

**Genetic engineering of soybean trypsin inhibitor gene
in groundnut (*Arachis hypogaea* L.) for induced resistance
to insect pests**

**A Thesis submitted in partial fulfillment of the requirements for the
degree of Master of Science in Biotechnology**

By

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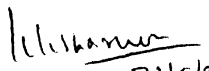
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CERTIFICATE

This is to certify that the dissertation entitled "*Studies on the expression of soybean trypsin inhibitor gene in groundnut for induced resistance to insect pests*" Submitted by Miss. T. Shashi Rekha in partial fulfillment of the requirement for the award of the Degree of Master of Science in Biotechnology of University of Calicut, is the bonafied work carried under our supervision, is an original one and has not been submitted earlier either to this University or to any other institution for the award of any degree or diploma.

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I. Introduction

I. INTRODUCTION

Plants have evolved with pathogens and insect pests for millions of years. Under certain environmental conditions, however, virulent pathogens can cause substantial damage to plant tissue both in the field and in storage. Plant pathologists and entomologists are faced with the job of preventing economic losses in these contrived situations. Considering the loss of yield of many crops accounting to about 30% by insects and diseases we attempted to transform groundnut with Soybean trypsin inhibitor (SBTI) gene in order to reduce the yield loss and also the cost on insecticides which is US\$ 6 billion annually.

Classical plant breeding by all accounts has been most successful in the last couple of decades. However, the large population growth, anticipated food and energy shortages, and the numerous ecological problems due partly to intensive agricultural practices of our present society have resulted in major limitations of classical plant breeding. Firstly, classical plant breeding is limited by sexual compatibility, which results in a limited gene pool. Secondly, inherent to the use of sexual crosses, the offspring consists of a 1:1 mixtures of the genes present in the wild relatives and in the elite plants that lead to many undesirable traits being incorporated. Hence an extensive time and labor consuming effort is required to eliminate the undesired characteristics by outcrossing that may take at least 10-15 years.

The advances made in the field of plant biotechnology during the last decade have the potential to overcome these limitations. These include: (1) the ability of transferring isolated and well defined genes into plant cells. (2) identification and characterization of regulatory structures present in plant genes, and (3) regeneration of intact and fertile transgenic plants.

Many inducible chemicals are the products of complex biochemical pathways, such as synthesis of phytoalexins and lignin that require the induced synthesis of several biosynthetic enzymes. Studies on single genes that code for individual enzymes, in pathways leading to the synthesis of these chemicals have been extremely useful. The transfer of isolated DNA into plant cells has become a routine procedure that is applicable to diverse crop species.

Several methods exist to transfer DNA into cells, which include, use of gram negative soil bacterium, *Agrobacterium tumefaciens* to introduce genetic material into the genome of most dicotyledonous plants and, direct transformation of plants by microprojectile bombardment, liposome fusion, microinjection etc.

The number of plant species and genotypes accessible to gene manipulation is limited by the inability to regenerate intact and fertile plants from their explants. The various strategies used to overcome this problem employ different vector and delivery systems for transferring the DNA into plant cells and are specific to the plant species and their ability to in vitro manipulation.

Biotechnological advances in entomology have occurred rapidly in the last decade, and their applications are wide for crops such as cotton (*Gossypium hirsutum* L.) , soybean (*Glycine max* (L.) Merrill) and corn (*Zea mays* L.), and it is now spreading for groundnut. Considerable research has been conducted on mass rearing and release, importation and manipulation of natural enemies of *Helicoverpa spp.* on cotton.

For several decades researchers have been studying the biosynthesis and regulation of specific chemicals associated with plant defense against pests and pathogens. For many years these were first considered to be “secondary plant compounds” but are now considered to be defensive chemicals that act either alone or in concert to contribute to the resistance of the plants against insect pests. These chemicals are found to be constitutive compounds or are synthesized in response to attacking pests or pathogens. The inducible chemicals, that can also occur constitutively, include complex substances such as antibiotics, alkaloids and terpenes as well as proteins like enzymes, enzyme inhibitors and lectins. (Janzen et al., 1977). These chemicals have provided interesting systems for studying inducible plant defense.

Induced defense responses to pests and pathogens are activated by signals released during the early stages of infection or insect attacks that include oligosaccharides derived from the cell walls of pathogens, insects and from the plants themselves, (Darvill and Albersheim 1984)., lipids such as eicosapentaenoic and arachidonic acid (Bryan et al. 1985),

glycoproteins (Keen 1980), enzyme proteins, action potentials, lipopolysaccharides, and traditional hormones such as IAA and abscissic acid (Thornburg et al. 1987). Many inducible chemicals are the products of complex biochemical pathways, such as the synthesis of phytoalexins and lignin, which require the induced synthesis of several biosynthetic enzymes (Dixon, 1986; Higuchi, 1985).

By transferring these genes from one plant species to another and expressing them either with their own promoters or with constitutive promoters, genetically modified plants can be readily obtained to test for their possible role in defending plants against specific pathogens or insect pests. For example, genes coding for hydroxy proline rich and glycine rich glycoproteins (Corbin,1987), chitinase(Broglie et al.,1986), β -glucanases(Kaufman et al.,1987) and protein inhibitors are used in the production of transgenic plants(Ryan,1989). Protease inhibitor proteins that are both developmentally regulated and induced in response to insect attacks are widely used for transformation of various crops for developing insect resistance.

Considering the constrains in producing transgenic plants for insect resistance, studies on transformation of groundnut were carried out with the following objectives:

- 1) Development of standard system of plant regeneration from leaflet explants of groundnut.
- 2) Transformation of the leaflet explants of groundnut with soybean trypsin inhibitor (SBTI) gene through *Agrobacterium tumefaciens*-mediated gene transfer.
- 3) Transformation of leaflets explants of groundnut with SBTI gene through biolistics.
- 4) Histochemical assay of SBTI in putatively transformed groundnut plants.

II. REVIEW OF LITERATURE

1. PROTEINASE INHIBITORS

Plants, microorganisms as well as animals contain a number of proteins which have the peculiar property of forming reversible stoichiometric protein-protein complexes with various proteolytic enzymes, thus bringing about competitive inhibition of their catalytic functions. They are present in multiple forms in numerous tissues. In addition to their role in regulating proteolytic activities, they are important for protecting fluids or tissues from degradation by unwanted or foreign proteolytic activities. High concentrations of protease inhibitors are often found in fluids and tissues that are particularly vulnerable to foreign proteases, such as blood serum (Travis et al., 1983), pancreatic acinar cells (Neurath, 1984) and storage tissues of plants (Richardson, 1980).

A. Protease inhibitor families in plants

In plants at least ten, protease-inhibitor families have been recognized, that are specific for each of the four mechanistic classes of proteolytic enzymes i.e. serine, cysteine, aspartic and metallo-proteases. Table 1 shows a list of known families of protease inhibitors found in plants (Garcia-Olmedo, 1987; Laskowski, 1980).

1. Soybean trypsin inhibitor (Kunitz) family.
2. Bowman-Birk inhibitor family.
3. Barley trypsin inhibitor family.
4. Potato inhibitor-I family.
5. Potato inhibitor-II family.
6. Squash inhibitor family.
7. Ragi I-2/ Maize bifunctional inhibitor family.
8. Carboxy peptidase A, B inhibitor family.
9. Cysteine proteinase inhibitor family (Cystatins).
10. Aspartyl proteinase inhibitor family.

Many inhibitors are products of multigene families and it is not unknown to find several isoinhibitor species exhibiting different specificities towards proteases. Proteinases can be classified as endopeptidases and exopeptidases i.e. enzymes that cleave internal peptide bonds and that cleave single amino acids from either C or N terminal end of the polypeptide chain respectively. A recent proposal concerning the nomenclature of proteolytic enzymes suggested that the term "protease" should be used to describe all enzymes that break peptide bonds. Endopeptidases be termed as "proteinases" and that exopeptidases be termed as "peptidase" (Barrett 1986).

B. Types of proteinase inhibitors

Proteinase inhibitors can be broadly divided into four main groups, that are characterized by the nature of their active sites and the reaction mechanism involved. These are as follows:

- i. Serine proteinase inhibitors.
- ii. Cysteine prteinase inhibitors.
- iii. Aspartic and metallo-proteinase inhibitors and
- iv. Bifunctional α -Amylase/ proteinase inhibitors.

i. Serine proteinase inhibitors: All serine inhibitor families from plants shown in Table 1 are competitive inhibitors and all inhibit proteinases with a similar standard mechanism. Serine proteinases have been identified in extracts from the digestive tracts of insects from many families, particularly *lepidoptera* whose growth is inhibited by proteinase inhibitors. In this order that includes a number of crop pests, the pH optima of the guts are in the alkaline range of 9-11 where serine proteinases and metallo-exopeptidases are most active.

Studies of the effects of proteinase inhibitors on insect diets first began in the 1950s when Lipke et al.,(1950) found that a protein fraction from soybean inhibited growth, as well as proteolytic activity in vitro, of the meal worm, *Tribolium confusum*. Trypsin inhibitors at 10% of the diet were toxic to larvae of the *Callosobruchus maculatus* and *Manduca sexta*. It

was suggested that inhibition of protein digestion alone does not cause the adverse effects of the inhibitors but causes hyperproduction of digestive enzymes and enhances the loss of sulfur amino acids by the insects.

Insect damage to the plant leaves can cause a striking increase in protease inhibitors in various plants, apparently as a defense response of the plants. A direct test of the roles of proteinase inhibitors in plant leaves as a defense against insects was first demonstrated by Hilder et al.,(1987).

In considering the transfer of proteinase inhibitor genes from one plant to another as potential defensive protein, the specificities and affinities of the inhibitors with target enzymes must be considered. Other studies have also shown that proteinase inhibitors can exhibit different affinities for members of homologous proteinase families from different organisms. Rascon et al.,(1985) have shown that three pure trypsin inhibitors SBTI, LBI and egg white inhibitor (EWI) inhibited trypsin and chymotrypsin from 12 animal species with a wide range of variability. The differences in the effects of various inhibitors in guts of animals, including insects, may be due to the different affinities of the inhibitors with proteinases.

