptidase.

1979 - First demonstrated that nucleus-encoded, cystosol-synthesized mitochondrial matrix proteins are synthesized as larger precursors, and developed the first cell-free system that mimics protein import into mitochondria. First

achieved the cell-free synthesis of a precursor for a lysosomal protein and translocation into microsomal vesicles. Elucidated, by Edman degradation, the primary structure of the first chloroplast stroma-targeted signal sequence.

1980 - Extended the signal hypothesis to a general hypothesis on intracellular protein traffic and membrane biogenesis, and proposed the concept of "topogenic" sequences. First isolated component-catalyzing, signal sequence-mediated translocation across the ER.

1981 - Showed that the isolated protein specifically recognizes signal sequences of nascent presecretory proteins, and named it the "signal recognition protein," or SRP. Postulated the existence of an "SRP receptor" in the microsomal membrane. Elucidated the primary structure of the first signal sequence for a lysosomal protein

1982 - Showed that SRP contains a 7S RNA molecule in the stoichiometry of one 7S RNA and one each of six different proteins. The term SRP now stands for "signal recognition particle."Purified the predicted SRP receptor from microsomal membranes.Elucidated the first primary structure for a signal sequence that targets proteins to mitochondria, this time by cDNA cloning rather than by Edman degradation.

Specific signals have been identified that are responsible for targeting prot to different organelles. The important subcellular components and their targeting signals are given below.

Sub cellular component / organelle	Signal
ER	a. HDEL/KDEL/RDEL motif
Vacuole	b. LQRD (Vacuolar targeting sequence)
Nucleus	c. Nuclear localization signal (NLS)
Peroxisomes/Glyoxisomes	d. SKL motif
Chloroplasts	e. N terminal transit peptide

Mitochondria

f. N terminal transit peptide

R – Arginine, H- Histidine, Q – Glutamine, D – Aspartic acid, S – Serine, E- Glutamic acid, L- Leucine, K - Lysine

a) Addition of this motif to other proteins may not be sufficient for retention in the ER but will slow transport.

b) May not be sufficient for targeting. Proper targeting may require protein folding to bring signal patches to surface of protein

c) NLS usually consists of a small region rich in basic amino acids (Arg & Lys) NLS are not absolutely conserved in either size or composition.

d) The addition of a C terminus SKL motif targets the protein to peroxisome in rice

e) The composition of transit peptide varies. They usually have no acidic residues but are rich in serine and threonine. Although the chloroplast has a genome, the majority of chloroplast proteins are encoded in nucleus, necessitating transport into the chloroplast.

f) They generally lack acidic residues and are enriched in alanine, leucine, serine and arginine.

These signals interact with specific receptors, either on the target organelle or on a carrier protein. There are 2 basic forms of targeting pathways.

• Post translational

Protein is transported after synthesis. This occurs in nucleus, mitochondria, chloroplast, peroxisomes.

- Co translational /secretory pathway

Transfer of membrane bound of secreted proteins into the ER during synthesis of the protein-ER, Golgi, lysosomes, plasma membrane etc.

In the absence of targeting signals, a protein will remain in the cytoplasm.

Nuclear targeting

It is an unusual pathway since 2-way traffic is involved. The trafficking of macromolecules between cytoplasm and nucleus through nuclear pore is mediated by specific carrier molecules such as members of the importin family (Stewart et al., 2001). DNA & RNA polymerases, trancription factors, histones etc. move into the nucleus and mRNA, t RNA, rRNA are trafficked out.

Proteins are transported through the complex nuclear pore. Proteins smaller than 20k Da move by diffusion. Those larger than 20k Da move by nuclear localization signals (NLS). They are a cluster of 4-8 positively charged amino acids eg. PKKKRLV. Signal sequence binds to importin. This complex then binds to Ran. Ran is a small GTP binding protein that assists in GTP hydrolysis. Ran mediates the passage through the nuclear pore. GTP is hydrolysed and protein is released. Importin and Ran are recycled to cytoplasm after use.

Mitochondrial Targeting

Mitochondria contain translocases for the transport of precursor proteins across their inner and outer membranes (Mishra, 2003) There are targeting sequences on the mitochondrial proteins that them get into the appropriate areas of mitochondrion after they are synthesized. These are specific receptor proteins on the outer membrane that bind these targeting sequences. The targeting sequences are a group of +vely charged at the N terminus of the protein, interspersed with some hydrophilic amino acids like serine and threonine which allow the protein to bind to mitochondrial receptor complex on the outer membrane. The receptor is

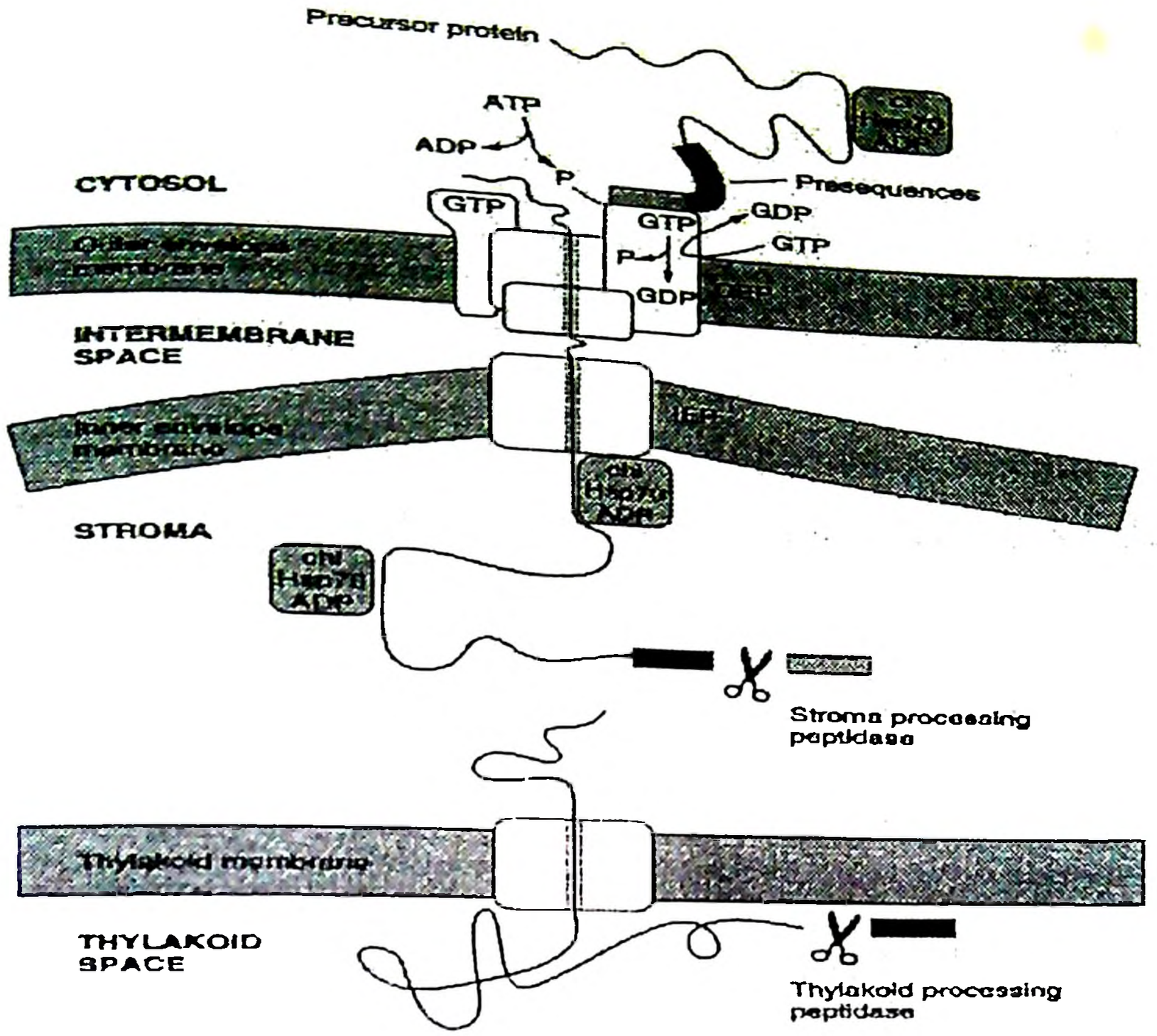
associated with a long pore that extends through both the outer & inner mitochondrial membrane. But these pores are narrow and a protein all folded up will not be able to pass through a pore unless it is unfolded. So mitochondrial proteins are prevented from folding, as they are being synthesized, by the chaperone proteins or chaperonins. In the cytosol, mitochondrial proteins are bound by chaperone protein typically hsp 70 or mitochondrial -import stimulating factor (MSF) which prevents folding and hydrolyses ATP for meeting the required energy. . Mitochondrial proteins are synthesized as precursor proteins inside the cell and are shuttled across the membranes by transport across outer mitochondrial membrane (TOM) and transport across inner mitochondrial membrane (TIM) complexes (Wiedemann *et al.*, 2003). The +vely charged signal sequence on the mitochondrial prot binds to receptor protein complex on the outer mitochondrial membrane. The binding part of the outer complex TOM is made up of two proteins called TOM (Transport across Outer Member) 70 & 37 (for their movements) & they then transfer the protein to two others Tom 22 & 20 which in turn transfer it to a channel made of TOM 40 protein. In short, five proteins make up the binding site on outer membrane pore of transfer complex. In the inner mitochondrial membrane is another protein complex TIM (Transport access Inner membrane). When the protein is driven through the pore, hsp 70 protein is displaced and a mitochondrial hsp70 chaperone binds to the protein at the expense of ATP. Proteins that will remain in the matrix are folded up by using ATP. The signal sequence is removed by a mitochondrial signal peptidase as it enters the matrix. Some proteins contain additional signal sequences that specify different locations, such as membrane or inter membrane space. The protein transport into the inner membrane space and inner membrane is by two ways :

- The protein is transferred first to the matrix, the N terminal signal sequence cleaved and the second signal sequence guides the protein to its location
- An alternative route, which avoids movement into the matrix but is blocked on the way. TIM 23 translocator in the inner member binds to the second signal sequence causing it to act as a stop transfer sequence. A

second signal peptidase in the intermembrane space removes the second signal sequence and releases the protein.

Chloroplast Targeting

Chloroplast biogenesis relies on the coordinate contributions of protein components from both plastid and nuclear genomes. The synthesis and import of nuclear-encoded chloroplast proteins is tightly regulated by communication between the two organelles. Protein import into chloroplasts is mediated by the activities of multimeric translocon complexes located in the outer (TOC complex) and inner (TIC complex) envelope membrane. However it is similar to the process in mitochondria. There are receptors of channels in the chloroplast outer & inner membranes, signal sequence on nuclear enclosed chloroplast protein, hsp 70 type chaperones that prevent premature folding and stromal chaperones that promote folding the plastid proteins are transported into chloroplast by recognition of a sequence of about 40-50 amino acids at the N terminal (transit peptide). This peptide directs translocation into stroma, where a specific peptide removes transit peptide. This is also a post translational targeting ie transported after synthesis. Transport process into stroma involves a complex protein import apparatus TOC (Transport across outer chloroplast membrane) and TIC(Transport across inner chloroplast membrane) using ATP & GTP. Targeting into thylakoids require a bipartite transit peptide. Removal of the stromal targeting signal sequence exposes a second transit peptide that acts as a luminal targeting peptide. This directs the protein across the thylakoid membrance into the thylakoid lumen, where it is also removed by a specific protease.



Peroxisomes

All peroxisomal proteins are encoded by nuclear genes. The signal for uptake into peroxisomal matrix is SKL (serine -lysine-leucine). The carboxy-terminal residues of several peroxisomal proteins were shown to act as a peroxisomal targetting signal. Studies have been conducted to test whether the C-terminus of glycolate oxidase, a key enzyme in the glycolate metabolism pathway, is functioning as a targetting signal that directs proteins into plant leaf peroxisomes. A chimeric gene coding for a fusion protein composed of the C-terminal- truncated beta-glucuronidase, a synthetic linker of four amino acids and the last six C-terminal amino acids of glycolate oxidase, was constructed. Transformation of tobacco plants with the chimeric gene resulted in expression of beta-beta-glucuronidase enzymic activity. About 50% of the transgenic beta-glucuronidase activity was localized to the peroxisomes. The results indicate that

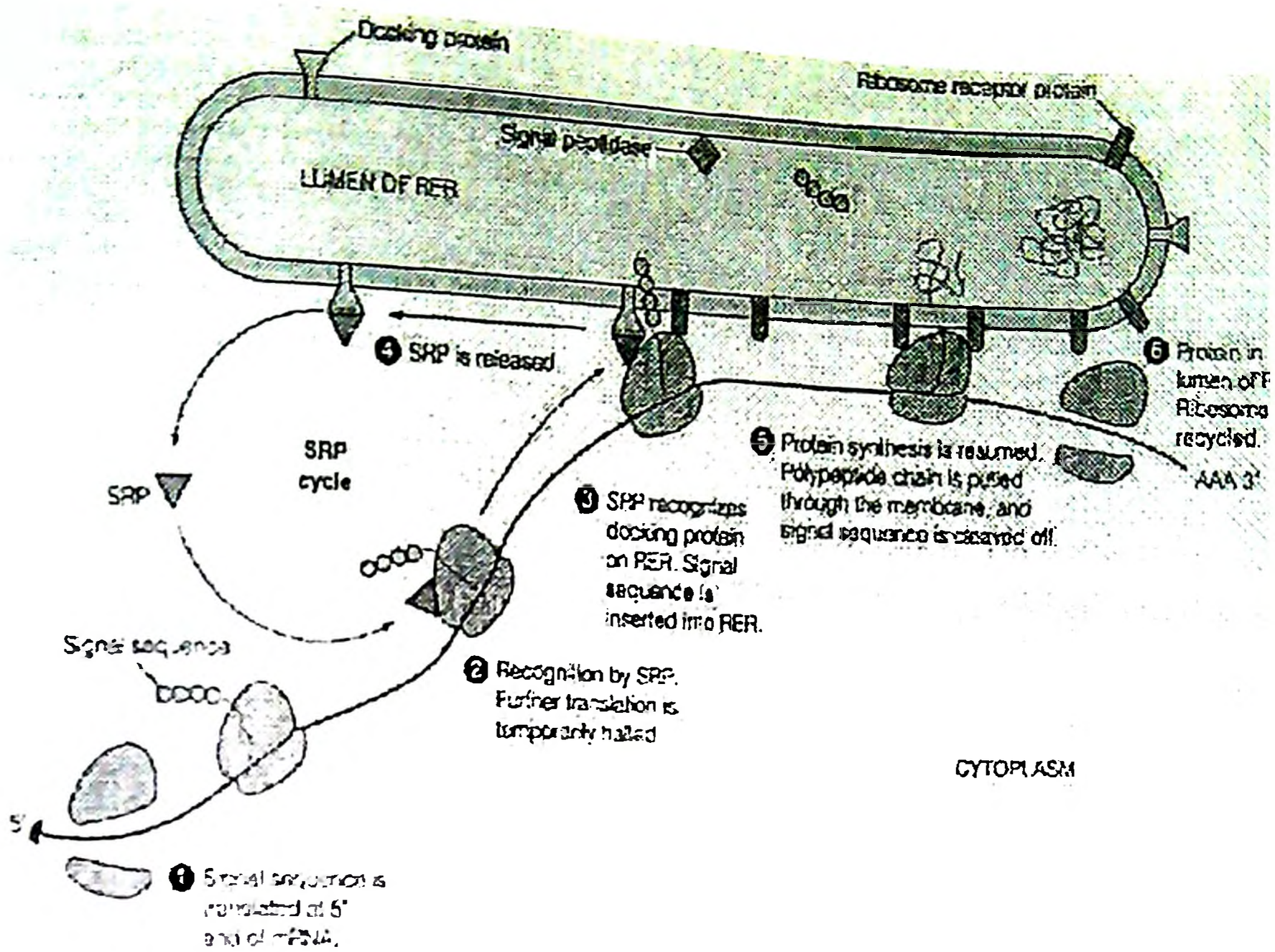
the six C-terminal amino acid residues of glycolate oxidase act as a targetting signal that is recognized by leaf peroxisomes (Volokita, 1991).