Several reports have shown that proteinase inhibitors are associated with resistance and that the inhibitor can inhibit proteinases of microbes including pathogens. Peng and Black (1976) found that levels of trypsin inhibitor increased more in leaves in varieties of tomato that were resistant to *Phytophthora infestans* than in susceptible varieties.

ii. Cysteine proteinase inhibitors: Proteinase inhibitors that inhibit the cysteine mechanistic class of enzymes have been known in plant tissues for many years and in animal tissues even longer. Cysteine proteinase inhibitors are now called “cystatins” as a class and consists of at least three distinct families (Barrett, et al., 1987). These are isolated from pineapple, potato, (Rodis et al., 1984), corn, rice, cowpea, mungbean, tomato, wheat, barley, rye, and millet (Tashins et al., 1986). Cysteine proteinases are common in animals, eukaryotic microorganism, bacteria and plants. In animals, they are sequestered in lysosomal

compartments or in the cytoplasm, where they are involved in intracellular protein turn over. Cysteine proteinases are not secreted as intestinal digestive enzymes in higher animals, but are found in midguts of several families of *Hemiptera* and *Coleoptera* where they appear to play important roles in the digestion of food proteins. These particular insects characteristically have mildly acidic pHs in their midguts near the pH optima of cysteine proteinases (pH ~5). Several members of the order *Coleoptera* are seed and leaf eating insects that are important pests of agricultural crops. None of these insects require serine proteinases but employ cysteine proteinases as major digestive enzymes (Gatehouse and Boulter, 1983).

Why some insects do not use serine proteinases as major digestive enzymes similar to most other insects and higher animals is a puzzle. The diversity among different species may have been a consequence of the evolution of digestive physiology of the insects in response to their ancient environment in which potentially toxic serine proteinase inhibitors could be used as food proteins. One hypothesis has been forwarded (Houseman et al., 1987), that the ancestors of insect species that use cysteine and aspartic proteinases lived for some time in an environment that did not require digestive proteolysis. As these ancestral organisms evolved its changing environments where proteolysis became necessary for survival, they may have isolated in niches where the serine proteinases were not useful, such as in the presence of high levels of serine proteinase inhibitors. Under these conditions the lysosomal enzyme systems, which are cysteine and aspartic enzymes in acidic compartmentalized environments, may have been shifted from a strictly intracellular role to include a secretory digestive role as well.

Cysteine proteinases isolated from insect larvae can be inhibited by both synthetic and naturally occurring cysteine proteinase inhibitors. The pH range of 5-7 which is the pH range of insects that were apparently using cysteine proteinases (Murdock and Brookhart et al., 1987 and Weiman , 1988).

iii. Aspartic and metallo-proteinase inhibitors : Knowledge of the role of aspartic proteinases in insect digestion is more limited than that of cysteine proteinases. In species of six families of *Hemiptera*, aspartic proteinases (cathepsin D like proteinases) were found along with cysteine proteinases (Houseman et al.,1983). The low pH of midguts of many members of the *Coleoptera* and *Hemiptera* provide more favorable environments for aspartic proteinases (pH optima ~ 3 ~ 5) than the high pH of most insect guts (pH ~ 8 ~11) where the aspartic and cysteine proteinases would not be active.

Wolfson and Murdock (1987) demonstrated that pepstatin, a powerful and specific inhibitor of aspartyl proteinases, strongly inhibited proteolysis by the midgut enzymes of the Colorado potato beetle. Plants have evolved two families of metallo-proteinase inhibitors,

1) The metallo-carboxy peptidase inhibitor family in potato and tomato plants and

2) The Cathepsin D inhibitor family in potatoes. The cathepsin D, is unusual as it inhibits trypsin and chymotrypsin as well as cathepsin D, but does not inhibit aspartyl proteases such as pepsin, renin or cathepsin E. The inhibitors of the metallo-carboxy peptidases from tomato and potato tissues are polypeptides that strongly and competitively inhibit a broad spectrum of carboxypeptidases from yeast and plants.

The inhibitor is found in tissues of potato tubers, where it accumulates during tuber development along with the potato inhibitor I and II families of serine proteinase inhibitors. This inhibitor also accumulates in potato leaf tissues along with inhibitor I and II proteins, in response to wounding (Graham et al., 1981 and Janzen, 1986). Thus, wounded leaf tissue and potato tuber tissues have the capacity to inhibit all five of the major digestive enzymes of higher animals and many insects (i.e. trypsin, chymotrypsin, elastase, carboxypeptidase A and carboxypeptidase B). It is of importance for research to assess the effects of varying the levels of the carboxypeptidase inhibitors in the presence of other proteinase inhibitors such as is found in potato tubers.

iv. Bifunctional - α - amylase / proteinase inhibitors: Proteins that inhibit α - amylases are found throughout the plant kingdom. Many of the abundant proteins in cereal seeds are

inhibitors of either α - amylases or proteinase inhibitors or both. The α -amylase inhibitory activities of these proteins are usually directed against α - amylases from animals, including a broad spectrum of insects or microorganisms but rarely against amylases from plant. The amylase inhibitors are considered to be parts of the protective chemicals of plants against pathogens and pests. In cereals, many of these inhibitor proteins are members of a large super family of storage proteins whose members are all thought to have arisen from the same thirty amino acid ancestor (Kreis et al., 1985).

Most members of these inhibitor families are only proteinase inhibitors, having appropriate reactive sites that determine their specificities. However, a few proteinase inhibitors also possess reactive sites that inhibit amylases and are therefore bifunctional inhibitors. These possess two types of independent reactive sites; one type specific for proteinases and the other for amylases that are located on separated regions of the proteins. Thus, the inhibitors can tightly bind and inactivate proteinase and amylase simultaneously.

The primary sequence of an α - amylase –proteinase inhibitor isolated from ragi is homologous with the barley trypsin inhibitor family. The bifunctional inhibitor possesses several disulfide bridges throughout the molecule that match with those of the barley trypsin inhibitor, indicating that the bifunctional inhibitor is not a fusion protein. The ragi bifunctional inhibitor contains two reactive sites whereas one reactive site is present in barley trypsin inhibitor (Campson, et al. 1983).

Gatehouse et al.,(1986) found that amylase inhibitors, purified from wheat were potent inhibitors of amylase found in the midguts of the larvae from two species of insects *C.maculatus* and *T. confusum*, which are storage pests of legumes. Inhibitors of proteases and carbohydrases (amylases), whether monovalent or bifunctional, may have evolved from the same ancestral gene to target multiple digestive enzymes of predators and pathogens. The acquisition of both bifunctional α -amylases / proteinase inhibitor genes and α -amylase inhibitor genes should provide some interesting opportunities for agricultural scientists to express these genes in transformed plants and to assess their defensive roles, either alone or in combination with other defensive genes, against insects and pathogens.

C. Occurrence and distribution

Occurrence of proteinase inhibitors in plants has been known since 1938 and they are extremely wide spread in their distribution throughout the plant kingdom. They appear to have been extensively studied in Leguminosae, Graminae and Solanaceae, probably because of the large number of species in these families which form important sources of food (Leiner et al., 1969).

The seeds of the Leguminosae have long been recognized as excellent sources of protein. It is perhaps not surprising therefore that a great deal of early work on protein inhibitors was on the now well- characterized inhibitors of trypsin and chymotrypsin from the widely grown soybean (*Glycine max*) and lima beans (*Phaseolus lunatus*) (Puztai, 1967).

Tubers of potato (*Solanum tuberosum*) are a notable source of a wide diversity of proteinase inhibitors. In this tissue, there are atleast seven different types of proteinase inhibitors which can be distinguished on the basis of their varying molecular weights, amino acid composition, N and C – terminal amino acids, stability during heating and specificity. Several of the inhibitor types may exist in the form of a number of closely related isoinhibitors.

The proteinase inhibitors also represent a quantitatively important fraction of the proteins found in most cereal grains. Mikos and Kirsi (1972) have demonstrated that several different inhibitors are present in high concentrations both in the embryos and endosperms of grains of barley, wheat, oats, and rye. They are also well known in the grains of rice, maize, and sorghum. A second survey for trypsin inhibitors in the plant tissue which are commonly found in human diets revealed that the potato and the sweet corn contained the highest levels, but they also were found in moderate amounts in spinach, broccoli, brussels, radish and cucumber. Negligible levels were found in fruits of peach, plum, and avacado (Chen and Mitchell, 1973).

D. Structure

The well characterized macromolecular proteinase inhibitors from plants are all proteins with little or no additional carbohydrate moieties. Generally they are relatively small with molecular weights falling in the lower part of the range 4000-80,000. The majority of the inhibitors are proteins containing between 70 – 90 amino acids and many of the large inhibitors are actually polymeric proteins whose protomers have minimum molecular weights of under 10,000 (Melville, et al., 1972). The few notable exceptions to this rule are the trypsin inhibitors from sweet potato (*Ipomea batatas* , 23,000- 24,000, Sugiura, 1973), oats 43,500,(Mikola,1972), and soybean Kunitz inhibitor (19, 900). The largest plant inhibitor known so far is the papain inhibitor from potato tubers, which is glycoprotein with a molecular weight of approximately 80,000.

The first details of the primary structure of the plant proteinase inhibitors started to appear in 1971 with the sequence of the lima bean inhibitor (Tan and Stevens 1971), and the soybean inhibitor. The complete amino acid sequences of other inhibitors including the soybean (Bowman–Birk) inhibitor (Odani and Ikenaka, 1972), the chymotrypsin inhibitor I from potatoes, the bromelain inhibitor from pineapples, the garden bean inhibitor and the carboxypeptidase inhibitor from potatoes have been determined. These sequences have revealed a striking degree of structural homology between some of the inhibitors.

A comparison of the primary structures of a number of the trypsin inhibitors clearly indicates their close structural similarities in fig 1. These details obviously permit comparisons to be made at several different taxonomic and phylogenetic levels. The figure illustrates intergenic (*Phaseolus / Glycine*), interspecific (*Phaseolus vulgaris / P. lunatus*) and intraspecific (iso inhibitors of *Phaseolus lunatus*) homologies.

In several plant proteinase inhibitors it has been noticed that the primary structures contain repetitive sequences or regions of internal homology. This phenomenon was first reported by Jan and Stevans (1971) in the lima bean inhibitor and also in soybean (Bowman–Birk) inhibitor and the garden bean inhibitor (see fig.1). These repetitive sequences have resulted from the extensions of shorter polypeptide chains by a process of

gene duplication. These regions of internal homology frequently contain the reactive (inhibitory) sites of the inhibitor which interact with the active site of the relevant proteinase.

Ozawa and Laskowski (1966) first proposed the reactive site model whereby the interaction of naturally occurring trypsin inhibitors with trypsin involved the cleavage of a single LYS – X, or ARG – X peptide bond in the inhibitor, and for chymotrypsin inhibitors the bond cleaved was LEU – X , TYR –X or PHE – X. The reactive sites are found to be located within a loop of the protein closed by a disulphide bridge. Different reactive (inhibitory) sites identified in plant proteinase inhibitors are shown in table 1.

ENZYME INHIBITED	REACTIVE SITE RESIDUES	SPECIES / INHIBITOR
Trypsin	ARG – ALA	Wheat , rye.
	ARG – ILE	Soybean (Kunitz).
	ARG – LEU	Maize.
	ARG – SER	Garden bean.
	LYS – SER	Soybean (Bowman- Birk)
Chymotrypsin		Chick pea, Lima bean etc,
	ARG – ILE	Soybean (Kunitz)
	LEU – SER	Lima bean, soybean.
	PHE – SER	Lima bean.
	LYS – SER	Potato II a / II b.
	LEU – ASP	Potato (Inhibitor I).
	MET – ASP	Inhibitor (Inhibitor I).
Elastase	ALA – SER	Garden bean.

In the case of Lima bean inhibitor the reactive site for chymotrypsin and trypsin inhibitor are in separate but homologous regions of the molecule and thus being called as

“Double headed”. Odani and Ikenaka (1973) have shown that it is possible by chemical and enzymatic means to cleave a double headed inhibitor into two smaller single headed fragments. In the case of Bowman- Birk Soybean inhibitor which contain 71 amino acid residues in its native form, treatment with cyanogen bromide and pepsin yielded two separate fragments of 38 and 29 residues. The larger fragment which contained the LYS – SER peptide bond retained 84%

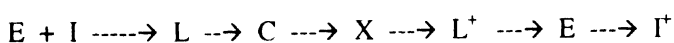
of the antitrypsin activity of the intact protein and the smaller one containing the LEU – SER bond had 16% of the anti chymotrypsin activity (fig. 2).

Recent findings by Laskowski et al.,1974, suggested that the ARG⁶³– ILE⁶⁴ reactive peptide bond in the soybean (Kunitz) trypsin inhibitor can be modified in various ways by chemical and enzymatic means without the inhibitor losing its activity.

E. The standard mechanism of proteinase inhibitors

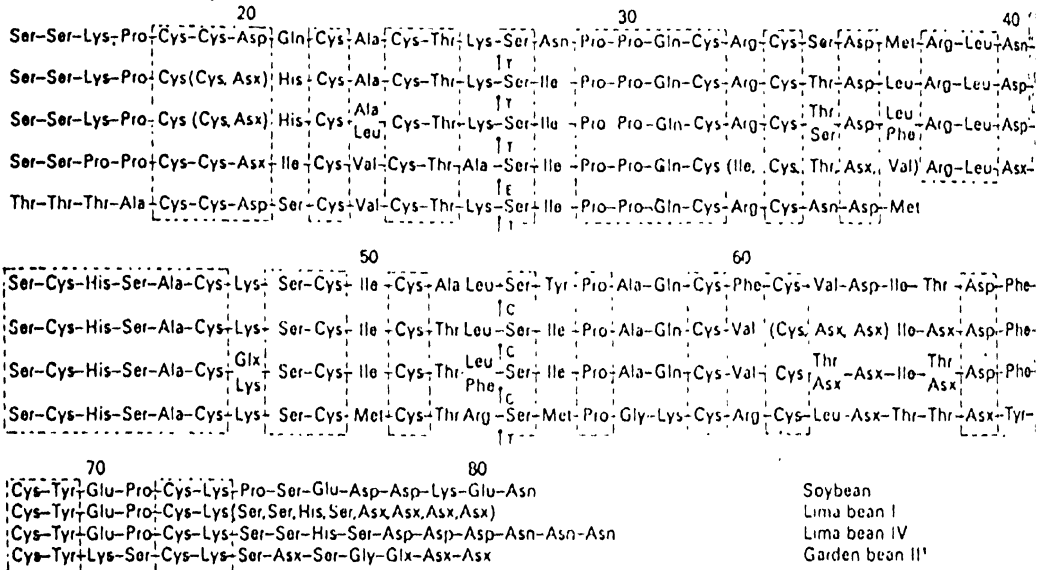
Inhibitors obeying the Standard mechanism are highly specific, limited proteolysis substrates for their target enzymes. On the surface of each inhibitor molecule lies at least one peptide bond called the reactive site, which specifically interact with the active site of the cognate enzyme. At typically used concentrations and neutral pH , the hydrolysis of the peptide bond is extremely slow. An additional property of the inhibitory reactive site is that their hydrolysis does not proceed to virtual completion, instead at neutral pH, the equilibrium constant between modified inhibitor (reactive site peptide bond hydrolyzed) and virgin inhibitor (reactive site peptide bond intact) is near unity (Estell, 1980). Since the same complex is found between the enzyme and either modified or virgin inhibitor both are thermodynamically equally strong inhibitors.

The overall mechanism of enzyme inhibitor interaction, including only those intermediates whose existence was definitely shown, can be written as follows,



Soybean [13]
 Lima bean I [14]
 Lima bean IV [14]
 Garden bean II* [20]
 Chick pea [90]

Asp-Asp-Glu-
 Asp-Glx-Pro-Ser-Glx-
 Ser-Gly-His-His-Glu-His-Ser-Thr-Asp-Glx-Pro-Ser-Glx-
 Glx-Pro-Ser-Glx-
 Ser-



Soybean
 Lima bean I
 Lima bean IV
 Garden bean II*

Fig. 1 Comparison of the amino acid sequences of the proteinase inhibitors from soybean (Bowman-Birk), lima bean and garden bean and a fragment of the chick pea inhibitor. Segments of identical (homologous) sequences in the proteins are enclosed in the dashed boxes. The reactive (inhibitory) sites of each protein are indicated by the arrows; T, site reacting with trypsin; C, chymotrypsin; E, elastase. The numbering of the residues shown refers to that proposed for the lima bean IV sequence by Stevens *et al.* [14]. The sequences are also arranged to facilitate comparison of the regions of internal homology (repetitive sequences) around the reactive sites (e.g. residues 14-36 and 41-63).

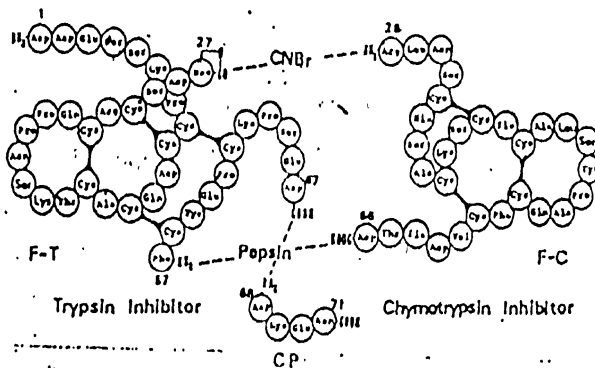


Fig. 2 Primary structures of two active fragments derived from the Bowman-Birk inhibitor, by sequential treatment with cyanogen bromide and pepsin (Odani and Ikenaka, 1978b). F-T is a trypsin inhibitor fragment, F-C a chymotrypsin inhibitor fragment, and CP is an inactive tetrapeptide fragment.

Where E : Enzyme; I : Virgin inhibitor; I⁺ : modified inhibitor; L : Loose complexes; L⁺:non covalent complexes; X : relatively long lived intermediate; C : stable enzyme inhibitor complex

i. Mechanism of action of serine proteinase inhibitors

The detailed molecular events at the active site of the enzyme and at the reactive site of the inhibitor are not clear (Huber and Bode, 1978). The mechanism of action of serine endopeptidase inhibitor involve a reactive site region which contacts the active site of the enzyme and instead of resulting in hydrolysis, results in formation of stable complex frozen in a transition state. A specific R group at the P1 position of the reactive site of the inhibitor is initially recognized by the specificity pocket of the enzyme. The carbonyl oxygen is pyramidalised as it is a substrate and a tetrahedral intermediate is formed when the serine oxygen of the enzyme attacks the carbonyl carbon of the inhibitor. The ensuing complex fits tightly and water is released from the active site region of the enzyme preventing hydrolysis and release of the cleaved inhibitor.

ii. The nature of the reactive site

In all the inhibitors that clearly obey the standard mechanism, the reactive site peptide bond is encompassed in at least one disulfide bond (Ozawa and Laskowski,1966), which ensures that during conversion of virgin to modified inhibitor the two peptide chains cannot dissociate. This requirement is not absolute since soybean trypsin inhibitor (Kunitz) with its Met⁸⁴ – LEU peptide bond hydrolyzed by subtilisin is still fully inhibitory and can be converted to modified inhibitor (ARG⁶³ – ILE reactive site hydrolyzed) without loss of 64 – 84 fragments (Laskowski, 1974).The aminoacid sequences surrounding the reactive sites of the various families of proteinase inhibitors are summarized in the table 2.

The reactive site residue, P1 generally corresponds to the specificities of the cognate enzyme. Thus inhibitors with P1 Lys – Arg tend to inhibit trypsin and trypsin like enzyme,

(Laskowski, 1971), those with P1 Tyr, Phe, Trp, Leu and Met inhibit chymotrypsin and chymotrypsin like enzymes.