Vacuole Targeting

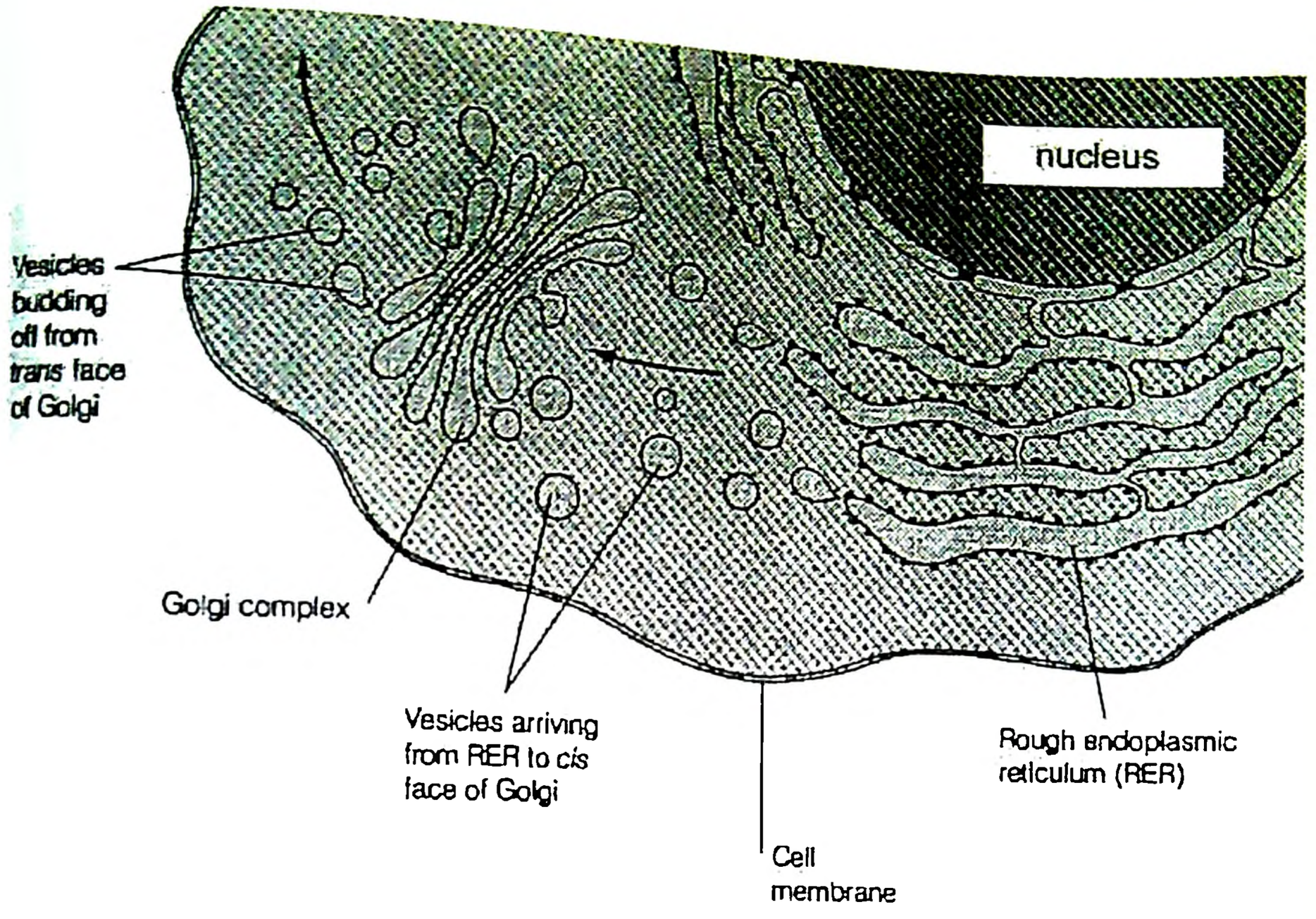
The mechanisms of protein sorting & transport from the ER into vacuoles are under study. Recently it has been revealed that plant cells have a complex vacuolar system. Some cells contain atleast two functionally distinct vacuoles; one with lytic function and other could be a storage compartment. Storage proteins such as legumin are stored within the cell in special vacuolar compartments that are distinct from the lytic vacuole known as storage protein vacuoles (Smith, 1999). So there are several types of vacuolar sorting determinants (VSD). A C terminal VSD of phaseolin and internal sequence specific VSD of ricin have been determined. Experiments are going on for finding the sorting receptor for the C - terminal VSDs.

Secretory Pathway

It is a co-translational tranloscation. The N terminal signal sequence is recognized by a signal recognition particle (SRP) while the protein is still being synthesized on the ribosome. The ribosome-protein complex binds to SRP and the translation is paused. The 6 protein subunits of the eukaryotic SRP are organized into two functional domains: a smaller one and a larger one. The smaller domain is responsible for translational regulation (halting), while the larger domain is responsible for signal sequence recognition and binding to the SRP receptor. SRP is the critical subunit that recognizes the signal peptide of the nascent protein chain. The SRP complex (SRP, signal sequence, mRNA, ribosome) docks with receptor on ER. Signal sequence crosses ER membrane. Translation continues and polypeptide chain is pulled into the ER lumen. By the time protein synthesis gets completed, there will be so many polypeptides in ER. They will be cleaved by signal peptidase and polypeptide gets detached from signal & signal sequence gets degraded.



This is followed by processing glycolysation ie adding sugar residues like mannose. It is then transported to the golgi bodies. The golgi transport the proteins to the final destination. The membranes of both golgi & ER are similar. So the vesicles pinched off from ER attaches to cis cisternae of golgi. Maturation of the protein occurs through cisternal progression upto trans cisternae by exocytosis. Exocytosis is the pinching of vesicles along with a part of the membrane. The last vesicle from trans cisternae is called the secretory vesicle, which gets transported, to the final destination. Many of them get integrated into plasma membrane and some get out of plasma membrane to other sites of action.



There are two different pathways - constitutive secretion and regulated secretion. In constitutive secretion proteins are packaged in vesicles and are secreted immediately via exocytosis around the cells. They secrete continuously and (unlike the regulated) no external signal is needed to stimulate the process. Cells that secrete constitutively have many golgi scattered around. Fibroblasts, osteoblasts and chrondocytes are those that use this pathway. In regulated secretion, proteins are packaged similarly, but secreted only in response to a specific signal, such as neural or hormonal stimulation. Cells that use this pathway are usually apical or polarized. Goblet cells (secrete mucus), β cells of pancreas (insulin) and odontoblasts (dentin) are those that use this pathway

How to study

Green fluorescent protein (GFP) obtained from the jellyfish - *Aequorea victoria* is a spontaneously fluorescent protein, which is finding wide use as a genetic marker that can be directly visualized in the living cells of many heterologous organisms. It is used to monitor gene expression, signal transduction, transformation, protein trafficking and localization, protein – protein interaction etc. GFP is mainly localised within nucleoplasm and cytoplasm of transformed *Arabdiopsis* cells and can give rise to high levels of fluorescence but it proved difficult to efficiently regenerate transgenic plants from such highly flourescent cells. However, when GFP is targeted to ER, transformed cells regenerate to give fluorescent plants (Haseloff *et al.*, 1997). The GFP reporter appears to be superior to others and provides a powerful visual tool to study organelle targeting in living cells and to select mutants with abnormal protein localization in intact plants (Chiu *et al.*, 1996).

Conclusion

Protein trafficking is an integral part of functioning of proteins. Protein trafficking begins with targeting of proteins to their proper destinations. It is complex in eukaryotes where a number of subcellular components are involved. Knowledge on protein trafficking plays an important role in many fields like molecular farming, metabolic engineering, enzyme engineering etc.

Discussion

1. What is Zellweger syndrome?

A) Zellweger syndrome is a rare congenital disorder characterized by reduction or absence of peroxisomes in cells of liver, kidney and brain. Symptoms are enlarged liver,high Fe and Cu levels in blood and vision disturbances. Lack of muscle movement is observed in infants. Sometimes it is associated with jaundice also.

2. What is the stop codon in transcription?

A) Stop codons are used for termination of translation. Termination signals are usually complementary palindromes. They exert their influence by enabling a stem loop to form in the growing RNA molecule. Possibly one of the transcription factors detaches from the complex at the end of the gene, destabilizing the complex and leading to the fall off the template at some later point.

3. How is GFP superior over GUS ?

A) GUS is large, requires an exogenous substrate and have leakage problems as the indigo dye often precipitates diffusely. It is a destructive analysis GFP , on the other hand , is spontaneously fluorescent and can be easily scored and used in living intact cells. It's expression is cell autonomous and independent of cell type and location. GFP is stable to photobleaching, oxidation and reduction and chemical reactions.

4. What is hsp 70?

A) Hsp stands for heat shock protein and 70 for the molecular weight. These are heat induced proteins and help to overcome heat stress

5. What is the application or importance of protein trafficking?

A) Protein trafficking can be utilized for transporting useful proteins to the desired plant parts. It is applied in molecular farming where

commercial products are synthesized by plants itself in the economic parts of the plant. Signal peptides have been manipulated using base analogs, which have found their application in cancer therapy. Transport and storage of different proteins to the vacuoles for storage are being studied. Study of adhesion proteins - cadherins which are important in tumor suppression and trafficking of Tumor Necrosis Factor (TNF) which are the major disease causing entity in inflammatory bowel disease, rheumatoid arthritis etc) are on the way.

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ABSTRACT

The eukaryotic cell has evolved highly elaborate subcellular organelles or structures that specialize in distinct biochemical and biophysical processes. The biogenesis of organelles, progression of localized biochemical reactions and cellular integration of these processes involve extensive subcellular traffic and localization of a wide variety of molecules. Within an organelle, spatially clustered biochemical reactants also require traffic and precise localization of all molecules involved. Thus organized traffic and localization are hallmarks of a living cell. Elucidating the underlying pathways and mechanisms of protein trafficking has vast importance in understanding how biological processes at the individual level are integrated at the whole plant level. This forms the basis of how a plant grows, develops and deals with biotic and abiotic stress.

Protein trafficking is the mechanism by which the biological cell transports proteins to its proper destination to have the desired effect. It is more complex in eukaryotes because of the presence of many intracellular compartments like nucleus, mitochondria, peroxisomes, chloroplast, endoplasmic reticulum, golgi bodies etc.

Signals direct proteins into a targeting pathway or redirect them once they have reached the initial target (Gillham, 1994). Nuclear targeting via the nuclear pore is done by nuclear localization signals. Mitochondrial proteins are synthesized as precursor proteins inside the cell and are shuttled across the membranes by transport across outer mitochondrial membrane (TOM) and transport across inner mitochondrial membrane (TIM) complexes (Wiedemann *et al*., 2003). The trafficking of proteins to the chloroplast has a similar mechanism and targeting into thylakoids requires a bipartite transit peptide (Slater *et al*., 2003). Secretory proteins and integral membrane proteins travel through the secretory pathway to a variety of destinations. Mutations that affect the trafficking of proteins are even associated with some human genetic diseases (Amara *et al*.,1992).

The feasibility of studying protein trafficking has gone a long way with the use of green fluorescent protein (GFP) from jellyfish *Aequorea victoria*. It is a vital spontaneously fluorescent marker to monitor signal transduction, transformation, protein trafficking and localization in living prokaryotic and eukaryotic cells. The GFP reporter appears to be superior to others and provides a powerful visual tool to study organelle targeting in living cells and to select mutants with abnormal protein localization in intact plants (Chiu *et al.*, 1996).

METABOLIC ENGINEERING

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COLLEGE OF HORTICULTURE

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DECLARATION

I, Shaneeja, V.M. (2004-11-33) hereby declare that the seminar entitled "Metabolic Engineering" have been prepared by me, after going through various references cited at the end and has not been copied from any of my fellow students.

Shaneeja VM

Vellanikkara

Date 26-11-2005

Shaneeja, V.M

2004-11-33.

CERTIFICATE

This is to certify that the seminar report titled "METABOLIC ENGINEERING" has been solely prepared by Ms. Shaneeja, V.M (2004-11-33), under my guidance, and has not been copied from any seniors, juniors or fellow students seminar reports.

Vellanikkara

Date 28.11.05

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INTRODUCTION

Biotechnology is the exploitation of living systems and organism for the benefit of man. Cell's metabolic networks that evolved in nature are not optimized for practical applications. In such cases, performance of metabolic pathway can be modified by genetic manipulation of cell. Manipulation of biosynthetic pathways in plants offers a number of exciting opportunities for plant biochemists and molecular biologists. This primarily aims at redesigning plant metabolism towards production of specific high value products for the sake of human welfare. This falls under the platform of second-generation transgenics. Recent advances in molecular biology techniques, analytical methods and mathematical tools have led to growing interest in using metabolic engineering to redirect the flow through metabolic pathways for industrial and medical purposes.

Metabolic engineering includes not only manipulation of endogenous metabolic pathways, but also the transplantation of metabolic pathways into new host organism. Metabolic engineering is generally defined as the targeted and purposeful alteration of metabolic pathways found in an organism in order to better understand and utilize cellular pathways for chemical transformation, energy transduction, and supra molecular assembly (Lessard, 1996). This multidisciplinary field draws principles from chemical engineering, computational sciences, biochemistry and molecular biology. In essence, metabolic engineering is the application of engineering principles of design and analysis to the metabolic pathways in order to achieve a particular goal such as the enhancement of process productivity (antibiotics, biosynthetic precursors, polymers etc), the extension of metabolic capability by the addition of extrinsic activities for chemical production or degradation etc Amino acids, vitamins, biopolymers, industrial chemicals, and chemical building blocks can be synthesized via metabolic engineering.

Previous strategies employed in metabolic engineering seemed to be more of an art with experimentation by trial and error. Later it was changed to a systematic and rational approach, which involves the use of recombinant DNA technology

and a better understanding of cellular physiology to modify intermediary metabolism (Bailey, 1991; Stephanopoulos and Vanillo, 1991).

Origin of metabolic engineering

The first stages of studies exploiting techniques to quantitatively analyze metabolic pathway information has been carried out with a technique using signal flow diagrams by researchers in the field of biochemical engineering in the 1970's. The technical term 'metabolic engineering' emerged in the 1990's and was defined as a targeted improvement methodology of product formation or cellular properties through the modification of specific biochemical reactions. The importance of analyzing the network rigidity in metabolic pathways (Stephanopoulos and Vanillo, 1991) and of recruiting heterologous activities, such as heterologous enzymes and transport systems (Bailey, 1991), has been emphasized.

Scope of metabolic engineering

Over the past decade, metabolic engineering has emerged as an active and distinct discipline characterized by its over-arching emphasis on integration. In practice, metabolic engineering is the directed improvement of cellular properties through the application of modern genetic methods. Although it was applied on an ad hoc basis for several years following the introduction of recombinant techniques metabolic engineering was formally defined as a new field approximately a decade ago. Since that time, many creative applications, directed primarily to metabolite overproduction, have been reported. In parallel, recent advances in the resolution and acquisition time of biological data, especially structural and functional genomics has amplified interest in the systemic view of biology that metabolic engineering provides (Stephanopoulose and Gill, 2001).

- In metabolic engineering, there is improvement of strains of living organism by mutation and selections, and there is opportunity to introduce heterologous

genes and regulatory elements. This makes metabolic engineering a very fascinating area of research. Scope of metabolic engineering are explained here with few examples:

- Modification of host cell or organism or the product formed by it.

Eg. Production of an erythromycin derivative, which is more stable at low pH of stomach by *Saccharopolyspora erythrae*

- Extended substrate range for growth and product formation.

Eg. Hemicellulose as substrate instead of glucose in alcoholic fermentation.

- Addition of new catabolic activities for degradation of toxic or unwanted chemicals.

Eg. Removal of acetate during bacterial fermentation.

- Modification of cell properties.