The most striking feature of P1 specificity is that exchange of Lys for Arg at this position, either by actual mutation or by semisynthetic replacement, leaves the inhibitor specificity and strength approximately the same. The exchange of Lys or Arg for a chymotrypsin specific residue generally changes the inhibitor from a good trypsin inhibitor to good chymotrypsin inhibitor. An exception to this is soybean trypsin inhibitor (Kunitz).

In most proteins, as stated earlier the active site residues are stubbornly conserved, where as in every inhibitor family there is appreciable variation in P1 residues. For example,

1) It is generally agreed that serine proteinases seldom hydrolyze with P¹ Pro. A silver pheasant ovo- mucoid withCys Asn Lys ↓ Ala..... reactive site sequence inhibits trypsin whereas highly homologous golden pheasant ovo – mucoid withCys Asn Lys ↓ Prodoes not.

2)There is stubborn conservation of P¹ Ser in all known Bowman–Birk. Semi-synthetic substitution study showed that Ser in this position was better than any other amino acid residue whose substitution was attempted.

Rather surprisingly a synthetic variant of pancreatic trypsin inhibitor (Kunitz) withCys Lys ↓ Phe reactive site is a much weaker inhibitor than the naturalCys Lys ↓ Ala inhibitor.

Semisynthetic modifications also led Kowalski et al., in 1976 to the conclusions that in soybean trypsin inhibitor (Kunitz) removal of the amino acid residue P¹ leads to inactivation of the inhibitor, presumably because reformation of the P1 –P² peptide bond is not possible. Similarly, insertion of an additional residue between P1 and P¹ also led to inactivation even though all the peptide bonds were reformed, because the required reactive site geometry was destroyed.

F. The inhibitor families

The number of known and partially characterized inhibitors of serine proteinases is enormous. They can be classified into atleast 10 and probably more families. The tentative list of such families is represented in the Table 3,two of which have been discussed here in detail.

1) Bovine pancreatic trypsin inhibitor (Kunitz) family.
2) Pancreatic secretory trypsin inhibitor (Kazal) family.
3) Streptomycin subtilisin inhibitor family.
4) Soybean trypsin inhibitor (Kunitz) family.
5) Soybean proteinase inhibitor (Bowman–Birk) family.
6) Potato I inhibitor family.
7) Potato II inhibitor family.
8) Ascaris trypsin inhibitor family.
9) Other families.

Bowman–Birk inhibitor family

These inhibitors are readily isolated from the seeds of all leguminous plants. It consists of two tandemn homology regions on the same polypeptide chain each with a reactive site (Krahn et al., 1970). It has 7 disulfide bonds and 71 residues. The homology regions are linked by interhomology region disulfide bonds (Odani, 1973). Ikenaka and co-workers split

the two homology regions by making two specific peptide bond cleavages (CN Br and a pepsin catalysed cut) (Odani, and Ikenaka, 1973). Both homology regions are separately active, but are weaker inhibitors than the parent molecule, due to partial loss of rigidity.

In most Bowman–Birk type inhibitors the P1 residue in the first (-NH₂ terminal) homology region is Lys and trypsin is inhibited, while the P1 residue in the second homology region is Leu and chymotrypsin is inhibited.

Many homologous Bowman–Birk inhibitors are isolated from species of beans or even from a single bean (Odani and Ikenaka 1977). Some of these forms appear to differ only in the length of their –NH₂ terminal sequence. The aminoacid sequence of Bowman–Birk inhibitor is shown in fig. 3.

Soybean trypsin inhibitor (Kunitz) family

The first plant inhibitor to be well characterized was soybean trypsin inhibitor (Kunitz). Its isolation and crystallization by M.Kunitz is one of the classic achievements of inhibitor chemistry (Kunitz, 1947). The polypeptide chain in single-headed Kunitz soybean inhibitor has 181 residues and only two disulfide bridges. The storage role for the inhibitor is supported by their presence in large quantities in seeds and tubers and represent about 6% of their protein.

Mickel and Standish (1947) observed that larvae of certain pests were unable to develop normally on soybean products. Lipke, et al.,(1954) studied the toxicity of soybean inhibitors on the complete development of *Tribolium confusum*, a common pest of stored grain.

The active site of Kunitz trypsin inhibitor is defined by two aminoacid sequences Arg–Ile in positions 63 and 64. This site when combined with the active site of trypsin and the peptide bond between Arg - Ile is cleaved, but still the trypsin remains bound to the inactive complex bound on the weak non-covalent interaction existing in the complex. The presence of inhibitors in the complex tissues may be correlated with different physiological stages of development. These inhibitors are associated with the resting stages and decrease during

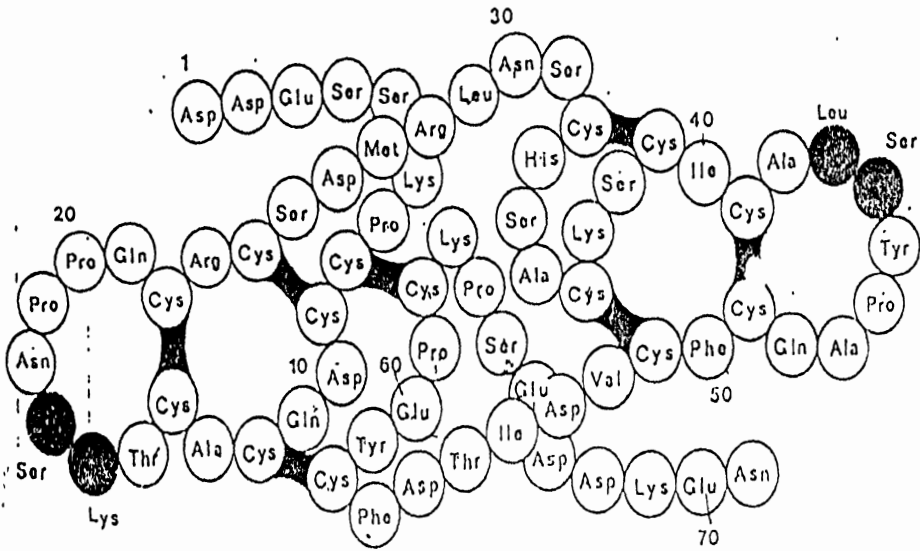


Fig 3 Amino acid sequence of Downman-Dirk inhibitor. The reactive sites for trypsin (Lys 16-Ser 17) and for chymotrypsin (Leu 44-Ser 45) are shaded. Source: Liener and Kakade (1980). Copyright by Academic Press, Inc.

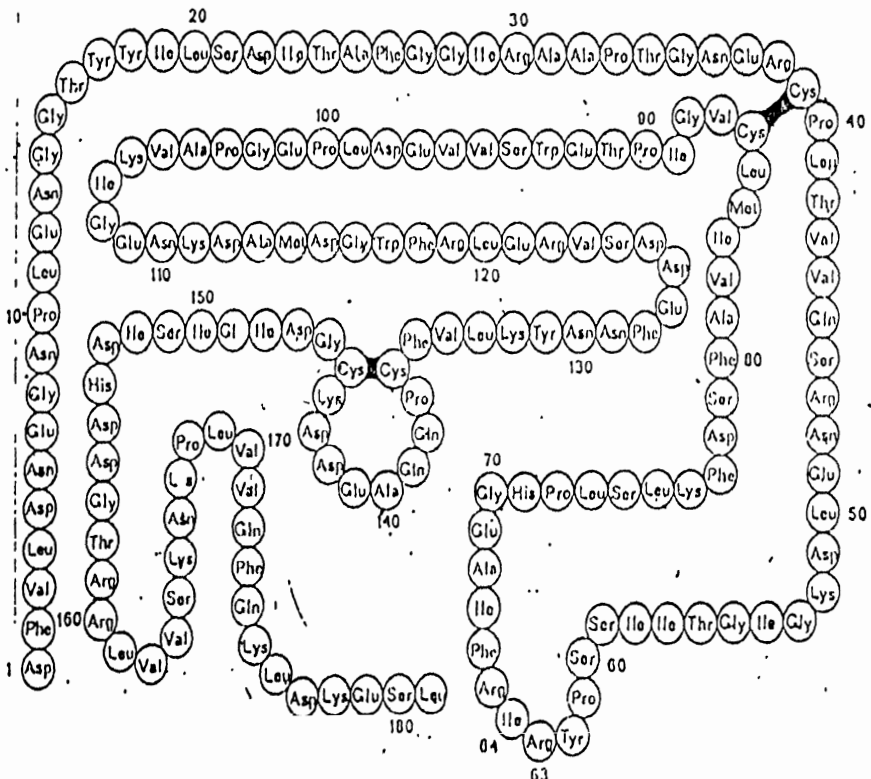


Fig 4 Amino acid sequence of Kunitz trypsin inhibitor from soybeans. Source: Liener and Kakade (1980) and Kim *et al.* (1985). Copyright by Academic Press, Inc.

germination. The amino acid sequence of the Kunitz inhibitor from soybean can be shown in the fig 4.

G. Effect of proteinase inhibitors on the digestive physiology of animals:

Most animals require proteolysis to degrade and use the component amino acids of the proteins they consume. Because of key roles of proteinases in the digestive processes of animal and microbes, considerable interest has been generated over the years about the effects of proteinase inhibitors that are often present in the food chain of humans. When such proteins are present in high concentrations in foods of some farm animals (Leiner, et al., 1988), they can significantly alter digestive processes and interfere with growth and development. Proteanase inhibitors do not pose a direct problem to humans, because food that contain high levels of these proteins are cooked, which inactivates the inhibitors.

Trypsin inhibitors in animal diets have been known to evoke increased pancreatic secretions, implying that inactive trypsin plays a role in the normal regulation of pancreatic functions. This regulation by trypsin involves the degradation of a "monitor peptide " (Fushiki, 1989; Iwai, et al., 1987), that is secreted into the gut where it regulates the release of a circulatory polypeptide hormone cholecystokinin (CCK). When CCK is released from the intestinal wall into the blood stream it controls various processes such as pancreatic secretions, gall bladder contraction, gut mobility and appetite. CCK is a family of polypeptides that range in size from 4-58 amino acids, but the small peptides proteolytically cleaved from the larger ones, are considered to be the active forms.

Interactions of the inhibitors with trypsin and other digestive proteases interfere with the normal degradation of the monitor peptide, which then abnormally activates the complex feedback mechanisms that produce major chronic physiological responses in animals. Thus the presence of high levels of proteinase inhibitors on a continued basis can lead to chronic hypersecretion by the pancreas, loss of proteolytic activity in the gut, loss of appetite, starvation and eventual death.

H. Application of proteinase inhibitors for insect resistance

A major tool of plant genetic engineering is the introduction of agronomically desirable phenotypic traits into crop plants. One such target is enhanced resistance to insect pests. A gene encoding a cowpea trypsin inhibitor (CPTI) which has been shown to give resistance to insect pests, confers, when transformed to tobacco, enhanced resistance to the insect pests. (Hilder, et al., 1987). Cowpea trypsin inhibitor (CPTI) are small polypeptides of 80 amino-acids belonging to Bowman-Birk type of double headed serine protease inhibitors. Feeding trials with purified CPTI, incorporated at physiological levels into artificial diets, have shown these to be anti-metabolic agents against *Heliothis*, *Spodoptera*, *Diabrotica* and *Tribolium* which cause major economic loss.

The CPTI gene was derived from plasmid pUSSRc3/2. A 550bp long AluI - ScaI restriction fragment containing 240bp coding sequence for mature inhibitor was transferred to SmaI site of *A. tumefaciens* Ti plasmid binary vector pROKII, (Bevar, M., 1985). This construct was used to transform leaf discs of *Nicotiana tabacum* cv. Samsun NN. Transformants were selected by their resistance to kanamycin and transformed plants were regenerated from shootlets by transfer to a root-inducing agar media containing kanamycin.

Levels of CPTI produced in the transformants were measured by dot-immunobinding assay using polyclonal antibodies raised in rabbit against total CpTI. The bioassay for insecticidal activity in insect feeding trials were done which have given good results. Incorporation of this gene into crops such as cotton, maize etc., are in progress.

Genes containing the cauliflower mosaic virus 35S promoter fused to open reading frames coding for tomato proteinase inhibitor I, tomato inhibitor II, and potato inhibitor II were expressed in transgenic tobacco plants (Johnson et al., 1989). The plant transformation plasmids were constructed containing either the inhibitor I or inhibitor II coding regions. Tobacco plants were transformed through *Agrobacterium* harbouring these plasmids and leaves from the transgenic plants were assayed immunologically for the presence of inhibitor I and II. Leaf extracts from the transgenic tobacco strongly inhibited both trypsin and chymotrypsin. Larvae of *Manduca sexta* (tobacco hornworm) which were fed a diet of

leaves from transformed plants grew more slowly than those fed on untransformed tobacco leaves. The presence of foreign tomato or potato inhibitor II in tobacco leaves at levels >100 µg/g of tissue severely retarded the growth of larvae that fed on them.

Potato inhibitor genes were obtained from a library of potato genes in Charon 4 phage by plaque hybridization. The constructed plasmids pRT45 containing potato inhibitor I-chloramphenicol acetyltransferase (CAT) gene was transferred into tobacco leaf tissue by the co-cultivation method (Thornburg, et al., 1987). Transformed callus tissue selected using kanamycin did not exhibit any detectable expression of CAT gene.

Over 80 plantlets were regenerated from the transformed callus cells and grown in sterile conditions which exhibited a very low CAT expression. The regenerated plants were grown to a height of 20-30 cm and tested for wound inducibility of CAT expression which showed that CAT gene is expressed in response to injury.

2) PLANT TRANSFORMATION

We are emerging from a period of plant transformation research dominated by the need to develop proven genetic transformation methods for the major environmental and economic plant species, into the era of applications of transformation as a core research tool in plant biology - a practical tool for cultivar improvement. The capacity to introduce and express diverse foreign genes in plants, first described for tobacco in 1984 (De Block et al., 1984) has been extended to over 120 species in at least 35 families (Paszowski et al., 1980). Successes include most major economic crops, vegetable, ornamental, medicinal and fruit yielding plants. Much of the support for plant transformation research has been provided because of expectations that this approach could, generate plants with useful phenotypes unachievable by conventional plant breeding, correct faults in cultivars more efficiently than conventional breeding and allow the commercial value of improved plant lines to be captured by those investing in the research.

A. Biological requirements for transformation

The essential requirements in a gene transfer system for production of transgenic plants is, (1) Availability of a target tissue including cells competent for plant regeneration, (2) A method to introduce DNA into those regenerable cells and, (3) A procedure to select and regenerate transformed plants at a satisfactory frequency.

The technique that has been proven to produce transgenic plants from a range of species is *Agrobacterium*-mediated transformation, bombardment with DNA coated microprojectiles and electroporation or PEG treatments of protoplasts. The stages and time courses for typical transformation strategies using *Agrobacterium* or DNA coated microprojectiles are shown in the figure 5.

Agrobacterium LEAF DISK

TRANSFORMATION.

Co-cultivate leaf disks with *Agrobacterium*.
cells.



1 - 2 days

Wash leaf disks.



Antibiotic treatment to kill *Agrobacterium*
and select transformed plant cells.



2 - 4 weeks

Surviving leaf disks onto fresh selection,
regeneration medium

MICROPROJECTILE CALLUS

TRANSFORMATION.

Initiate and proliferate embryogenic
cells.



8 - 12 weeks

Microprojectile bombardment.



Callus grown without selection.



4 days

Callus grown under escape free
selection.



6 - 10 weeks

Root excised on selection media.



8 - 12 weeks

Plant regeneration and rooting
on Escape free media



4 - 6 weeks

Potted transgenic plants.



8 - 12 weeks

Potted transgenic plants.

Agrobacterium mediated transformation: *Agrobacterium* is gram negative bacterium belonging to the family Rhizobiaceae. They are classified according to the phytopathogenic characteristics as follows:

1. *A. tumefaciens*, which induce crown gall disease.
2. *A. rhizogenes* which induce hairy root disease
3. *A. radiobacter* which is avirulent.

They act on the dicotyledonous plants and a very few monocotyledonous plants of Liliaceae and Amaryllidaceae. Once initiated by *Agrobacterium* tumour growth can continue in the absence of bacterium and the tissue grows anexically in tissue culture media lacking exogenous supplies of auxins and cytokinins which are normally required to promote growth of plant tissues in vitro. Tumour tissues synthesize novel amino acid and sugar derivatives known collectively as "opines". The types of opine synthesized in the tumour is dependent on the strain of *Agrobacterium* that initiated tumour formation. The *Agrobacterium* selectively catabolises the opine whose synthesis it has induced using it as a source of carbon and nitrogen.

Both tumour induction and opine synthesis are associated with the presence of a megaplasmid, the Ti (tumour inducing) plasmid in the case of *A. tumefaciens* and the Ri (root inducing) plasmid in *A. rhizogenes*. The structural formulae of the characteristic opines can be shown in the fig 6.

Ti plasmid of *Agrobacterium tumefaciens* : Ti plasmids of *A. tumefaciens* are 200-250 kb in size and are stably maintained in the *Agrobacterium* at temperatures below 30°C. It has four important regions as follows:

- 1) T- DNA (transfer DNA) region .
- 2) Vir (virulence) region.
- 3) CON region.
- 4) ORI region.

The first two are involved in tumour formation and the last two is conjugative transfer and replicative maintenance of the plasmid within the *Agrobacterium*.

B. Genetic colonisation

The process of crown gall induction consists of a large number of discrete essential steps.

- 1) Wounding of the plant is necessary to allow entrance of bacteria and to make available compounds that induce its virulence system.
- 2) The bacteria multiply in the wound sap and attach to the walls of plant cells in the wound.
- 3) T–DNA is transferred and expressed in the plant cells even before integration.
- 4) After integration T-DNA expression is maintained at a particular stable level depending on the position of integration.
- 5) Tumors develop due to cell divisions triggered by the continuous production of auxin and cytokinin via T-DNA encoded enzymes.
- 6) The resulting tumors consist of a mixture of transformed and normal plant cells.

7) The T-DNA containing cells produce and excrete opines that are consumed specifically by the infecting *Agrobacterium*.

An opine act not only as an inducer of its catabolic genes but also as an aphrodisiac and activate the conjugative transfer system of the Ti-plasmid. The genetic map of octopine Ti-plasmid is shown in the figure 7.

C. Applications

i. Vector systems

Although besides T-DNA no other parts of the Ti-plasmid become integrated into the genome of plant cells, it has long been debated whether the entire plasmid or just the T-DNA segment was introduced into the plant cells. Genetic experiments showed that the T-region separated from the rest of the Ti-plasmid was maintained on independent replicons and did not form a cointegrate vector again. This firmly established that no physical linkage between the two is necessary for T-DNA transfer. Transfer system is determined by the *Vir* and *Chv* genes and the 24 bp direct repeat which flanks the T region. On the basis of these considerations vectors for transformation of plants have been developed. These can be distinguished into two types as follows,

1) Cis systems in which new genes are introduced via homologous recombination into an artificial T-DNA already present on Ti-plasmid.

2) Binary systems in which new genes are cloned into plasmids containing an artificial T-DNA which are introduced into *Agrobacterium* harboring a Ti-plasmid with *Vir* region but lacking T region.

Transgenic plant cells with Ti T-DNA are tumors and cannot be regenerated. However plants transformed with disarmed T-DNA are non tumorous and can be regenerated. In order to select transformed plant cells, new markers have been developed which are based on the sensitivity of the plant cells to antibiotics and herbicides. The expression of bacterial genes encoding for enzymes capable of detoxifying such compounds in plant cells can lead to such

resistance. Vectors are now available which allow selection, for instance for kanamycin resistance via neomycin phosphotransferase (NPT II) gene from the bacterial transposon Tn 5, hygromycin resistance via hygromycin phosphotransferase (HPT) gene from *Escherichia coli* etc. Screening for transformants can be done by using the genes for opine synthase activities, luciferase which give light emission, β - galactosidase and β - glucuronidase.