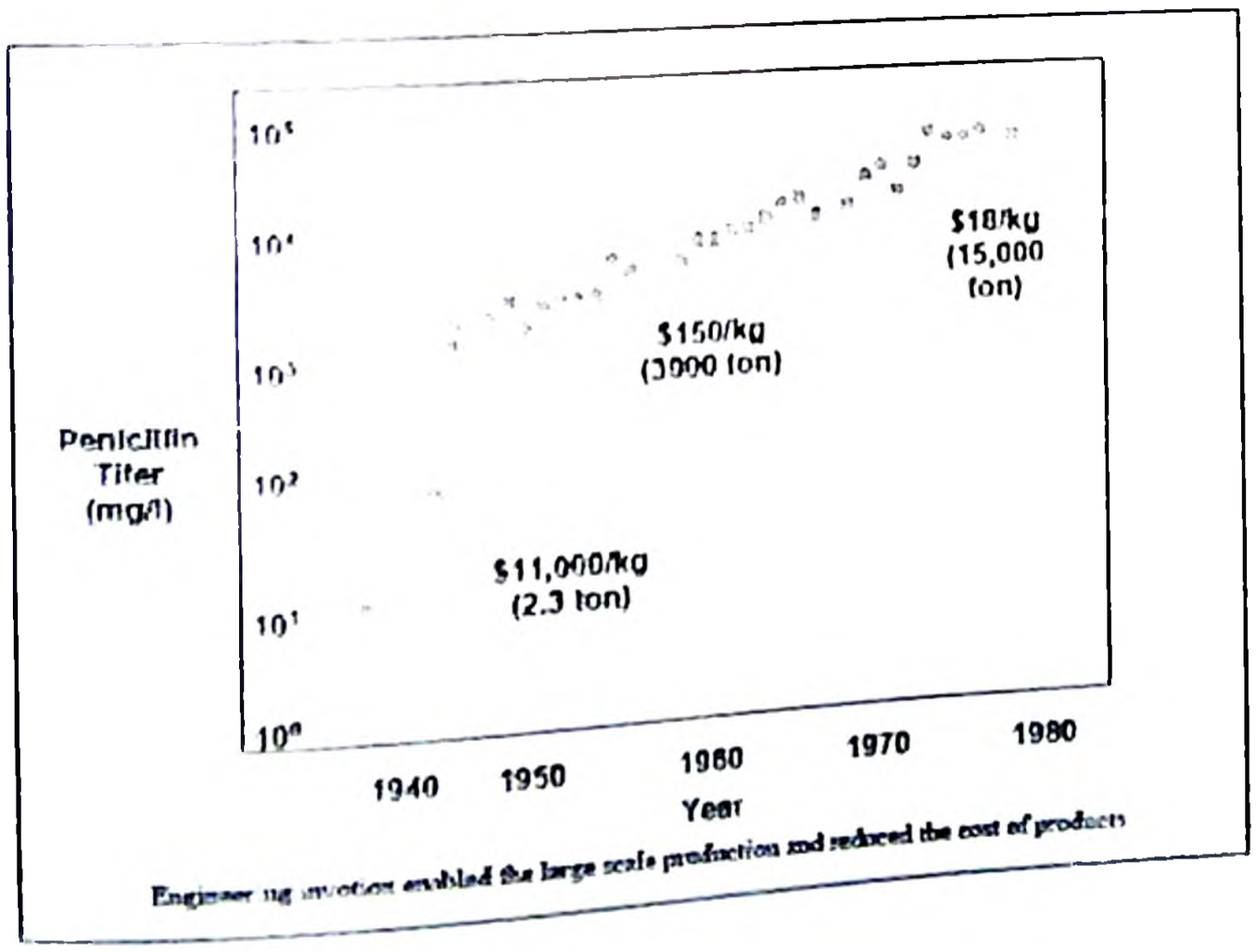
Eg. Strains having adaptation to various physical and chemical conditions.

- Production of chemicals new to the host organism by completion of partial pathways or by the transfer of entire biosynthetic pathway.

Eg. Melanin production in *E. coli*.

- Improved production of selective chemicals already produced by the host organism.

- Eg. Production of antibiotics like penicillin by *Penicillium chrysogenum*



Engineering invention enabled the large scale production and reduced the cost of products

Producing beer, wine, cheese, pharmaceuticals and other biotechnology products often involves metabolic engineering.

Basic concept

Cells will have an optimal use of their resources for their survival. Metabolic pathways are networks regulated to optimally distribute their fluxes. Metabolic engineering is to overcome the regulation to produce the product of our interest or to create a new product that the host cells normally don't need to produce.

It is based on concept of rigid network and flexible and rigid nodes (node means each step of the reaction network). This concept was firstly introduced by Stephanopoulos and Vanillo (1991). The rigidity of a network or its resistance to variations in metabolic change is due to control mechanisms established to ensure balanced growth For an engineering strategy to be successful, a better understanding of the host cell is necessary. The effects of genetic manipulation on growth and possible effects on 'unrelated' systems should be examined.

APPROACHES IN METABOLIC ENGINEERING

Classical approach:

In this approach, detailed study of enzyme kinetics, the system network, and intermediate pools involved is conducted and the genetic manipulation is proposed for some presumed benefits.

Inverse metabolic engineering:

Here, firstly a desired phenotype is identified and the environmental or genetic conditions that confer this phenotype are determined. Based on this, the phenotype of the selected host is altered by genetic manipulation (Delgado and Liao, 1997)

Eg. Expression of oxygen binding protein VHb in *E. Coli* the observed phenotype of high heme cofactor levels in an obligate aerobe *Vitreoscilla* under oxygen limitation suggested that synthesis of hemoglobin could improve growth of other organisms under similar limitations (Bailey *et al.*, 1996).

Strategies of improving the production of a metabolite (Nielson, 2001):

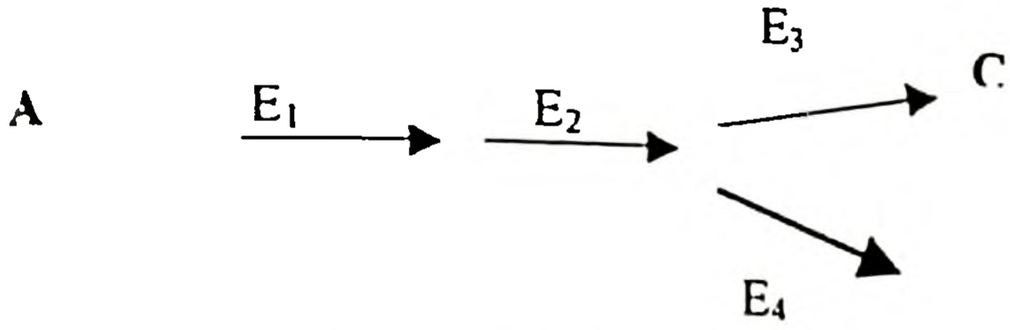
Strategies of improving the production of a metabolite (Nielson, 2001):

A). Modification of pathway

1. Amplification of rate limiting enzyme – Removal of bottleneck

Eg. In the following network E_2 is the rate limiting enzyme

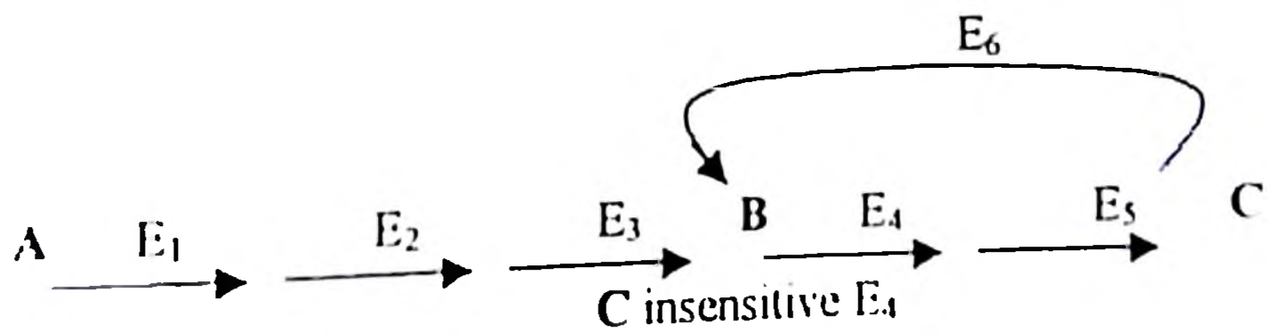
2. Amplification of the branch point enzyme – Metabolic conversion



Suppose in this reaction if we want to increase the concentration of B then amplify enzyme E_4.

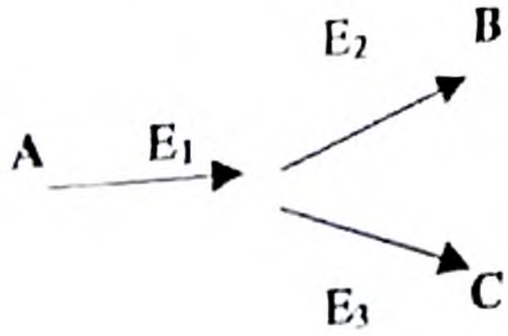
3 Remodeling the regulatory element of the protein by protein engineering or using a heterologous enzyme – By pass of bottleneck.

In the following example enzyme E_4 is sensitive to the concentration of product C and C is converted to B by an enzyme E_6. So to have an increased production of C, we will replace the E_4 by a C- insensitive E_4.



4 Introduction of heterologous enzyme with different catalytic mechanism – Redirection of metabolic flow

In the following example we will introduce an enzyme E_3 that can act on the substrate A to have a new product C



5. Amplification of the first enzyme in terminal pathways – augmentation of carbon flow and identification of potential bottlenecks.

6. Replace the enzyme(s) with other(s) that are energetically or kinetically more favorable.

B). Enhancing the precursor and energy supply by engineering the central metabolism.

C). Engineering the transport system.

1. Engineering the substrate and precursor uptake by increasing the rate of uptake or by changing the specificity towards the substrates or precursors.

2. Engineering the product secretion.

D). Engineering the tolerance to its own product or high substrate concentration.

E). Decoupling the growth and production.

General methodology for the development of efficient strains:

Identification of target phenotype

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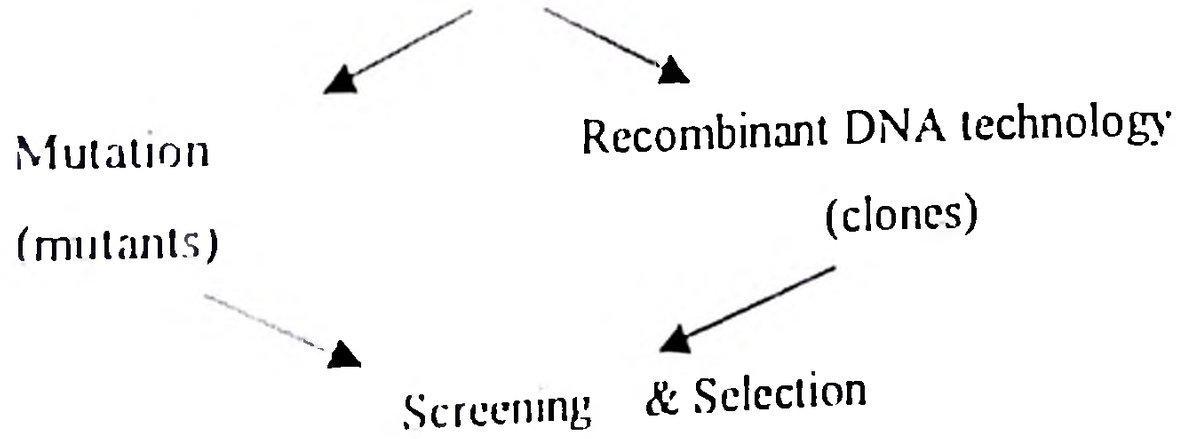
Increase the frequency of occurrence of genes that may confer the phenotype in the selected host.



Plant Metabolic engineering

Plants are the raw material for the world's feed supply as well as biobased industrial products. Providing an ample food supply for human kind is a serious challenge for our society. Plants as a biorenewable resource have the potential for providing society with many of our basic goods as well as energy. The design of the plants to meet these potentially competing demands is a scientific and

engineering challenge. Plant cellular activities can be improved by manipulation of enzymatic, transport and regulatory functions of cell with the use of recombinant technology. Some of areas of plant biotechnology, in which the genetic engineering and modification of metabolic pathways have played a major role, are discussed here.

A. Modification of plant nutritional content:

One of the goals of plant genetic engineering has been to create crops that are tailored to provide better nutrition for humans. To improve the dietary intake of all essential nutrients and to improve the consumption of various health promoting compounds, researchers have been developing new ways to increase the nutritional quality of plants. The major focus of this agricultural technology is the identification and isolation of the genes that are needed to synthesize a beneficial compound so that its level can be increase in modified crops.

1.Amino acids: Amino acid pathways are important targets for plant metabolic engineering. Since plants represent the major global food supply, large efforts are devoted to increasing the content of "essential" amino acids, which are absolutely required in human foods and animal feeds. Engineering of amino acids is also undertaken to improve plant growth and stress tolerance. Many of the pathways of amino acid metabolism in plants have been elucidated, and genes encoding most of the enzymes are now available. The expression of recombinant genes in transgenic plants, coupled with genetic and biochemical approaches, has contributed significantly to the understanding of regulatory networks of the metabolism of amino acids and their incorporation into proteins. This knowledge is now being extensively applied to metabolic engineering of crops (Galili and Hofgen, 2002). Examples are as follows:

Lysine: One novel way to increase the lysine content of seeds is to enhance production of lysine in transgenic plants by deregulating lysine biosynthetic pathway. The amino acid lysine threonine, methionine and isoleucine are all derived from aspartic acid (Fig 5).

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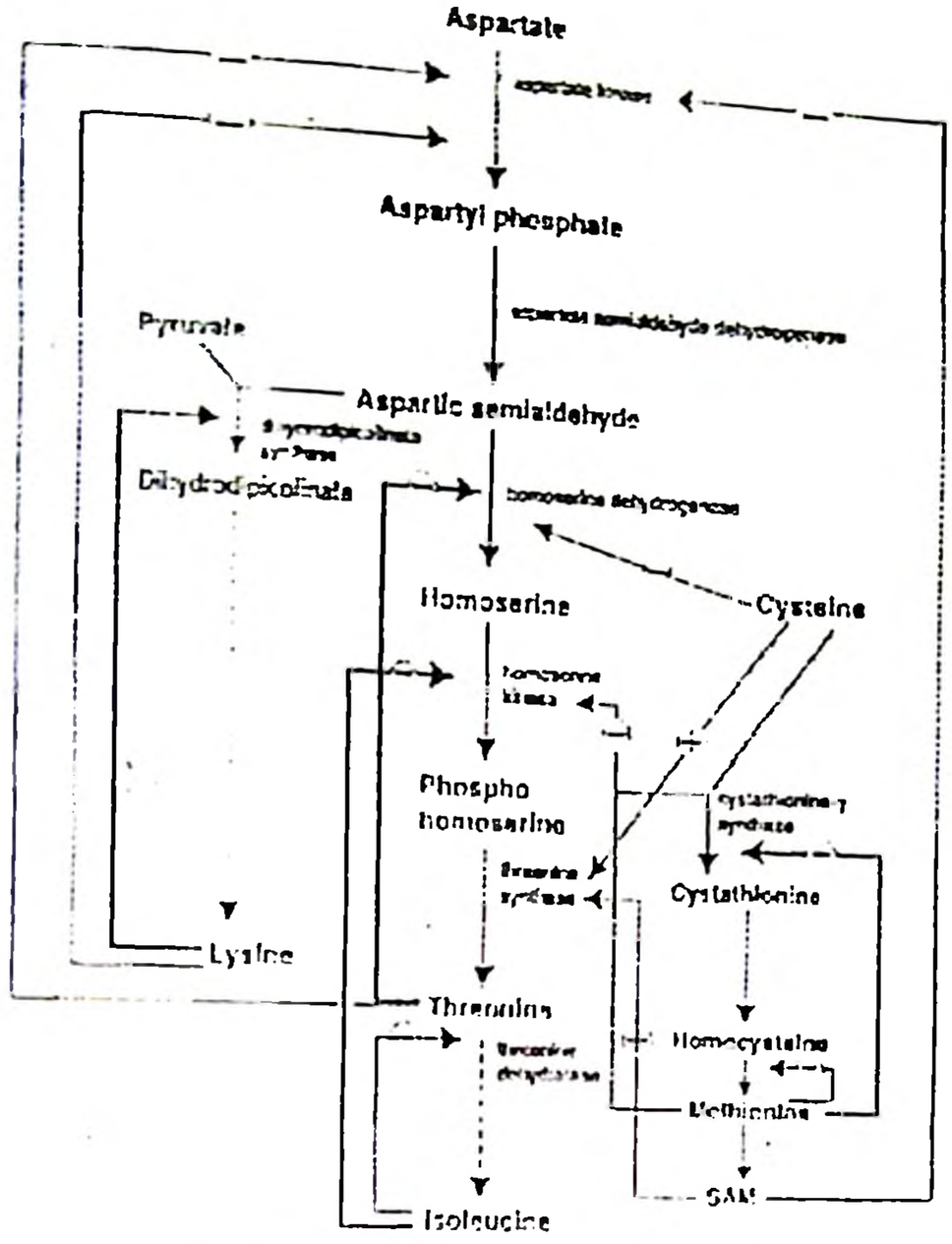


The first step in the conversion of aspartic acid to lysine is the phosphorylation of aspartokinase (AK) to produce β aspartyl phosphate. The condensation of aspartic β semialdehyde with pyruvic acid to form 2,3-dihydropicolinic acid, catalysed by dihydropicolinic acid synthase (DHDPS is the first step commited to lysine biosynthesis. Both AK and DHDPS are feed back inhibited by lysine. Thus to overproduce lysine, it is necessary to abolish the feedback inhibition of these two enzymes. This was accomplished by cloning genes for lysine feedback insensitive DHDPS and AK from *Corynebacterium* and *E. coli* respectively fusing each of these genes to a chloroplast transit peptide, placing each gene under the control of a seed-specific promoter, and then introducing the two genes using a Ti plasmid binary vector, into canola and soybean plants. Transgenic canola and soybean plants had more than a 100 fold increase in the lysine in their seeds with an

overall doubling of the total seed lysine content in canola and a 6 fold increase in total lysine content in soybean.