Histological staining for the reporter enzymes can be done using 5-bromo-4-chloro-3-indolyl derivatives which release a compound after enzymatic activity that is quickly converted into indigo (blue) with oxygen. In order to avoid expression of β - glucuronidase by *Agrobacterium*, gene constructs were made in which gene lacked a bacterial ribosome binding site or contained an intron in its coding sequence. The *Agrobacterium* vector system is being used extensively now for the transfer of various traits to plants such as follows,

1) Resistance to viruses, 2) herbicide tolerance, 3) male sterility, 4) cold tolerance, 5) resistance to pathogenic bacteria, 6) resistance to insects etc.

Construction of plant vectors in which one genes in the T-region are replaced by genes that do not disturb plant development are shown in the figure 8.

ii. The GUS gene fusion systems

The use of gene fusion is becoming an increasingly common and powerful strategy in the study of gene activity. Gene fusions are DNA constructions in which DNA sequences from two genes are combined such that the coding sequence of one gene are transcribed or translated under the direction of another gene. The *E.coli* gene encoding β - glucuronidase(GUS) has now been developed for use as a reporter gene in transgenic plants, animals and bacteria. GUS catalyses the hydrolysis of a wide variety of β - glucuronides. GUS is an exo-hydrolase, it will not cleave glucuronides in internal positions within polymers. The enzyme is stable and can tolerate many detergents and varying ionic conditions. It has no cofactor and ionic requirements. β - glucuronidase from *E.coli* is a monomer of molecular weight of 68.2 kDa. Its active form is a tetramer. GUS is encoded by

the *gusA* locus of *E.coli*. There are many substrates for GUS but the most used is 5-bromo-4-chloro-3-indolyl β -D-glucuronide (X-glu).

D. Direct gene transfer

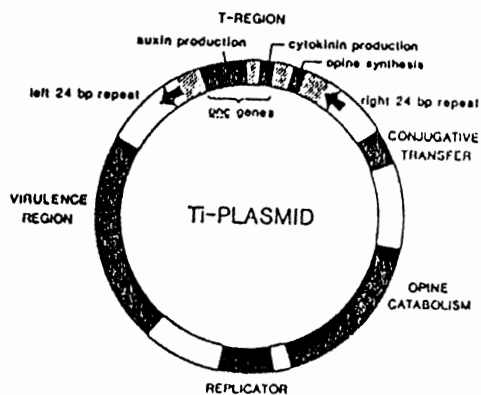
Transformation through Biolistics

The biolistic process has proven useful in the transformation of numerous and diverse organisms, including bacteria, algae, yeast and filamentous fungi. Animal cells and tissues have also been transformed. The process has been most useful to date for the transfer of DNA into a wide variety of plant species.

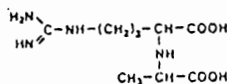
Microprojectile bombardment in plant cells was first described by Sanford et al in 1987. A variety of metals such as tungsten, gold, platinum and palladium can be used with the biolistic apparatus. Currently used biolistic methodologies involves chemical precipitation of plasmid DNA onto microspheres of tungsten and gold. General conditions for efficient binding of DNA to microprojectiles which include the amount of DNA, precipitating agents such as CaCl_2 or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, polyamines and amount of particle have been determined. Several alternate polyamines including spermine, caldine and thermine are used. Spermine dramatically increases the frequency of both transient and stable transformation in tobacco.

The microprojectiles are then accelerated into the target cells and tissues using various devices such as those powered by a gun powder charge. The helium powdered device gives improved transformation rates for suspension cultured tobacco cells.

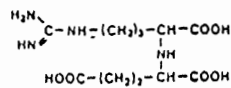
The genes thus entered into plant cells and gets stably integrated which can be selected by using the specific reporter gene such as GUS by the histochemical assay as described earlier.



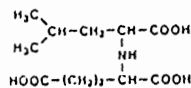
Genetic map of an octopine Ti plasmid.



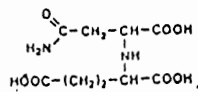
OCTOPINE



NOPALINE

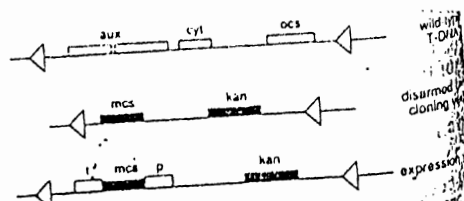


LEUCINOPINE



SUCCINAMOPINE
(ASPARAGINOPINE)

Structural formulae of four characteristic opines



Construction of plant vectors in which the opine genes in the T region are replaced by genes that do not disturb plant development. mcs, multiple cloning site; kan, kanamycin resistance gene for plants; p, promoter; t, terminator.

III. Materials and methods

III. MATERIALS AND METHODS

1) Standardisation of tissue culture with leaflets of *Arachis hypogea* L.

A. Plant material used: Groundnut (*Arachis hypogea* L.) JL-24 was used as the experimental source material.

B. Culture media and culture conditions

Murashige and Skoog (1962) basal medium (MS) with different modifications were used. The various combinations were as follows:

- 1) MS + B5 organics + 5 μ M BAP (1 mg / L) + 0.5 μ M IAA (0.1 mg / L).
- 2) MS + MS organics + 8.9 μ M BAP (2 mg / L) + 5.38 μ M NAA (1 mg / L).
- 3) MS + MS organics + 13.5 μ M BAP (3 mg / L) + 5.38 μ M NAA (1 mg / L).
- 4) MS + MS organics + 17.8 μ M BAP (4 mg / L) + 5.38 μ M NAA (1 mg / L).
- 5) MS + MS organics + 17.8 μ M BAP (4 mg / L) + 2.69 μ M NAA (0.5 mg / L).
- 6) MS + MS organics + 22.2 μ M BAP (5mg / L) + 0.5 μ M NAA (0.1 mg / L).

The culture media was adjusted to pH 5.8 with 1N HCl or 1N NaOH prior to autoclaving and was solidified with 0.8% agar. BAP and NAA were added directly to the media before autoclaving whereas IAA was filter sterilized and added after autoclaving.

C. Culture initiation

Seeds removed from dry pods were disinfected with 0.1% HgCl₂ for 7 min. Subsequently the seeds were washed 3-4 times with sterile distilled water under aseptic conditions. The seed coats of the d₀ seeds were removed and the leaflets were excized. The leaflet explants were placed on the shoot induction media with the basal cut end in contact with the media. The seeds for d₁, d₂, and d₃ were placed on MS basal media for germination to obtain leaflet explants from seedlings of different age. This was to check the age dependent regeneration frequency of the explant. After induction of shoot buds within 3 weeks the cultures were transferred to fresh media of same composition for further development.

2) Plasmid used for transformation

Agrobacterium tumefaciens strain C58 harbours a binary plasmid pHS 737: SBTI which was used for plant transformation studies. It was developed as a binary vector for use in *Agrobacterium tumefaciens* mediated gene transfer. It is 14380 bp in size with SBTI gene of 600 bp. The SBTI gene was cloned by K. K. Sharma into Xho I (blunted) and Bam HI sites of pHS 737 from pRT : SBTI. It is driven by a single 35S promoter and is also provided with termination sequences. The gus : : npt fused gene driven by another 35S cauliflower mosaic virus and provided with polyadenylation sequences can be used as a reporter gene.

A. Isolation of the plasmid

The Alkaline – lysis method (Birnboim and Doily, 1979) was used for plasmid isolation. A single colony of the bacteria was grown in 25 ml of liquid YEB medium (Appendix A) with kanamycin at 50 mg / L concentration for selection, for overnight at 28°C at 175 rpm. 1.5 ml of this culture taken in an eppendorff tube was centrifuged at 12000 rpm for 30–60 sec at room temperature. The supernatant was removed having the bacterial pellet as dry as possible. Pellet was suspended in 100 µl ice cold GTE (Appendix A) and kept on ice for 5 minutes. 200 µl of freshly prepared lysis buffer (Appendix A) was added and the tube was inverted to mix the contents gently and left for 5 min on ice. 150 µl of 5M potassium acetate (Appendix A) was added and the tube was inverted several times and left on ice for 5 min. Centrifugation at 12000 rpm for 15 minutes at 4°C was added and the supernatant transferred to a fresh tube. Care is taken not to have any precipitate in the supernatant. An equal volume of phenol: chloroform (1:1) was added and the tubes inverted several times gently. The samples were then centrifuged for 2 min and the upper layer was transferred to fresh tube. The DNA was precipitated with 0.6 vol. of isopropanol. It was allowed to stand for 2 min at room temperature. The pellet was washed with ice cold 70 % ethanol. Ethanol was removed by aspiration and the pellet air dried. The pellet was dissolved in 250 µl of TE (pH–8.0) buffer containing 20 µg / ml (Appendix A) Dnase free Rnase.

B. Electrophoresis of pHS : SBTI

The plasmid thus isolated was run in 0.8% gel containing ethidium bromide at 0.5µg/ml at 50V, in 1X TAE electrophoretic buffer. The bands were examined by long wavelength U.V illuminator.

C. Restriction analysis of the plasmid pHS 737 : SBTI

pHS 737 : SBTI was restricted with several enzymes that have unique sites in the plasmid. A 20 µl reaction was used in which 16.5 µl of DNA, 2 µl of buffer and 1.5 µl of restriction enzyme was used. All the additions done on ice and the tubes were incubated for 3 hrs at 37°C in hot water bath . The reaction was stopped after electrophoresis with the addition of 0.5M EDTA (pH 8.0). The fragments were then visualized after electrophoresis in 0.8 % agarose run at 50V in 1X TAE, by transilluminator.

3) Transformation through *Agrobacterium*

A. Culture initiation

i. Preparation of inoculum for transformation through *Agrobacterium* : 5 ml of *Agrobacterium* culture harboring pHS 737 : SBTI plasmid was transferred into a 25 ml tube under aseptic conditions and centrifuged at 4000 rpm for 10min at 4°C. The pellet was resuspended in 5 ml of sterile ½ MS and centrifuged for 10 min at same rpm and temperature. This step is repeated once again. The pellet dissolved in 25 ml of ½ MS which was used for transformation.

ii. Sterilization of leaf discs of tobacco : The leaves of tobacco were surface sterilized by sequential treatments by dipping them in 70% ethanol for 30 sec and then wiping them with sterile tissue paper. These were transferred to 15% clorax solution for 10 min. These leaves were then washed in a series of sterile distilled water for 3-4 times. Leaf discs were cut with a sterile leaf disc borer and placed on MS media supplemented with 2.25 mg / L of BAP and 0.1 mg / L of NAA.

iii. Co-cultivation with *Agrobacterium tumefaciens*

Co-cultivation of tobacco leaf disks with *Agrobacterium* : The tobacco leaf discs were dipped in *Agrobacterium* inoculum thus prepared for 5 min. This facilitates the adhesion of the bacteria to the cut ends of the leaves. Transfer the leaf discs to the same media with their abaxial side in contact with the media. Co-cultivation was for 2-3 days after which the leaf discs were transferred to the same induction media supplemented with 250 mg/L of cefatoxime. (cefatoxime being bacteriostatic arrests the growth of bacteria). After induction for 2-3 weeks, the explants were transferred to the selection media of the same composition but supplemented with 50 mg/L of kanamycin.. This facilitates the growth of only those shoots which were transformed thus providing good selection.

Co-cultivation of leaflets of groundnut with *Agrobacterium* : The leaflets from the sterilized seeds were dipped in the inoculum of *Agrobacterium* after incizing them at their basal region from which the regeneration takes place. The leaflets were then placed on MS basal media supplemented with 3 mg/L of BAP and 1 mg/L of NAA. Co-cultivated for 2-3 days and then transferred on to the same media containing cefatoxime. After induction of organogenesis for 2-3 weeks, they were transferred on to the selection media (same media) supplemented with 50 mg/L of kanamycin for the specific growth of transformants.

4) Transformation through biolistics

Biolistic-mediated gene transfer was carried out by using the gene gun PDS 1000 He from Bio-Rad and operated as per the manufacturers protocols. Biolistics involves the direct transfer of DNA into the plant tissue. Two plasmids were used pHS 737 : SBTI. pHS 737 : SBTI has been described earlier.

pRT 99GUS : pRT99GUS contains β -glucuronidase (GUS) gene driven by a strong cauliflower mosaic virus 35S promoter and also a NPT II gene used for kanamycin selection is also driven by another 35S promoter. This plasmid has been used to optimize the bombardment conditions. Both these genes were provided with polyadenylation sequences and multiple cloning site. The plasmids pRT99GUS and pHS737:SBTI are shown in the

figs.9 and10. The two plasmids pHS:737 and pRT:SBTI from which pHS737:SBTI was constructed are shown in the figs.11 and 12.

A. Transformation technique

The explants were sterilized as described earlier. The explants were placed compactly at the center of the induction media in a petriplate. Transformation through biolistics involves the prior preparation of microcarrier and macrocarrier.

i. Microcarrier preparation

In a 1.5ml microfuge tube 60 mg of microprojectiles were weighed. To that 1ml of freshly prepared 70% ethanol was added. It was vortexed on a platform vortexer for 3-5 min and incubated for 15 min. The microparticles were pelleted by spinning for 5 sec in a microfuge and the liquid discarded. The following steps were repeated 3 times.

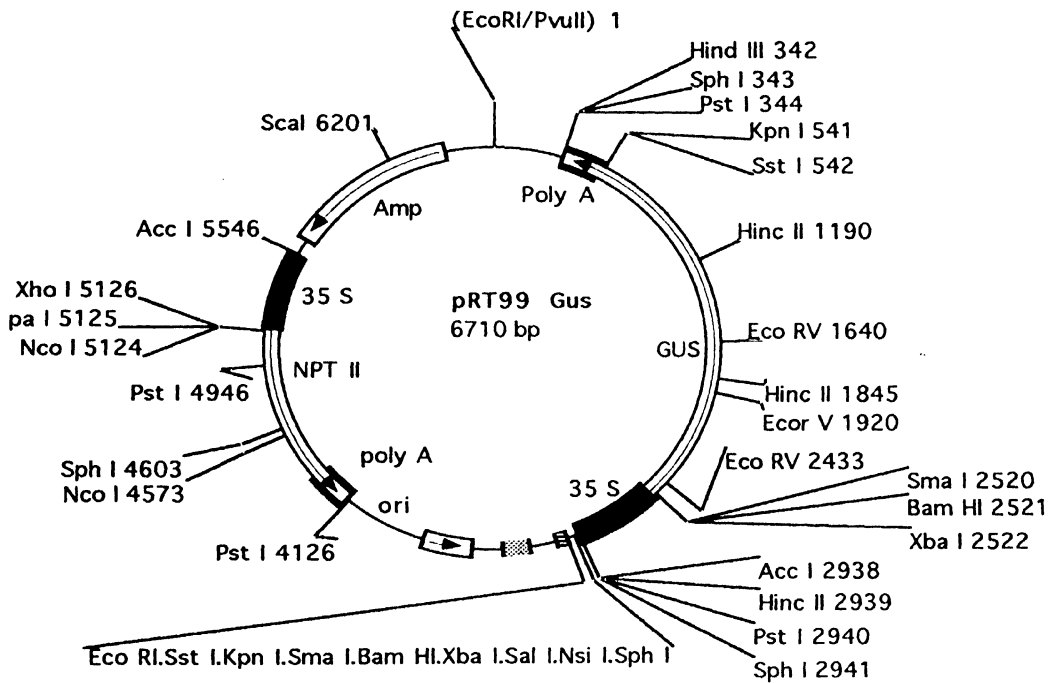
a) 1 ml of sterile water was added b) vortexed for 1 min c) the particles were allowed to settle for 1 min d) The microparticles were pelleted by spinning for 3 sec in a microfuge.e)The liquid was then discarded.

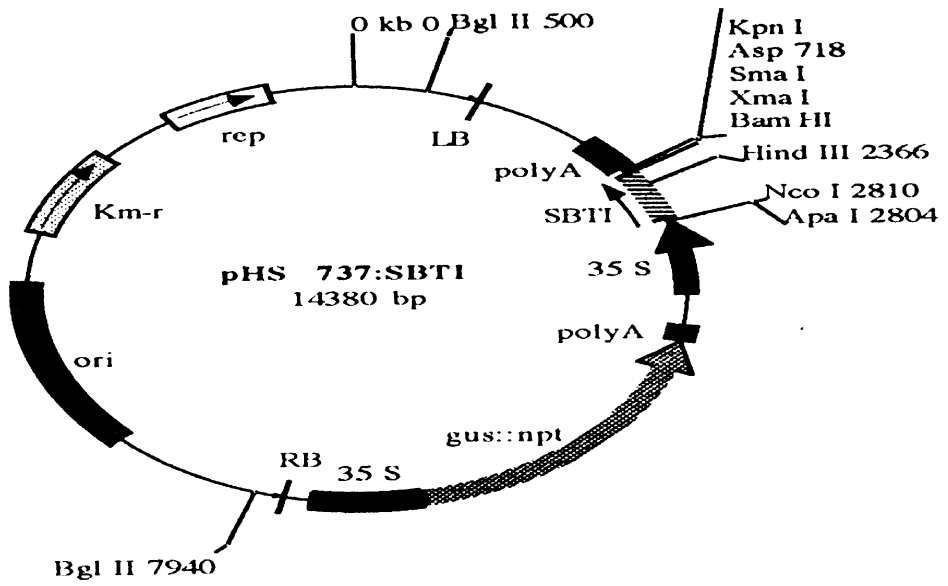
After these steps 1 ml of sterile 50% glycerol was added to bring the microparticle concentration to 60 mg/ml. The microparticles were then stored at room temperature for upto 2 weeks for further use.