Methionine and cysteine: These two amino acids containing reduced sulfur, are not only an important substrate of protein biosynthesis but are also precursors of various other metabolites such as glutathione, phytochelatines, S-adenosylmethionine, ethylene, polyamines, biotin, and are involved as methyl group donor in numerous cellular processes. While methionine is an essential amino acid due to an inability of monogastric animals and human beings to synthesize this metabolite, animals are still able to convert methionine consumed with their diet into cysteine. Thus, a balanced diet containing both amino acids is necessary to provide a nutritionally favorable food or feed source. Because the concentrations of methionine and cysteine are often low in edible plant sources, e g potato, considerable efforts in plant breeding and research have been and are still performed to understand the physiological, biochemical, and molecular mechanisms that contribute to their synthesis, transport, and accumulation in plants. During the last decade molecular tools have enabled the isolation of most of the genes involved in cysteine and methionine biosynthesis, and the efficient plant transformation technology has allowed the creation of transgenic plants that are altered in the activity of individual genes. The physiological analysis of these transgenic plants has contributed considerably to our current understanding of how amino acids are synthesized. It was found that threonine synthase is the regulatory element in methionine synthesis (Nikiforova *et al*; 2002).

A different approach has been used to increase the amount of sulfur amino acids, where a gene for a naturally occurring, sulfur-poor, seed protein is isolated and modified to encode a protein with an increased S-amino acid composition. Seed specific expression of a feedback insensitive AK alone resulted in 17-fold increase in free threonine and a 3-fold increase in free methionine in seeds of tobacco (Balasubrahmanyan and Lodha, 1999).

2.Vitamins:

Humans depend on plants for most of the vitamins. So metabolic engineering to improve vitamin is important.

Vitamin E: Tocochromanols (tocopherols and tocotrienols) are important lipid soluble antioxidants and are an essential part of the mammalian diet. Oilseeds are particularly rich in tocochromanols with an average concentration 10-fold higher than other plant tissues. Tocotrienols are the principal form of Vitamin E in the seed endosperm of cereal grains. The cDNAs for homogentisate geranylgeranyl transferase (HGGT) were isolated from barley, wheat, and rice. This enzyme catalyzes the committed step in the tocotrienol biosynthetic pathway. Expression of this enzyme in model plant systems conferred tocotrienol biosynthetic ability and resulted in large increases in total Vitamin E content (Cahoon, 2004).

Vitamin C:

Humans cannot synthesize vitamin C. It is an antioxidant and precursor for several metabolic functions including collagen synthesis. Vitamin C is synthesized from L-ascorbic acid. Modification of pathway resulted in increased level of vitamin C (Agius *et al.*, 2003).

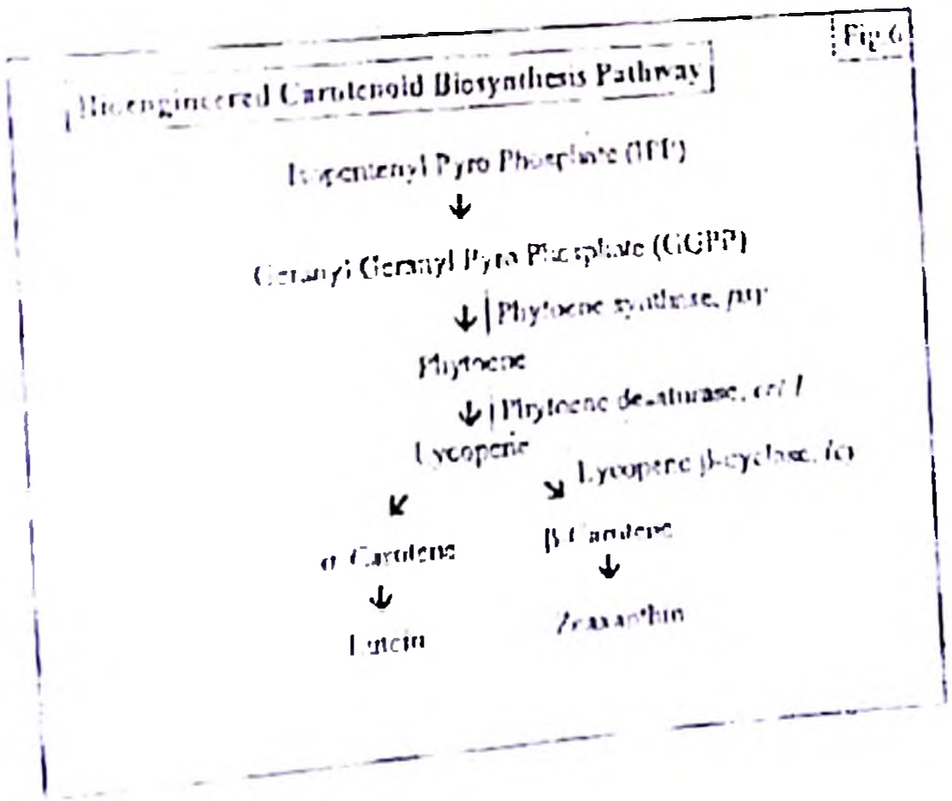
Folic acid

Humans depend on plants as a major source of dietary folates. Inadequate dietary levels of the vitamin folate can lead to megaloblastic anaemia, birth defects, impaired cognitive development, and increased risk of cardiovascular disease and cancer. The biofortification of folate levels in food crops is a target for metabolic engineering. Folates are synthesized de novo from pterins and para-amino benzoic acid, which are subsequently combined to form dihydropteroate, the direct precursor to dihydrofolate. GTP cyclohydrolase-1, which catalyzes the first committed step in pterin biosynthesis, s a rate-limiting step in pterin synthesis in plants and, therefore, in folate synthesis. The expression of an unregulated bacterial GTP cyclohydrolase-I in plants would increase pterin biosynthesis with a concomitant enhancement of folate levels. The *folE* gene encoding GTP cyclohydrolase-I was cloned from *Escherichia coli* and introduced into *Arabidopsis thaliana* through plant transformation. The expression of bacterial

GTP cyclohydrolase-I in transgenic *Arabidopsis* resulted in a 1,250-fold and 2- to 4-fold enhancement of pterins and folates, respectively (Hossain *et al.*, 2004).

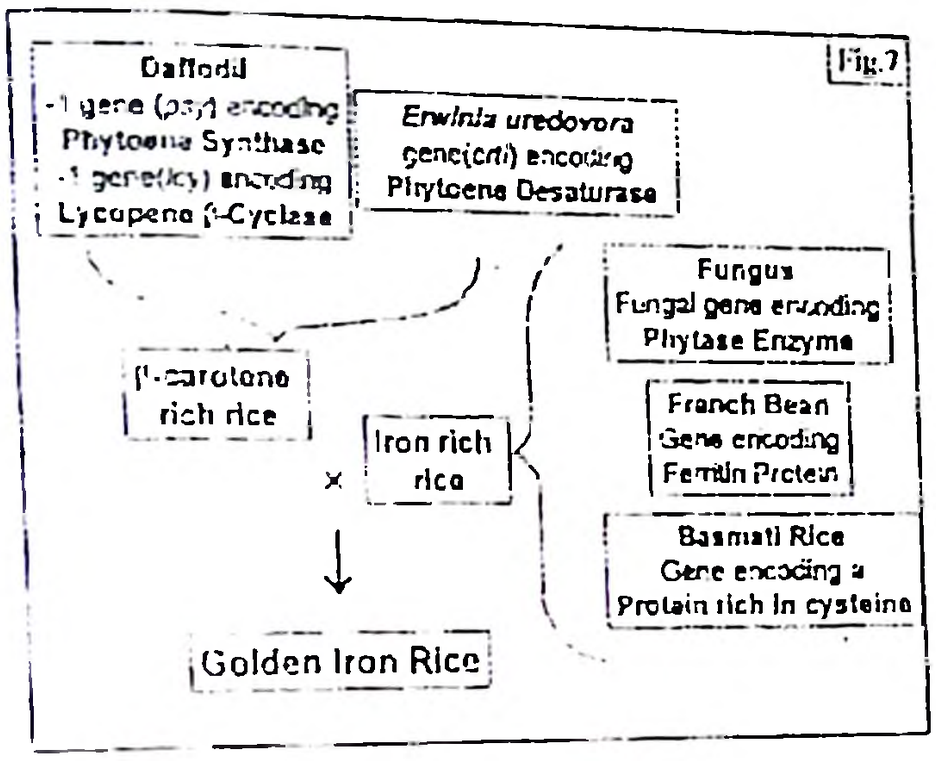
Vitamin A:

A successful example is the genetic modification of rice in order to enhance its provitamin A (β carotene) and iron content. Rice in its naturally milled form does not contain β carotene or any of the immediate precursors. The immature rice endosperm synthesizes the carotenoid precursor geranyl diphosphate (GGPP), which can be converted to β carotene. This conversion involves the use of 3 essential enzymes (Fig.6) that are not found in rice kernel. Scientists led by Dr. Ingo Potrykus were able to insert the 3 genes encoding these enzymes into the rice and modify it to properly use the enzymes and produce beta-carotene. The three enzymes are phytoene synthase and lycopene β cyclase from daffodil, and phytoene desaturase from *Erwinia uredovora* bacterium. The rice grains containing β carotene are golden or saffron coloured and popularly known as 'golden rice'. Transformant rice seeds were found to contain 1.6 micro grams of β carotene per g seed weight (Ye *et al.*, 2000).



People with high rice diet are very likely to experience iron deficiency since rice contains a molecule called phytate, which entraps 95 per cent of dietary iron there by depriving the human body from absorbing it. To increase the iron content of rice, three genes were introduced into the rice genome. The first gene encode for the enzyme phytase, which breaks down phytate. This enzyme can also withstand

high temperature and so will not be denatured when rice is cooked. The second gene encode for iron storage protein ferritin, which doubles the iron level in rice grains. The third gene encode for one protein, which is rich in the amino acid cysteine and helps in the iron absorption in human digestive system (Fig. 7).



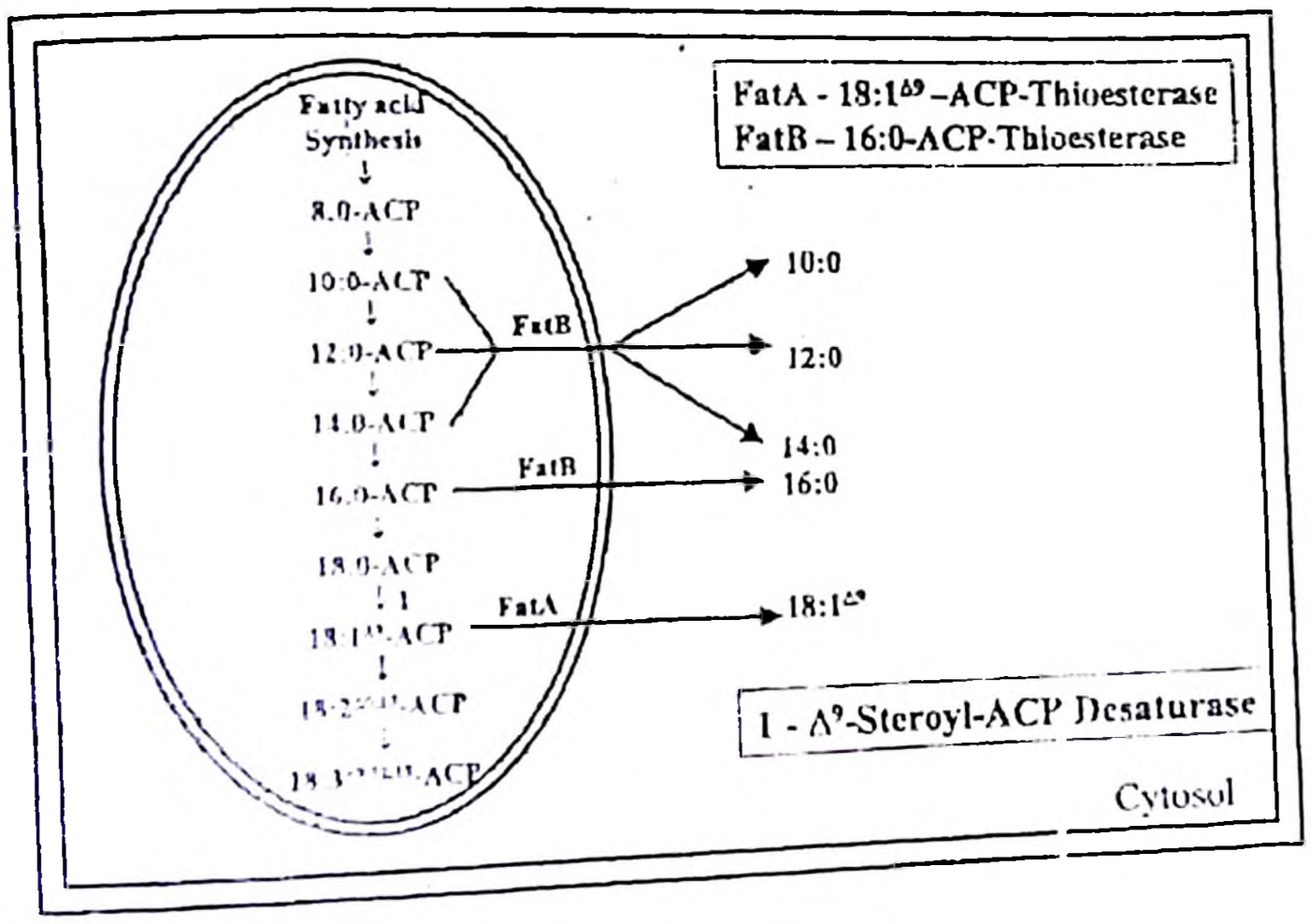
The final 'golden – iron ' strain was engineered by cross breeding the iron rich strain with the golden rice to form hybrids that contained all of the new genes. Rice was also successfully modified in another study to increase its iron content by the addition of the soybean ferritin gene to rice genome. The iron content of the modified rice seeds was as much as 3 times greater than the original seeds.

3.Lipids:

Fatty acids are the most abundant form of reduced carbon chains available from nature and have diverse uses ranging from food to industrial feedstocks. Plants represent a significant renewable source of fatty acids because many species accumulate them in the form of triacylglycerol as major storage components in seeds. With the advent of plant transformation technology, metabolic engineering of oilseed fatty acids has become possible and transgenic plant oils represent some of the first successes in design of modified plant products. Directed gene down-regulation strategies have enabled the specific tailoring of common fatty acids in several oilseed crops. In addition, transfer of novel fatty acid biosynthetic genes from noncommercial plants has allowed the

production of novel oil compositions in oilseed crops. These and future endeavors aim to produce seeds higher in oil content as well as new oils that are more stable, are healthier for humans, and can serve as a renewable source of industrial commodities (Thelen and Ohlrogge, 2002). It is currently possible to change the degree of unsaturation and modify the chain length of fatty acids in plants by genetic engineering (Fig.8).

Thioesterases and Desaturases in Fatty Acid Synthesis Fig.8



The transgenic stearic acid variety of canola contains an antisense copy of a *Brassica* stearate desaturase gene, which inhibits the expression of normal canola gene and leads to the accumulation of stearic acid rather than the desaturation of stearic acid to oleic acid and the seed stearate content was increased from a mere 2 per cent to as high as 40 per cent. In another study, seed specific achieved an increase in stearate content to 11 per cent over expression of a specific long-chain thioesterase gene from soybean. Crossing this line with a transgenic line producing 13 per cent stearate due to down regulation of stearic acid desaturase (SAD) gene resulted in cultivars with about 45 per cent stearate. This result demonstrates that SAD and thioesterase compete for stearoyl-ACP. Simultaneous

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inhibition and over expression of two enzymes respectively, produce the combined effect of enhancing stearate levels.

β-keto synthase (KAS II) enzyme that converts palmitate to stearate is over expressed in transgenic *Brassica napus* resulting in reduced seed palmitate levels (Knutzon *et al.*, 1992)

The first non food products of plant genetic engineering is the commercial production of rape seed plant variety Laurical that was modified to produce lauric acid, a 12 carbon fatty acid used to make soaps and detergents. One gene was introduced from California bay tree. That gene shut off fatty acid synthesis after 12 carbons rather than allowing the acids to grow to 18-carbon length normal for the plant.

γ Linoleic acid (GLA) is the first intermediate in the bioconversion of linoleic acid to arachidonic acid GLA is important in alleviating hyperchholestromia and many coronary heart diseases. It is not produced in oilseed crops. This conversion is catalyzed by δ6 desaturase and the gene encoding this enzyme has been cloned from *Synechocystis*. Constitutive expression of this gene in transgenic tobacco resulted in the production of GLA (Reddy and Thomas, 1996). Recently, this desaturase gene has been cloned from filamentous fungi *Mortierella alpina* and borage transferred to canola and tobacco respectively (Sayanova *et al.*, 1997; Knutzon *et al.*, 1998)

B. Modification of terpenoid biosynthesis:

Terpenoids are a chemically diverse group of compounds, that play essential role in maintaining membrane fluidity (sterol), electron transports (ubiquinone, menaquinone and plastoquinone), glycosylation of proteins (dolichol) and the regulation of cellular developments (McCaskil and Croteau, 1998). The increasing knowledge of terpenoid biosynthesis and important functions of terpenoid compound have made the biosynthetic pathway of terpenoid excellent target for metabolic engineering (Bohlmann *et al.*, 1998).

Plants have been estimated to collectively synthesize more than 30,000 different terpenoids, of which many have useful applications in the manufacture of foods, industrial compounds, and pharmaceuticals. Terpenoids are synthesized from the condensation, in a head to tail fashion, of 5-carbon isoprene (or hemiterpene) units. Major terpenoid classes include mono-, sesqui-, and diterpenes, which are mostly secondary metabolites, as well as tri- and tetraterpenes, which are generally primary metabolites. This large family of compounds includes essential molecules such as carotenoids, gibberellins, abscissic acid and brassinosteroids, sterols, and the phytol chains of chlorophylls, tocopherols, and quinones.

Until recently, it was thought that the synthesis of terpenoids in higher plants was by a cytosolic route that is derived from mevalonate. However, during the past few years it has become clear that plants also use a parallel plastid pathway that converts pyruvate and glyceraldehyde3- phosphate to 1-deoxyxylulose5-phosphate (DXP), which is metabolized in a series of steps to isopentenyl diphosphate and dimethylallyl diphosphate the common precursors of all terpenoids. This latter pathway, termed the DXPS pathway, is prevalent in bacteria but has not been found in fungi or most animals. Plants use the mevalonate-dependent pathway to synthesize sesquiterpenes and triterpenes, whereas other major terpenoids derive from the DXPS pathway (Lichtenthaler, 1999). The gene encoding the first step enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXPS) has been constitutively over expressed in bacteria and *Arabidopsis*. In both cases, increased enzyme activity caused an increase in accumulation of downstream terpenoids, indicating that DXPS is rate limiting.

In mint, it is thought that essential oil monoterpenes, which accumulate in glandular trichomes, derive from the DXPS pathway. Mahmoud and Croteau (2001) exploited a gene that they had previously isolated, encoding deoxyxylulose phosphate reductoisomerase (DXR), which converts DXP to 2-C-methylerythritol4-phosphate, and constitutes the first committed step in the DXPS pathway of terpenoid biosynthesis. They substituted a strong constitutive promoter for the DXR promoter, and introduced the modified *DXR* gene into peppermint

plants. The result was striking: Most transgenic plants accumulated more oil than control plants, with increases of up to 50 per cent.

C. Metabolic engineering for biosynthesis of isoflavanones:

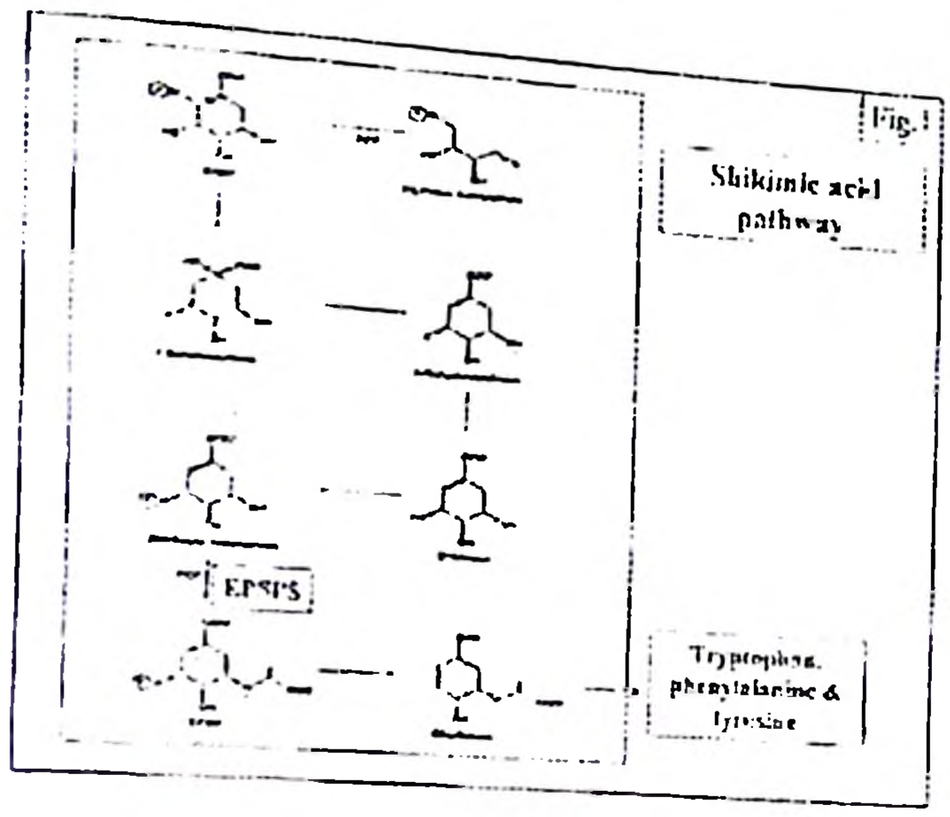
Isoflavonoids are distributed predominantly in leguminous plants and play critical roles in plat defence and root nodulation. A cytochrome P450 (P450), 2-hydroxyisoflavanone synthase, is the key enzyme in their biosynthesis. Isoflavanones are synthesized from flavone, naringenin and liquitergenin. So by engineering key enzymes to non leguminous plants would provide food manufactures an alternative to legumes (Dixon and Stele, 1999).

D. Engineering herbicide tolerance in plants:

Approximately 10 per cent of global production is lost through weed infestation every year. In addition many herbicides do not discriminate weeds from crop plants, many must be applied before the weeds take hold and some persist in the environment. The creation of herbicide resistant crop plants is one way to overcome some of these drawbacks. Herbicide tolerant plant may reduce the need for tillage to control weeds, thereby effectively reducing soil erosion. One herbicide is N- phosphonomethyl glycine commonly referred to as Glyphosate. It is a non-selective, non-residual herbicide with agricultural horticultural forestry and domestic applications.

The herbicide glyphosate acts as an inhibitor of 5-enol pyruvyl shikimate-3- phosphate synthase (EPSPS), which is an enzyme in the shikimate pathway and plays an important role in the synthesis of aromatic amino acids both in bacteria and plants. This target enzyme condenses phophoenol pyruvat (PEP) and 3- phopho shikimic acid (S3P) to form 5 enol pyruvyl shikimate 3 phosphate (EPSP) that is the precursor for the biosynthesis of aromatic amino acids (tryptophan, phenylalanine, tyrosine) (Fig. 1) vitamins and many secondary

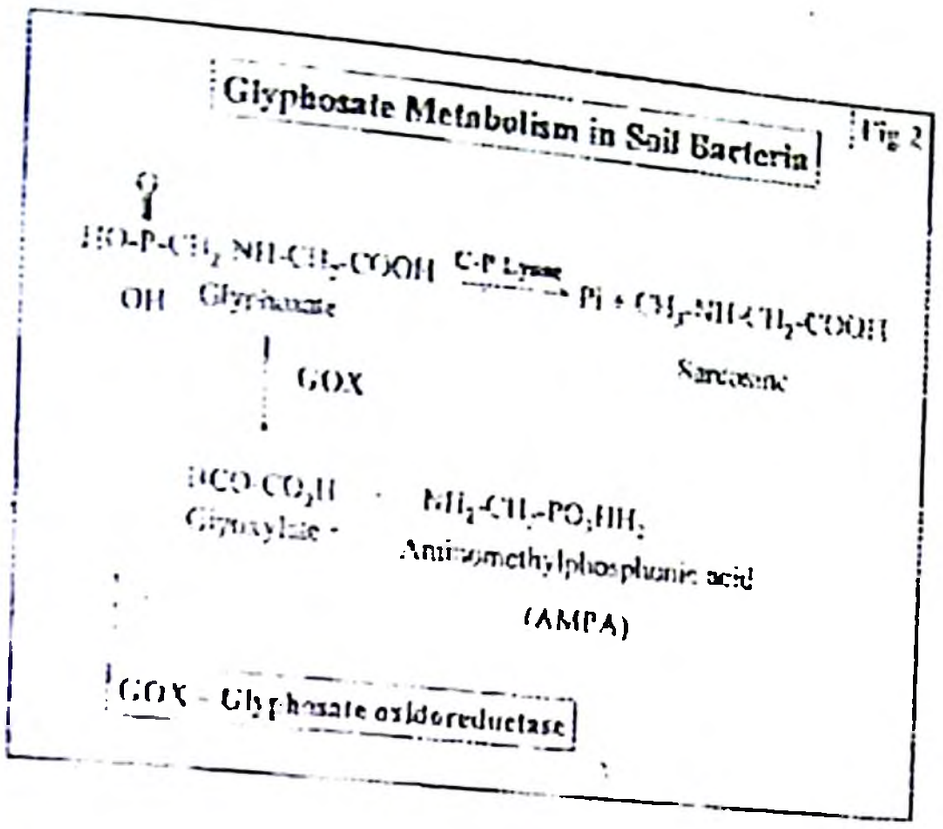
metabolites. As a result of inhibition of aromatic amino acid biosynthesis, protein synthesis is disrupted resulting ultimately in the plant's death (Dekker and Duke, 1995).



Till date, two approaches have already been used to construct plants that contribute to resistance to glyphosate.

1 **By expressing genes of glyphosate tolerant EPSPS:** Over expression of EPSPS gene in transgenic plants results in the production of more EPSPS enzyme, thereby enhancing their tolerance to glyphosate. Out of the isolated EPSPS, the one that has evolved over a billion years with wild type or higher affinity towards PEP was the best solution. Such an enzyme was CP4 EPSPS from *Agrobacterium* sp. strain CP4. This gene has been used to obtain transgenic glyphosate tolerant soybean, cotton etc.

2 **By expressing glyphosate oxidoreductase (GOX) gene in the plants:** Genes for enzymes involved in glyphosate metabolism are abundant in soil microbes. The enzyme GOX, catalyses the cleavage of the C-N bond of glyphosate, yielding aminomethyl phosphonic acid (AMPA) and glyoxylate as reaction products (Fig. 2). GOX was cloned from *Achromobacter*, which uses GOX enzyme for using glyphosate as carbon or phosphorus source.



The target of glyphosate action in the plants, the EPSPS enzyme is located in the chloroplast. The expression of *gox* gene may augment glyphosate tolerance if the GOX protein could be delivered into the chloroplast. Crop plants tolerant to glyphosate were produced by stably inserting both the CP4 EPSPS and *gox* genes into their chromosomes. Several examples of plants engineered with *Agrobacterium* EPSPS and GOX that have received regulatory approval for commercial use include canola in Canada and Japan and corn in USA (Dekker and Duke, 1995)

E. Engineering for increasing shelf life of fruits:

A major problem in the fruit marketing is the premature ripening and softening during transport. These changes are part of natural ageing process of fruit. The understanding of biochemical and physiological basis of fruit ripening has helped in engineering tomatoes with increased shelf life. The following two approaches can be used to develop plants with longer shelf life.

- **Engineering the genes involved in ethylene production:** The plant growth regulator ethylene induces the expression of a number of genes that are involved in fruit ripening and senescence. Ethylene is derived from amino acid L methionine via S- adenosyl methionine (SAM), and 1- aminocyclo propane-1-

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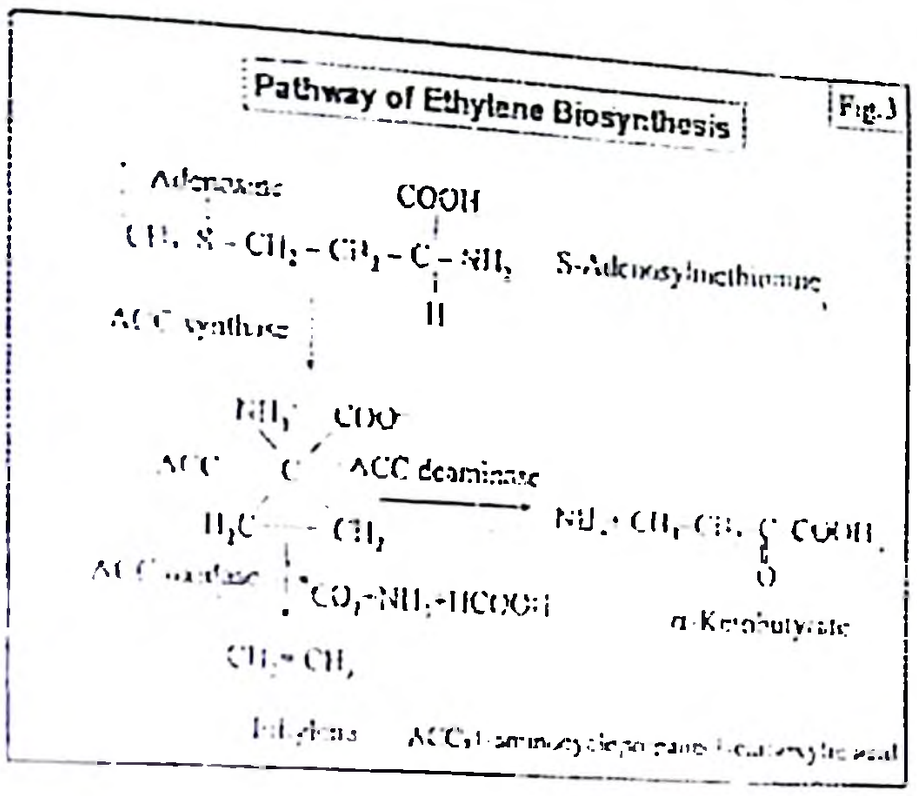
carboxylic acid (ACC). The latter step is catalysed by ACC synthase. ACC is oxidized by ACC oxidase to ethylene and formic acid (Fig. 3).

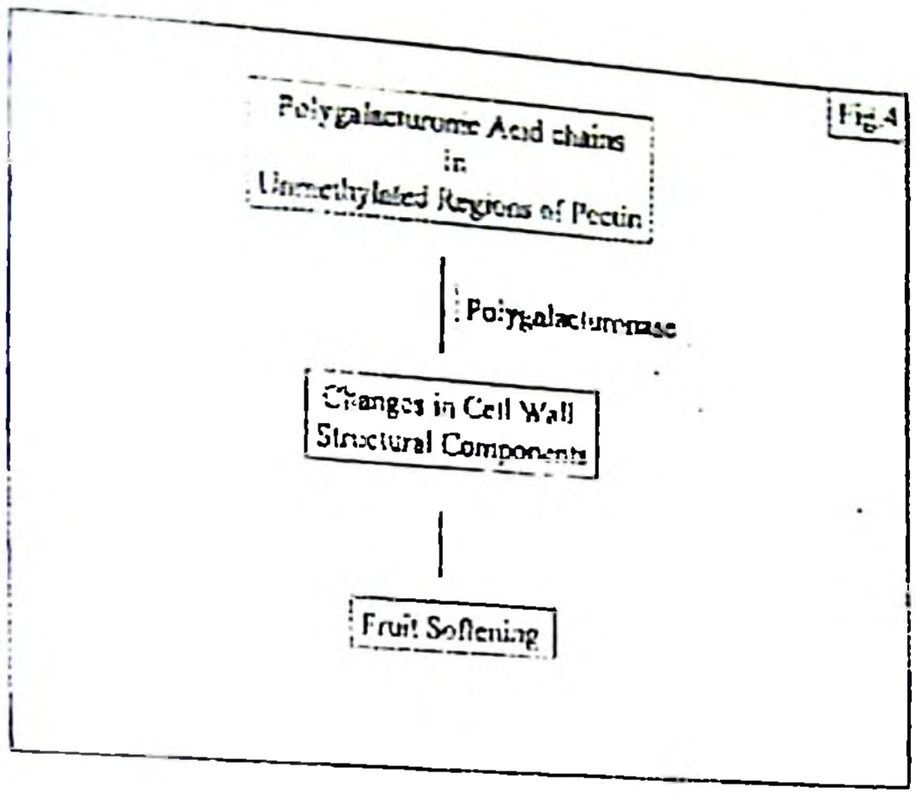


The transgenic plants that have been engineered to contain antisense RNA versions of either ACC synthase or ACC oxidase enzymes, whose activities are essential to plant's synthesis of ethylene, have much lower than normal levels of ethylene Therefore the fruit that is produced by those plants has an extended storage life

Another way of reducing ethylene production is to over express foreign genes for S- adenosyl methionine hydrolase (SAMase) or ACC deaminase, which deplete plant cells of the required precursors of ethylene. The gene for enzyme ACC deaminase was isolated from a soil bacterium, fused to 35S promoter from CaMV and expressed in tomato plants. The transgenic plants synthesized a lower level of ethylene than did normal plants, and again the fruit of the transgenic plants had a significantly longer shelf life (Gray *et al.*, 1992).

- **Reduction in polygalacturonase activity by Antisense gene expression:**

Some of the genes that are induced during ripening encode the enzyme cellulase and polygalacturonase (Fig 4). By interfering with the expression of one or more of these genes, ripening can be delayed.



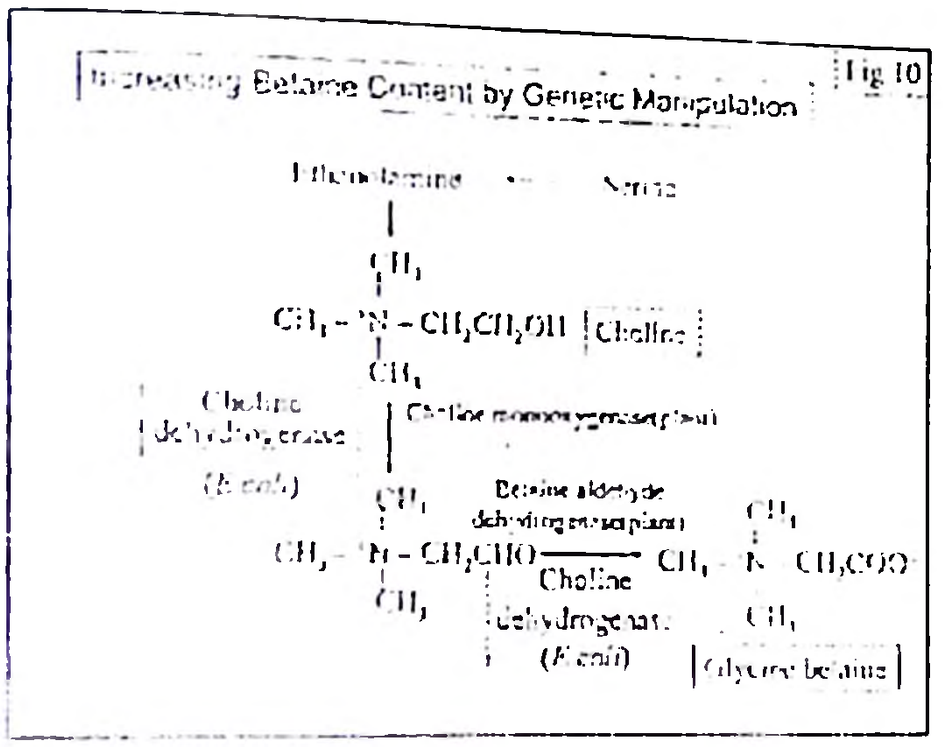
Transgenic tomato plants with single or multiple copies of the polygalacturonase cDNA inserted in the Antisense orientation were constructed, and in many such plants the expression of major isoenzyme was suppressed by over 90 per cent. PG Antisense plants were shown to contain reduced levels of PG mRNA and enzyme activity. The effect was demonstrated to be due to post transcriptional degradation of PG mRNA in cells in which the endogenous gene and antisense genes were both transcribed i e in ripening fruit. In these plants fruit softening was delayed and modified, but not eliminated, indicating the participation of other enzymes, and probably the importance of other cell wall structural components in the softening process. Flavr Savr is one such genetically engineered tomato.

F. Development of salt tolerant plants by metabolic engineering:

To proliferate under conditions of salinity, many plants synthesize low molecular weight nontoxic compounds, which are collectively called osmoprotectants. These compounds facilitate both water uptake and retension and also protect and stabilize cellular macromolecules from damage by high salt levels. Some well-known osmoprotectants include sugars, alcohols, proline, and quaternary ammonium compounds like betaine.

Glycine Betaine is a potent osmoprotectant that is well-established target for metabolic engineering of osmotic resistant plants (Nuccio *et al.*, 1999). It is synthesized from choline in two steps in both bacteria and plants. In plants like

spionach, choline is converted to betaine aldehyde by enzyme choline monooxygenase and then to betaine by betaine aldehyde dehydrogenase (BADH). In bacteria such as *E coli*, both these steps are catalyzed by the same enzyme, choline dehydrogenase (Fig. 10). Therefore to create a more salt tolerant tobacco, researchers have used the *E.coli betA* gene, which encodes choline dehydrogenase. In laboratory tests, tobacco plants expressing *E. coli bet A* gene were upto 80per cent more tolerant of a high (i.e. 300 mM) salt concentration than were non transformed tobacco plants (*Nuccio et* al., 1999).



Molecular farming:

Genetic engineering of plants to produce totally new-use, non-food products, such as biopharmaceuticals, industrial enzymes and bioorganic compounds is called molecular farming. Most of the products will be derived from atmospheric CO_2 and biodegradable biomass

Eg 1 Bioplastics -poly hydroxy alkanoates in plants

Eg 2 Lauric acid is an ideal surfactant since it provides balanced solubility in both aqueous and nonaqueous environments Seeds of California bay tree, *Umbellularia californica*, whose major component is laurate, possess high levels of 12 0-ACP thioesterase Thioesterase cDNA specific to lauroyl-ACP from seeds of *U. californica* was expressed under the control of an early seed specific promoter in transgenic *Arabidopsis* and canola The transgenic seeds in field trials, had upto 25 per cent laurate in *Arabidopsis* and upto 45per cent in canola.

Commercial cultivation of the transgenic canola over thousands of acres in Georgia, USA and sale of the oil from harvested seed by Calgene Inc. to the soap industries, represents a good example of success of plant molecular farming (Murphy, 1996).

Industrial applications of metabolic engineering:

Polyhydroxyalkanoate (PHA): is a class of biopolymers accumulated in intracellular granules by many environmental microbes for the storage of carbon and reducing power. These compounds have received considerable interest because they have properties similar to common thermoplastics and elastomers, making them a potential source of biodegradable plastics that are produced biologically from renewable resources. The copolymer poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) was produced commercially under the trade name Biopol TM by ICI and then Zeneca Bio Products. By varying the composition of the copolymer (e.g., amount of 3-hydroxyvalerate (HV) in the polymer), one can vary the polymer properties. However, in industrial processes, copolymer composition was controlled by varying the ratio of glucose to propionate in the medium, increasing the cost of the polymer.

Aldor and Keasling (2001) metabolically engineered a pathway to produce poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), a biodegradable thermoplastic with proven commercial applications, from a single, unrelated carbon source. An expression system was developed in which a prpC strain of *Salmonella enterica serovar typhimurium*, mutant in the ability to metabolize propionyl-CoA, served as the host for a plasmid harboring the Acinetobacter polyhydroxyalkanoate synthesis operon (phaBCA) and a second plasmid with *E. coli sbm* and *ygfG* under an independent promoter. These two genes encode a novel (2R)-methylmalonyl-CoA mutase and (2R)-methylmalonyl-CoA decarboxylase, respectively, which convert succinyl-CoA, derived from the TCA cycle, to propionyl-CoA, an essential precursor to 3-hydroxyvalerate (HV). The *S. enterica* system accumulated PHBV with significant HV incorporation when

grown aerobically with glycerol as the sole carbon source. It was possible to vary the average HV fraction in the copolymer by adjusting the arabinose or cyanocobalamin (precursor to coenzyme B12) concentrations in the medium.

Isoprenoid biosynthesis:

Isoprenoids are among the most diverse groups of compounds synthesized by biological systems and are important in maintaining membrane fluidity, electron transport, protein prenylation, cellular and organismal development, as fragrances and essential oils, and as antibacterial and antifungal agents. Recently, many of the genes responsible for isoprenoid synthesis have been cloned from plants and microorganisms, and many of these genes can be expressed in functional form in *Escherichia coli* and other microorganisms (Martin *et al.*, 2001).

Production of blue dye Indigo:

Cloning of a single gene from *Pseudomonas putida*, that encodes naphthalene deoxygenase, resulted in the generation of an *E. coli*. Strain able to synthesise indigo in a medium containing tryptophan. A fermentation process was developed for production of indigo from glucose using recombinant *Escherichia coli*. This was achieved by modifying the tryptophan pathway to cause high-level indole production and adding the *Pseudomonas putida* genes encoding naphthalene dioxygenase (NDO). In comparison to a tryptophan-over-producing strain, the first indigo-producing strain made less than half of the expected amount of indigo. Severe inactivation of the first enzyme of aromatic biosynthesis, 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase (the aroGfbr gene product), was observed in cells collected from indigo fermentations. Subsequent *in vitro* experiments revealed that DAHP synthase was inactivated by exposure to the spontaneous chemical conversion of indoxyl to indigo. Indigo production was thereafter improved by increasing the gene dosage of aroGfbr or by increasing substrate availability to DAHP synthase in vivo by either amplifying the tktA (transketolase) gene or inactivating both isozymes of pyruvate kinase. By combining all three strategies for enhancing DAHP formation in the cell, a 60per

cent increase in indigo production was achieved. Metabolic engineering was then further applied to eliminate a byproduct of the spontaneous conversion of indoxyl to indigo, thereby solving a serious problem with the use of bio-indigo in the final denim dyeing application (Berry *et al.*, 2002).

Conclusion:

The interest in metabolic engineering is stimulated by potential commercial applications where improved methods for developing strains which can increase production of useful metabolites. Recent endeavors have focused on using biologically derived processes as alternatives to chemical processes. Such manufacturing processes pursue goals related to "sustainable developments" and "green chemistry" as well as positioning companies to exploit advances in the biotechnology field Some examples of these new processes include the microbial production of indigo (Genencor) and propylene glycol (DuPont) and others involve improvements in the more traditional areas of antibiotic, and amino acid production by a large number of firms. The extension of metabolic engineering to production of desired compounds in plant tissues and to provide better understanding of genetically determined human metabolic disorders broadens the interest in this field beyond the fermentation industry and bodes well for its future importance

DISCUSSION

1. What is the difference between molecular farming and metabolic engineering?

A. Molecular farming can be done using metabolic engineering and genetic engineering. Metabolic engineering is a part of genetic engineering; it is more complex than genetic engineering.

2.How bioinformatics tools can be utilized in metabolic engineering?

A. Bioinformatics can be used to analyze the metabolic pathway.

3.Can metabolic engineering be utilized for imparting insect resistance in plants?

A. Metabolic engineering of terpenoids and other plant compounds are reported to impart insect resistance.

4.What is the disadvantage of creating herbicide tolerant plants?

A. There may be a super weed that is having resistance to the herbicide due to horizontal transfer of genes.

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ABSTRACT

Manipulation of biosynthetic pathways in plants offer a number of exciting opportunities for plant biochemists and molecular biologists. This primarily aims at redesigning plant metabolism towards production of specific high value products for the sake of human welfare. This falls under the platform of second generation transgenics. Recent advances in molecular biology techniques, analytical methods and mathematical tools have led to growing interest in using metabolic engineering to redirect the flow through metabolic pathways for industrial and medical purposes.

Metabolic engineering is generally defined as the targeted and purposeful alteration of metabolic pathways found in an organism in order to better understand and utilize cellular pathways for chemical transformation, energy transduction, and supra molecular assembly (Lessard, 1996.) This multidisciplinary field draws principles from chemical engineering, computational sciences, biochemistry and molecular biology. In essence, metabolic engineering is the application of engineering principles of design and analysis to the metabolic pathways in order to achieve a particular goal such as the enhancement of process productivity, the extension of metabolic capability by the addition of extrinsic activities for chemical production or degradation etc. Amino acids, vitamins, biopolymers, industrial chemicals and chemical building blocks can be synthesized via metabolic engineering

Previous strategies employed in metabolic engineering seemed to be more of an art with experimentation by trial and error. Later it was changed to a systematic approach. By metabolic engineering, production of vitamins, fatty acids, terpenoids, alkaloids as well as minerals can be improved. A significant accomplishment in the metabolic engineering of carotenoids towards the nutritional value of rice crop has been achieved.

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Humans depend on plants as a major source of dietary folates. Inadequate dietary levels of vitamin folate can lead to megaloblastic anaemia, cancer and cardiovascular diseases. Target of metabolic engineering is biofortification of folate level in plants. Folates are synthesized de novo from pterins and para-amino benzoic acid, which are subsequently combined to form dihydropteroate, the direct precursor to dihydrofolate. The *folE* gene encoding for GTP cyclohydrolase-1 (enzyme which catalyses first committed step in pterin synthesis) was cloned from *E.coli* into *Arabidopsis thaliana* through plant transformation. The expression of bacterial GTP cyclohydrolase-1 in transgenic *Arabidopsis* resulted in a 1,250fold and 2 to 4 fold enhancement of pterins and folates respectively. These results helped to identify other potential factors regulating folate synthesis; suggesting ways to further enhance folate levels in food crops

The interest in metabolic engineering is stimulated by potential commercial applications where improved methods for developing strains which can increase production of useful metabolites. Recent endeavors have focused on using biologically derived processes as alternatives to chemical processes. Such manufacturing processes pursue goals related to "sustainable developments" and "green chemistry" as well as positioning companies to exploit advances in the biotechnology field

EDIBLE VACCINES

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NISHA.G

(2004-11-10)

M.Sc. Agri. Plant biotechnology

SEMINAR REPORT

(PRESENTED On 29-10-2005

IN PARTIAL FULFILLMENT FOR REQUIREMENT OF THE

COURSE NO. PBT-651)

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

COLLEGE OF HORTICULTURE

KERALA AGRICULTURAL UNIVERSITY

VELLANIKKARA, THRISSUR-680656

KERALA

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DECLARATION

I, Nisha.G. (2004-11-10) hereby declare that the seminar entitled "Edible vaccines" have been prepared by me, after going through various references cited at the end and has not been copied from any of my fellow students.

Vellanikkara
Date 26-11-2005

Nisha, G.
2004-11-10

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CERTIFICATE

This is to certify that the seminar report titled **"EDIBILE VACCINES"** has been solely prepared by Ms. Nisha.G (2004 – 11 – 10), under my guidance, and has not been copied from any seniors, juniors or fellow student's seminar reports.

Vellanikkara

Date

Dr.D.Girija

Major advisor

Assistant professor

CPBMB

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INTRODUCTION

Humans have always had a great fascination with the world around them and the scientific explanation for the phenomena that affect their lives. Disease and sickness have plagued man since his origin, and man has always sought a way to remedy the contracted ailment. As humans progressed and advanced their medical treatment for diseases, the idea arose "What if we can not just treat the ailment, but prevent the contraction of the disease altogether"? This idea led to the discovery of vaccines, which revolutionized the medical health care system.

History of vaccination

Some Indian Buddhists drank snake venom in the 7th century in an attempt to become immune to its effect. They may have been inducing toxoid like immunity. The ancient practice of variolation by Chinese for preventing natural small pox by inoculating pus from small pox patients was introduced in England in early eighteenth century.

Edward Jenner laid the first mile stone in vaccination in 1796 with a systematic, successful attempt at eradication of small pox. Subsequently Louis Pasteur accidentally observed that chicken cholera bacillus cultures left on the bench for two weeks lost their pathogenisity and retained the ability to protect birds against subsequent infection by them lead to discovery of process of attenuation and the development of live vaccines.

In 1885 Pasteur developed first Rabies vaccine successfully providing attenuated germs when injected into the body confers immunity against the disease. Louis Pasteur is one of the pioneers in the field of vaccinology and it was he who coined the term for vaccination for such prophylactic measures.

The weakened form of small pox was called a 'vaccine' from vacca, the Latin word for 'cow' subsequently it was Louis Pasteur who suggested that all the inoculation to be called vaccination to honor Edward Jenner.

Chronology of important human vaccines

Year	Disease	Vaccine
1721	Small pox	'Variolation' with live virus
1798	Small pox	'Naturally attenuated' cow pox virus
1885	Rabies	Attenuated live and inactivated virus
1896	Typhoid fever	Inactivated intact bacteria
1896	Cholera	Inactivated intact bacteria
1897	Plague	Inactivated intact bacteria
1923	Diphtheria	Partially purified formalin toxoid
1926	Pertussis	Inactivated intact bacteria
1927	Tuberculosis	Live attenuated BCG strain
1955	Polio	Inactivated virus IPV "Salk"
1961	Polio	Live attenuated virus OPV "Sabin"
1963	Measles	Inactivated and live attenuated virus
1974	Japanese encephalitis	Inactivated virus
1976	Rabies	Tissue culture inactivated virus
1981	Hepatitis B	Inactivated plasma derived
1986	Hepatitis B	Inactivated, Recombinant virus

How do vaccines work?

The organisms that cause a disease or materials produced from these organisms are weakened or killed and then made into vaccines. These vaccines are injected into the body or are taken orally. The body reacts by making disease fighting called antibodies, which build up in the system and guard against the disease for a long time, often for a lifetime. Thus immunization helps the body to defend itself against a particular disease.

Vaccination have accomplished near miracles in the fight against infection diseases. It stands as one of the modern medicines greatest success stories. They have consigned small pox to history and should do the same for polio. By the late 1990's an international campaign to immunize all world's children against 6 devastating diseases was reportedly reaching only 80% of infants and was reducing the annual death toll from this infection by roughly 3 million. WHO estimates that 10 million children die in

developing countries each year from infection diseases that could be prevented with vaccine.

Therefore developing an inexpensive and easily transportable vaccine has been the major objective of vaccinologists.

In addition, developing safe vaccines has been another important object in vaccine research since conventional vaccines having viable attenuated microbial components pause small risk to the world population. For this season, vaccine makers today favour subunit vaccines that contain primarily the antigenic epitopes from pathogen as vaccine component instead the whole attenuated live or killed pathogen in the vaccine. Then antigenic epitopes have no way of establishing an infection but offer protection against infectious pathogens. However, producing this subunit vaccine is expensive currently because they are biotechnology produced using the cultures of microorganism. At the end user the use of syringes and needles add to the cost of vaccine dose.

There fore a need for a new technology for the production and delivery of safe and less expensive vaccines has lead the researchers to attempt the expression of vaccination components in plants that are consumed as food. This new technology is called edible vaccine technology. (Sivanandham and Vani, 2004)

What are edible vaccines?

The concept of edible vaccine is to endow plants with the genetic ability to synthesize protein as antigen normally present on the surface of various infection disease agents. An animal or human ingesting antigen as foreign and mount an immune responses that would protect against infection by pathogen.

In the early 1990's the potential of this concept was announced to the world at large

The first report of production of edible vaccines comes a surface protein from *streptococcus* in tobacco at 0.02% of total leaf protein level, appeared in 1990, in the form of a patent application under the International Patent Corporation Treaty.

In 1992 Artzen and his group announced first successful report of expression of hepatitis B surface antigen in transgenic tobacco.Dr.Charles Arntzen is the pioneer scientist in the field of edible vaccines.

organs such as seeds are frequently stable at room temperature eliminating the need for refrigeration during transport and storage.

Delivery of multiple antigens - plants have the capacity to express more than one transgene allowing delivery of multiple antigens.

Reduce dependence on foreign supply.

Antigen protection through bioencapsulation.

Subunit vaccine means improved safety. Unlike live attenuated vaccines, plant derived vaccines are submit vaccines. They contain only a small part of the pathogen and are unable to establish an infection. This offers additional level of vaccine safety.

Integration with other vaccine approaches.

Mechanism of the induction of immunity by edible vaccines

The major mechanism of the induction of immunity by edible vaccines is the induction of mucosal immune system although the induction of systemic immune system exists to certain degree. The induction of mucosal immunity with this type of vaccination involves using of antigens that stimulate the mucosal immune system components known as M cells that are present in the intestinal liming. M cells take the antigens that have entered the small intestine and pass them to other cells of the immune system, such as antigen presenting cells (APCs) including macrophages and macrophage derived dendritic cells. Then APCs process these antigens and display the resulting protein fragments on the cell surface. B cells are stimulated by the native antigenic protein as well as M cell - processed proteins. Helper T cells are stimulated by APCs to produce helper cytokines for the proliferation of the stimulated T and B cells. The plasma cells derived by the B cell stimulation through the above method produce antigen - specific membrane and secretary antibodies. Some of the stimulated B cells are transformed to memory cells. Some of the intestine processed antigens get into blood circulation and target the other immune priming system such as lymph nodes, spleen etc. to drive a good systemic immunity resulting in the production of IgG types antibodies.

Development of Edible vaccines

The process of developing an edible vaccination begins by selecting a suitable vaccine antigen of a pathogen. Some examples of the candidate antigen genes are

presented in the table.

Genes	Diseases
1. *E. coli* heat labile enterotoxin B sub	*E. coli* enteric diarrhoea
2. Cholera toxin B sub	Cholera
3. Norwalk virus capsid protein (NV-CP)	Norwalk virus enteric diarrhoea
4. Hepatitis B virus surface antigen (HBs Ag)	Hepatitis B
5. Hepatitis C antigen Gene	Hepatitis C
6. Rabies virus G protein	Rabies
7. Measles hemagglutenin gene	Measles
8. Rota virus VP7	Rota virus diarrhoea
9. HPV major capsid protein L_1	Human cervical cancer
10. 83 KDa protein antigen	Anthrax
11. RSV fusion protein	Respiratory disease with respiratory syncytial virus
12. VPI gun	Foot and mouth disease

Antigens - considerations

1) Is the antigen safe and non-pathogenic in all circumstances?

2) Can the antigen induce protection immune response?

3) Is the antigen suitable for expression in plant?

Why plants?

Vaccines made from plants are safe due to absence of contamination with animal pathogen or toxins that could be present in vaccine expressed in animals or bacterial cells. Given the ease with which plants produce plentiful biomass on an agricultural and industrial scale, the approach would considerably cut down the cost. Vaccines made from plants would not depend on cold chain, which is required today for conservation and distribution, which could enable oral administration as an alternative to intravenous injection.

On the other hand transgenic plant materials containing antigen are the superior mean of both inducing a primary immune response and injection of antigen. Reports indicated that plant cell denaturation occurs near an immune effector's site in the gut known as payer's patches. It also produces long lasting secondary immune response

Plants are ideal because they can synthesize and assemble large amount of proteins to provide huge quantities of soluble protein at relatively low cost.

Plants could be grown locally and cheaply using the standard growing methods of a given region. Because many food plants can be regenerated readily, the crops could potentially be produced indefinitely without the growers having to purchase more and more seeds or plants year after year. Mass production is in expensive since the plant material is used directly for vaccination.

Developing transgenic plants that can express the antigenic proteins will eliminate the need for fermentation facilities.

If an edible plant is used to produce a vaccine purification to remove host toxins should not be necessary.

Choice of plant species for vaccine delivery?

1. Is it able to be eaten raw and unprocessed?
2. Is it suitable for infants?
3. Can it be widely and easily grown?
4. Can it be easily stored? Is it resistant to spoiling?
5. Is it amenable to transformation and regeneration?
6. The plants must have short duration (Prakash, 1996)

SUIUTABLE CROPS

Some of the plants under study to host vaccines include banana, potato and tomato (Rao, 2003)

Banana

Advantages

1. Most third world countries, which would benefit most from edible vaccination are in tropical climate that are suitable for growing bananas.
2. They are sterile, genes do not pass from banana to banana.
3. Also it is appealing to children.
4. They can be eaten row as compared to potato or rice that need to be cooked
5. Bananas can also be consumed in pure form.

Limitations

1. Fruits spoil fairly rapidly after ripening.
2. Also it has got a long fruit bearing period.
3. Difficult to perform transformation work.

4. Fruits are difficult to transport and storage.

Potato

Advantages

1. Many potato cultivars are pollen sterile. It appears that inadvertent gene flow from this crop can be easier to minimize.
2. They are easily grown without technologies that are lacking in under developed countries.
3. It is easily propagated from eyes.
4. Can be stored for longer periods. They do not require refrigeration

Limitation

1.Poor palatability of raw potato. Heating denatures protein.

Tomato

Advantages

1. Most commercial cultivars are self-pollinated in nature; the subject of inadvertent gun escape is reduced to certain extent.
2. Tomato is quite versatile and can be processed into many different products. It is easily transformed.
3. It grows faster.
4. Presence of vitamin A helps in the transcription of antibody genes.

Limitations

1. Difficult to storage and transport (Mor *et al.*, 1998)
2. They spoil in couple of days.

Maize.

Advantages

1. They can be preserved for longer period due to low moisture content.
2. Cost of production is low.
3. It is easy to transport.
4. High protein content in edible parts

Limitations

1. It is difficult to regenerate plants from modified single cell.(Mason and Arntzen,1995)

2. The plants are difficult to handle in tissue culture

Other crops - Rice, Lettuce, Lupine, Soybean, Tobacco.

Techniques of development of edible vaccines

Agrobacterium mediated transformation still remains the method of choice for dicots. A general method, the biolistic method is being used for transformation of plants including monocots (Sharma *et.al.*, 1999). The first step in the process of creating an edible vaccination is the construction of a binary vector that is a plasmid with two genes. One gene codes for the vaccine antigen and the other for antibiotic resistance. The binary vector is then transferred into a bacterial host usually through soil bacterium *Agrobacterium tumefacians*. This Agrobacterium contains a Ti (tumor-inducing) plasmid, which can act as a vector in the construction of transgenic plants. The Ti plasmid is genetically inactivated so that it is only capable of integrating DNA into the host cell's genome and it can no longer cause tumor. The general process of making a transgenic plant vaccine is briefly described below.

A leaf from the plant that is to be transformed is cut and propagated in tissue culture These tissue cultured plant cells are exposed to agrobacteria having Ti plasmid coded with an antigen gene. The Ti plasmid in the Agrobacterium randomly integrates the encoded gene into the plant's genome. The plant cells are then exposed to a plate of antibiotic for selection. Any plant cell that has not been effectively transformed will not have the gene for antibiotic resistance; and there fore will die. The plant cells that have been transformed with Ti plasmid are allowed to grow into callus that sprout into shoots and roots. These calluses are the planted and they grow as transgenic plants that yield the desired food product with the vaccine components in it.

Another step is engineering tissue specific promoters in plants so that the antigen get expressed only when the fruits get ripened. Such medicinal fruit may also spot a different colour, thanks to the engineer pigmentation gun that distinguish it from the normal fruits. These genes with antigenic property can be linked with a protein that will be produced in abundance during particular stage of the crop growth. Once we get enough of the desired protein in plants, next challenge is to make the animal immune system respond to those proteins. Many antigens that one eats in food do not give an immune response because the body perceives the antigens as food and the immune response repressed. To alert the immune system the researchers may try to pair the vaccine disease protein with very strong oral immunogens like cholera toxin. These adjutants help in stimulating an immune response.

Particle gun method

This method was introduced by Klein and coworkers in 1988. Here 1-2 µm gold or tungsten particle coated with DNA is used for transformation. Particle acceleration is achieved by pressurized Helium gas.

The main components of a helium pressure device are; gas acceleration tubes, rupture disc, stopping screen, and macro carrier carrying particles coated with DNA and target cells. These components are enclosed in a chamber to enable the creation of partial vacuum, which facilitates particle acceleration and reduces damage to the plant cell. After creation of partial vacuum sufficiently pressurized Helium gas is released in the acceleration tube to break the rupture disc. This generates Helium shock waves, which accelerate the macro projectiles to which DNA coated micro projectiles are attached. The macro projectile is stopped by stopping screen and the micro projectile pass through this screen and become embedded in the cells kept about 10 mm below the stopping screen.

Successful stories of edible vaccines

1. Rabies virus coat glycoprotein gene in tomato
2. Norwalk virus capsid protein in tobacco potato and tomato
3. Hepatitis B virus surface antigen in tobacco and potato
4. *E. coli* heat labile enterotoxin B subunit in tobacco and potato
5. Cholera toxin B subunit (CT-B) in potato
6. Tobacco plants vaccine against non-Hodgkin's lymphoma
7. Cytomegalovirus specific antigen in tobacco.
8. Tobacco to produce surface protein antigens of streptococcus mutants. principal cause of tooth decay.
9. Alfalfa expressing *E. coli* antigen to control diarrheal disease.
10. Vaccine against measles in tobacco plants
11. Structural protein VP1 of foot and mouth disease virus in Arabidopsis (Carillo *et al*., 1998)
12. Potato for delivery of human insulin antigen.
13. Yu and Langridge produced first multi component vaccine in potato against 3 enteric disease including cholera, rotavirus and ETEC.
14. Lupine and Lettuce were used to express a hepatitis B surface antigen.
15. Corn was used for the production of an LT-B subunit vaccine.

16. RSV-F antigenic protein gene expressed in tomato. RSV - respiratory syncytial virus - causing bronchiolitis and pneumonia.
17. Tobacco plants expressing CTB subunit of toxin (Hein *et al.*, 1995)
18. Potatoes to serve as vaccine against human papilloma virus (HPV), which causes cervical cancer
19. Last July, Prodegene, a Biotech Company has got patent for edible vaccines against Hepatitis B.

Some plant vaccines

Diseases	Plant vaccines
1. Tooth plague	Tobacco
2. Hepatitis B	Tobacco, lettuce, potato (Thanavakam *et al.*, 1995)
3. Diarrhoea	Tobacco, potato (Haq *et al.*, 1998)
4. Rabies	Tomato, Alfalfa (Modelska *et al.*, 1998)
5. Foot and mouth	Arabidopsis (Yusibov *et al.*, 1998)
6. Norwalk virus	Potato (Mason *et al.*, 1996)
7. HIV-I	Cowpea, maize (Porta *et al.*, 1994)
8. HIV-14	Cowpea (Porta *et al.*, 1994)
9. Gastroenteritis	Maize (Mor *et al.*, 1998)
10. Malaria	Tobacci (Mor *et al.*, 1998)
11. Cholera	Tobacco, potato (Hein *et al.*, 1995)
12. Shipping fever	White clover (Lee, 2001)

Animal experiments

Before scientists could study the effect of edible vaccinations in people, they will have to obtain positive answers to the following questions in model studied.

a) Can plants be engineered to carry functional antigen genes for a specific disease?
b) Can the antigens retain immunogenic property in the oral and GI physiology?
e) Will they stimulate the immune system?
d) Will the immune response be strong enough to depend the hosts against the infection?

Several laboratory experiments have been conducted to address the above questions. They are

1) Tobacco containing B sub unit of heat labile enterotoxin

Two groups of mice were given each dose of 12.5 µg of LT-B from crude soluble extract of tobacco leaves and purified rLT-B.

Response: Serum and mucosal immunoglobulin from animals immunized with plant or bacterially expressed antigen neutralized the biological activity of LT to the same extent.

2) Potato containing LT-B

Mice were given 5 g of tubes per dose (12.5 µg) containing 15-20 g of rLT-B.

Response: The immune response to purified bacterial rLT-B was greater than to transgenic potato samples. This result indicates that some factors in the plant interfere with the antigenicity of LT-B antigen.

3) Transgenic potato containing CTB

Mice were fed with 3 g of potato per dose containing 30 µg of CTB.

Response: Both the groups of mice fed with potato and gavaged with similar dose of bacterial CTB exhibited the same extent of intestinal protection levels.

4) Transgenic tobacco and potato containing NVCP.

Mice were fed with t-rNV and potato tubers containing with a similar dose rNV.

Response: Both showed systemic and immune responses. But the response with potato tubers was lower than the response with r NCVP.

5) Transgenic lupine containing HBs Ag.

Two groups of mice were fed with the vaccine. One with 5 g of callus (150 mg/g) for 1 days and the other with 1 g of callus on each of 5 consecutive days.

Response: Mice that received 5 g of transgenic lupine callus over the course of one day developed a better immune response to HBV than fed in multiple doses of 1 g of the tissue.

6) Transgenic potato containing HBsAg

Mice were fed with 5 g tuber per dose with 10 g of CT as adjuant.

Response: Peak response was observed four weeks after the third dose.

Clinical trials with edible vaccination

Subsequent to the encouraging results obtained in animal model studies, clinical trials have been conducted with edible vaccines to check the toxicity and the efficacy of these vaccines in the human. Summary of these trials are,

1) Transgenic potato containing LT-B

Study: 11 volunteers were given 50-100 g of raw transgenic potato and 3 control participants were given 50 g of raw untransformed potato.

Response: A significant rise in LT-B antibodies was displayed by 10 of the 11 participants, where as no LT-B specific antibodies were detected in the control participants.

Inference: Plant derived LT-B delivered in edible plant tissues was stable in GI tract and proved capable of inducing an immune response in humans

2) Transgenic potatoes containing NVCP

Study: 20 volunteers received 2 or 3 doses of transgenic potato and 4 volunteers received 3 doses of wild type potato. Each dose contained 150 g of potato containing 215 and 751 µg of NVCP.

Response: Four of 20 developed specific serum and 6 of 20 developed specific stool antibodies.

Inference: The efficacy of this vaccine to induce immune response was tested.

3) Transgenic lettuce containing HBs Ag.

Study: 3 individuals received transgenic lettuce leaves twice: 200 g at first and within 2 month 150 g (0.1-0.5 µg/100 g of fresh tissue). Two control individuals were given same amount of non-transgenic lettuce to the same schedule.

Response: 2 weeks after the 2nd feeding, sera from all the three volunteers showed HB Ag specific antibodies. Two of 3 showed more than protection levels of antibodies.

Inference: This clinical study was based on the results obtained from pre-clinical studies of transgenic lupine containing HBsAg. However immune response was acceptable only after second feeding but not after the first feeding

Human trials

Year	Scientist	Institute where research was conducted	Plant vaccine	Disease
1997	Dr. Arntzen	Boyce Institute & University of Maryland, New York	Potato	Diarrhoea
1998	Hilary Koprowski	National Institute of Allergy and Infectious disease (NIAID) and Thomas Jefferson University, Philadelphia	Lettuce	Hepatitis B
1999	Yasmin	Rosewell Park Cancer Institute,	Potato	Hepatitis B

	Thanavala	New York		
2000	Arntzen	Boyce Institute	Potato	Norwalk virus

Institutes working on plant vaccines

Institute	Plant vaccine
1. Boyce Thompson Institute, New York, USA	Tomato with Norwalk DNA
2. Prodigene Seeds (A division of Novartis)	Corn with Gastroententis antigen
3. Stanffer Seeds (A division of Novartis)	Hepatitis B vaccine
4. Axis Genetics, UK	Hepatitis B vaccine
5. Large Scale Biology Corporation, USA	Hodgkins lymphoma vaccine
6. Meristem Therapeutics, USA	Therapeutic proteins in plants
7. Scripp's Research Institute	HIV vaccine using cowpea mosaic virus
8. Poland & U S. Thompson Jefferson University	Hepatitis B in Lettuce

Patents in plant vaccines

Patent Holders	Claim
1. Rhibozyme-Pharm	Nudeic acid vaccine
2. Found. Advan. Mil. Med. (USA)	Shigellosis vaccine
3. Rubicon Lab	Retrovirus vaccine
4. Biosource (Large scale Biology)	HIV vaccine and Malarial vaccine
5. Applied phytologies	Vaccine production in rice, barley, wheat, corn
6. Texas University	Hepatitis B vaccine
7. University of Yale	Vaccine against invertebrates
8. Biocem, Rhone-Merieux	Rabies vaccine
9. Pasteur Institute	*E. coli* vaccine

10. University of Texas A & M Tulane University	*E. coli* vaccine
11. USDA/Philadelphia University	Rabies vaccine in tomato
12. Cornell University	Increasing foreign protein expression
13. Scripp's Research Institute	Vaccine production in lettuce, spinach, tobacco, kidney bean
14. Prodigene	Gastroenteritis vaccine in tomato and potato
15. Purdue Research Foundation	Modified viruses for vaccine production
16. Mycogen/Washington University	Vaccine production Techniques

Issues and concerns

GMO debate

No matter how great of an argument we make for these medicinal foods, they are still genetically modified organisms. There fore we enter the whole debate of whether genetically modified organisms are safe in our environment. Critics of GMO technology argue that not enough testing has been done to ensure that genetically altered organisms will not have negative environmental consequences. Organizations like Green peace or Friends of the earth would argue that GMOs are too dangerous to use and therefore would be opposed to edible vaccination and the like

Who will he the regulatory authorities?

A major obstacle for taking these edible vaccines to utility in the human is the confusion among authorities to regulate this product. The questions that need to be addressed are.

a) Who should be the licensing authority for the edible vaccination?

b) Is that drug or agricultural authority?

c) Which component of vaccine needing licensing?

d) Is it the whole vector with the antigen, the antigen alone or the vaccine part of the plants or the genetically engineered fruit?

Evaluation of dosage requirement

One major challenge in the development of edible vaccination is dosage. It has been difficult to determine the correct amount of vaccine producing fruit or vegetable to eat and controlling the amount of vaccine produced in crops that grow to different size.

Since we are unsure of dosage levels, there is large concern for people who receive too much which could be toxic to induce oral tolerance or too litter which result in non-immunized people walking around thinking that they have immunity.

Products are ground up freeze dried and poured into gelatin capsules. which assures that the vaccine is given in uniform doses and make it likely to be approved by regulatory agencies.

Survival in gut / in ability to enter through digestion

There is some debate as to whether or not medicinal foods when consumed will be able to deliver the medicinal cargo via. the digestive system.

- Will the full dosage enter the blood stream?
- Will the digestion enzymes or acids in the stomach destroy the proteins prior to entering the blood strain?

It is said that the tough outer wall of plant cell apparently serves as temporary armor for the antigen, keeping them relatively safe from gastric secretions Experiments on animals or human clinical trials have shown that the medicinal cargo does find its way into the blood stream.

Oral antigen immunogenisity

Oral vaccine requires a higher antigen dose than either intranasal or parenteral vaccination. A question frequently asked is whether realistic quantities of edible plant material will be able to supply sufficient antigen to generate protection immunity. Many antigens that one eats in food do not give an immune response because body perceives the antigen as food and the immune responses get repressed. To alert the immune system the researcher may try to pair the vaccine disease protein with very strong oral immunogens or adjutants like cholera toxin. They help in stimulating an immune response.

What are the issues with oral tolerance?

Phenomenon of oral tolerance has shown that ingesting certain proteins can at times cause the body to shut down its responses to those proteins. The induction of oral tolerance is both time dependent and dose dependent. The antigen dose necessary to induce protection is generally smaller than that required to produce tolerance. In addition repeated or continuous exposure is usually necessary to induce oral tolerance. In this setting we believe it is unlikely that an edible vaccine would lead to oral tolerance.

Horizontal gene flow?

Concern about transfer of genes to non-target organism remains to be addressed. There are two reasonable ways to keep the transgene away from neighboring plants. The one way consists in the usage of plants with male sterility as characterized in potato. The other way to present transgene transmission is the integration of transgene into the chloroplast genome. In most plant species chloroplast genome is maternally inherited. This means that the transgene or any protein expressed following chloroplast transformation are not present in pollen; thereby reducing the risk of transmission of the transgene to neighboring crop or weed species by cross pollination. Expression of transgene from the chloroplast genome also results in the accumulation of significantly greater quantities of protein; since there is only one nucleus in the cell, but many chloroplasts so transgene that are inserted into chloroplasts exist at much higher level

How can the expression of antigen components be stabilized in the edible vaccination?

One of the methods would involve inserting antigen gene into a specific chromosomal position instead of integrating randomly into any where in the genome. But successful human clinical trials have shown that adequate doses of antigen can be achieved with plant-based vaccine.

Delivering the vaccine in intact plant material rather than in plant extract may enhance antigen immunogenisity as bioencapsulation of the antigen within the high plant cell wall and membrane compartment increased protection from intestinal digestion

How to increase the amount of antigen component in an edible vaccine?

When compared with the edible vaccine the purified recombinants induced a better immune response. The amount of vaccine dose can be increased to increase the bioavailability of the vaccine compound. Additionally linking antigen genes with strong promoters known to produce the gene products more readily can increase the expression of vaccine components. But previous attempts at boosting the dose of antigen in potato plants often led to stunted growth of the plants and reduced tubes formation. Too much mRNA from the transgene was causing gene silencing in the plant genome. The solution is to device a gene amplification system that is silent until the latest possible stage in the plant development.

What is the best food for this approach?

Is it banana, potato, tomato or some other crop?

Possible plant cell interference with antigen presentation.

To the moment there is not much knowledge about post translational modifications, glycosylation, acetylations, and correct folding of the polypeptides when introduced to the plant cell. Further interaction of the antigen with the plant cell metaboiism are not yet studied intensively and need to be evaluated.

Strategies

Most of the countries have set up guidelines for the testing and production of genetically engineered crops. In most of the countries these guidelines have not been put to the test. There is need therefore to proceed with well-defined protocols for growing the antigen expressing crop, containment and monitoring risk.

A strong public education plan should be an essential part of the strategy. Bringing agriculture to medicine will definitely have a strong positive impact on the predominantly agricultural economy, positioning them better to handle their health care and other needs

Conclusion

There are 3 important aspects in the vaccines issues. They are

(1) Affordability of the vaccine

(2) Safety of the vaccine

(3) Versatility of preparation, distribution and administration of the vaccines.

These three aspects are satisfied to a greater degree by the edible vaccine approach. The feasibility of edible vaccine approach has been shown in animal model studies and in clinical trials. Transition from a model system into a practical reality still has some way to go, including managing issues of oral tolerance, safety of genetically modified organism, effective vaccine doses and horizontal gene flow. Successful edible vaccines have the potential to transform health policy and practice in both developed and developing countries. The growing understanding of the microbiology and immunology of these vaccines together with the power of molecular biology and biotechnology would certainly make the edible vaccine approach in the front-runner.

"*Let your food be your medicine*" is an excellent summary of development of edible vaccine.

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DISCUSSION

1. How do you distinguish plantibodies and edible vaccines?

Plantibodies provide passive immunity whereas edible vaccines provide active immunity.

2. Is there any commercially released variety?

A Cherry tomato variety against respiratory syncitial virus.

3. What is epitope?

It is a small portion of the antigen where antigenisity is confined

4. How do you achieve site-specific integration of transgene?

Chloroplast transformation is a method to achieve this.

5. The work that is being done on edible vaccines at TNAU?

TNAU is now working on plant vaccine against rabies.

6. Some naturally available medicinal plants?

Phyllanthus neruri, Ocimum sanctum,

7. How do you identify the vaccine producing fruit?

We can engineer some pigmentation gene during transformation process to distinguish it from the normal ones.

8. How edible vaccines become cheaper?

They do not require extensive fermentation and purification procedure as that of conventional vaccines

ABSTRACT

Vaccines have accomplished near miracles in the fight against infectious diseases. A vaccine is administered in advance to stimulate the production of antibodies and to give body the time to set active immunity before the invasion by a pathogen. In developing countries four million children die every year due to vaccine preventable diseases. This is due to difficulties in transporting these vaccines and economic difficulties in buying and distributing these vaccines to remote places. The need for a new technology for the production and delivery of inexpensive and safe vaccines has led the researchers to attempt the expression of antigen component in plants. This new technology is known as edible vaccine technology (Sivanandham and Vani, 2004)

Edible vaccines are genetically engineered plant food substances that contain the genes of immunogenic antigen, that are not toxic to the host. The advantages of edible vaccines would be enormous. They are cost effective, safe, easy to administer and have the potential to address many of the limitations faced by traditional vaccines (Stoger *et. al.*, 2000)

Agrobacterium mediated transformation still remains the method of choice for dicots. A general method, the biolistic method is being used for transformation of plants including monocots (Sharma *et.al.*, 1999). Some of the plants under study to host vaccines include banana, potato and tomato (Rao, 2001). The major mechanism of induction of immunity by edible vaccines is the induction of mucosal immune system although induction of systemic immune system exists to certain degree.

The feasibility of edible vaccine approach has been shown in animal model studies and in clinical trials. Transition from a model system into a practical reality still has some way to go, including managing issues of oral tolerance, safety of genetically modified organism, effective vaccine doses and horizontal gene flow. Successful edible vaccines have the potential to transform health policy and practice in both developed and developing countries.

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