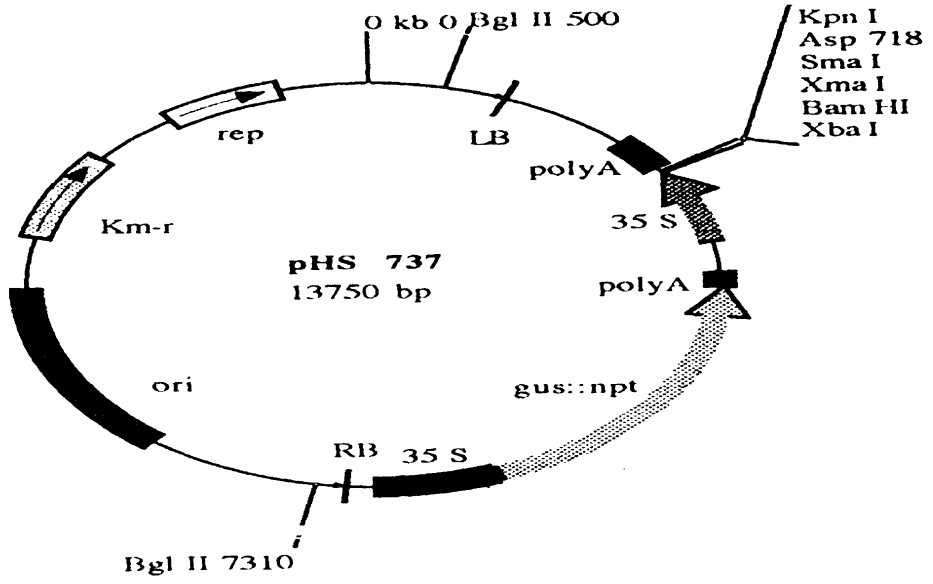
ii. Coating DNA onto microcarrier

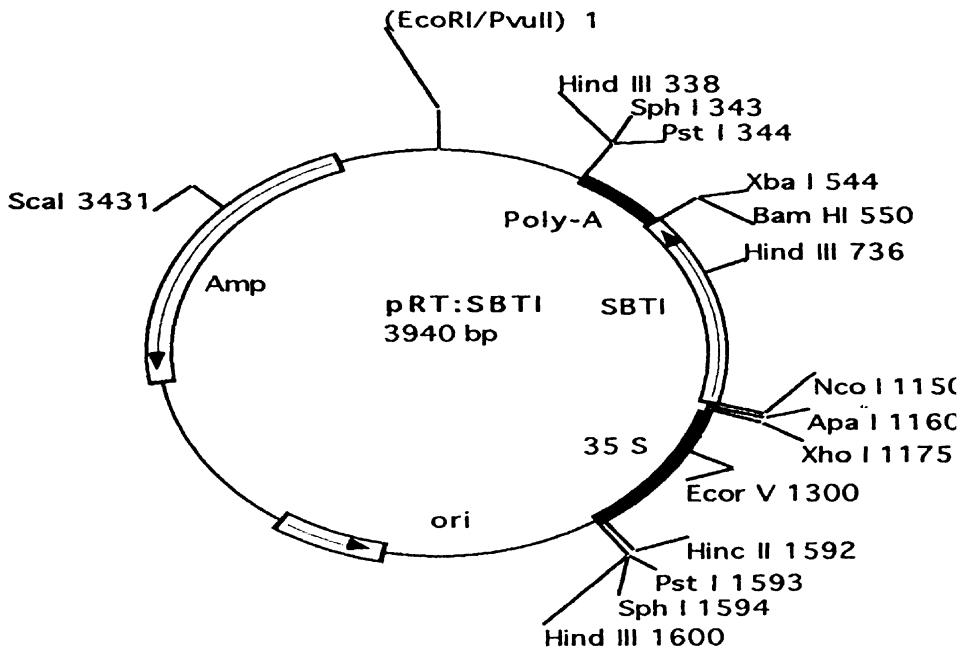
The microcarriers thus prepared were vortexed for 5 min on a platform vortexer to resuspend and disrupt agglomerated particles. 50 μ l (3mg) of microcarriers was removed to a 1.5 ml microfuge tube. The following components were added in the order while vortexing the tube, 5 μ l DNA (1 μ g/L), 50 μ l CaCl_2 (2.5 M), 20 μ l spermidine (0.1 M).

Again vortexed for 2-3 min. The microcarriers were allowed to settle for 1 min and pelleted by spinning for 2 sec in a microfuge tube. The liquid is then discarded. 140 μ l of 70% ethanol was added without disturbing the pellet and the liquid discarded. 140 μ l of 100% ethanol was added without disturbing the pellet and the liquid removed .The pellet









was resuspended in 140 μ l of 100% ethanol by tapping the side of the tube several times and then by vortexing at a low speed for 2 sec. 6 μ l of the microcarriers was taken and then spread onto the macrocarriers evenly.

5. Selection of the transformants

After 24 hrs of bombardment, the explants were spread evenly on the same media and allowed to regenerate for a week. Then the regenerating explants were transferred to the same media containing 50 mg/L of kanamycin and allowed them for further development. Only those which were transformed had regenerated which can be confirmed through GUS assay.

A. GUS assay for the putative transformants : GUS assays are done both for the transient and permanent expression of the gene transferred. Transient expression is checked after 24 hrs of bombardment for biolistics. GUS assays for the permanent gene integration was done after 20 days which is ideal.

The explants were dipped in 100 μ l of the X-glu present in an eppendroff tube, which were covered with aluminum foil to prevent light and kept at 37°C for 24 hrs during which the enzyme glucuronidase cleave the substrate into a blue complex which was visualized on the transformed tissue. The assay mixture was removed and the tissue was cleared of chlorophyll by sequential changes of 70-95% ethanol until no chlorophyll persists in the tissue. The tissue was mounted in glycerol and observed under the microscope.

IV. Results and Discussion

IV. RESULTS AND DISCUSSION

Since the regeneration of the transformed tissue has been found difficult, standardization of the regenerative system had been taken up by many scientists. Different concentrations of the auxin and cytokinin had been used in order to check for the regeneration frequency of the embryo leaflets of groundnut. The age of the explant has also been taken into consideration to check for the regeneration frequencies with different concentrations of phytohormones.

The following table represents the percentage response of the different aged leaflets as explants to the various concentrations of phytohormones.

Table 1. Effects of different concentrations of BAP, IAA and NAA on leaflet explants of groundnut cv. JL 24. For d₀ leaflets it is as follows:

Media Composition	No. of explants	No. of explants showing response	No. of shoots in each explant.	% of explant responding.
MS+B5organics+1mg/ LBAP+0.1mg/L IAA	77	28	2-4	36.36
MS+Msorganics+2mg/ LBAP+1mg/L NAA.	30	22	3-5	73.33
MS+Msorganics+3mg/ LBAP+1mg/L NAA.	24	18	2-4	75.00
MS+Msorganics+4mg/ LBAP+1mg/L NAA	48	30	2-6	62.50
MS+Msorganics+4mg/ LBAP+0.5mg/L NAA	45	29	2-4	64.44
MS+Msorganics+5mg/ LBAP+0.1mg/L NAA.	37	26	2-4	70.27

Table 2. Effects of different concentrations of BAP, IAA and NAA on the leaflet explants of groundnut cv. JL 24. For d₁ leaflets it is as follows:

Media Composition	No. of explants cultured	No. of explants responding	No. of shoots per explant	% of explant responding
MS+B5organics+1mg/ LBAP+0.1mg/L IAA	30	26	2-4	86.66
MS+Msorganics+2mg/ LBAP+1mg/L NAA	30	21	8-10	70.00
MS+Msorganics+3mg/ LBAP+1mg/L NAA	46	40	8-10	86.95
MS+Msorganics+4mg/ LBAP+1mg/L NAA	55	46	6-8	83.63
MS+Msorganics+4mg/ LBAP+0.5mg/L NAA	45	28	3-6	62.22
MS+Msorganics+5mg/ LBAP+0.1mg/L NAA.	34	18	3-6	52.94

Table 3. Effects of different concentrations of BAP, IAA and NAA on the leaflet explants of groundnut cv. JL 24. For d₂ leaflets it is as follows:

Media Composition	No. of explants	No. of explants showing response	No. of shoots in each explant.	% of explant responding.
MS+B5organics+1 mg/ LBAP+0.1mg/L IAA.	37	31	4-6	83.78
MS+Msorganics+2mg/ LBAP+1mg/L NAA.	40	38	8-10	95.00
MS+Msorganics+3mg/ LBAP+1mg/L NAA.	39	38	8-10	97.43
MS+Msorganics+4mg/ LBAP+1mg/L NAA	53	49	6-8	92.45
MS+Msorganics+4mg/ LBAP+0.5mg/L NAA.	44	35	4-6	79.54
MS+Msorganics+5mg/ LBAP+0.1mg/L NAA	43	24	4-6	55.81

Table 4. Effects of different concentrations of BAP, IAA and NAA on the leaflet explants of cv. JL 24. For d₃ leaflets it is as follows:

Media Composition	No. of explants cultured	No. of explants responding	No. of shoots per explant	% of explant responding
MS+B5organics+1mg/ LBAP+0.1mg/L IAA	40	18	3-5	45.00
MS+Msorganics+2mg/ LBAP+1mg/L NAA	36	15	3-5	41.66
MS+Msorganics+3mg/ LBAP+1mg/L NAA	36	20	2-6	55.55
MS+Msorganics+4mg/ LBAP+1mg/L NAA	33	18	2-6	54.54
MS+Msorganics+4mg/ LBAP+0.5mg/L NAA	36	20	2-4	55.55
MS+Msorganics+5mg/ LBAP+0.1mg/L NAA	52	22	2-4	42.30

The percentage of regeneration was found to be the highest for 3 mg/L BAP+1 mg/L NAA than for other compositions which is evident from the figs.13, 14, 15, 16, 17, and 18. These results are similar to those given by Livingstone and Birk (1995) in which the embryonic leaflets produced more number of shoot primordia on MS medium containing 3 mg/L BAP+ 1 mg/L NAA than on 5 mg/l BAP+1 mg/L NAA.

As already stated the regeneration frequency is also depended on the age of the explant and it was confirmed from experiments that d₂ leaflets gave better regeneration than d₀,d₁ and d₂ leaflets.This can be represented graphically for the concentration of 3 mg/L BAP+1 mg/L NAA in the fig.19.

The regeneration frequency of the explant with different phytohormone concentrations can be represented graphically for the d₂ leaflets in the fig.20 given which shows that 3 mg/L BAP+1 mg/L NAA gave better regeneration than for other compositions with the increasing concentrations of BAP showing effect on the induction of the explant.



Fig. 13. Response of the d2 leaflet explant of groundnut to MS media with 1mg/L BAP+0.1mg/L IAA.

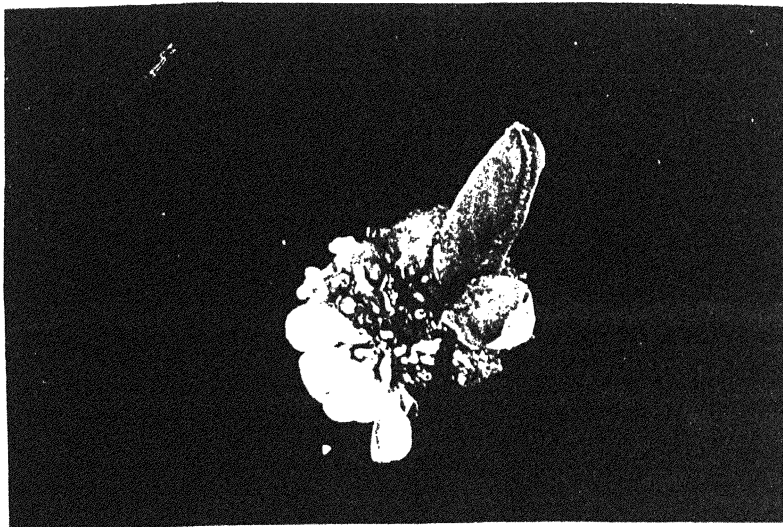


Fig. 14. Response of the d2 leaflet explant of groundnut to MS media with 2mg/L BAP+1mg/L NAA.



Fig. 15. Response of the d2 leaflet explant of groundnut to MS media with 3mg/L BAP+1mg/L NAA.

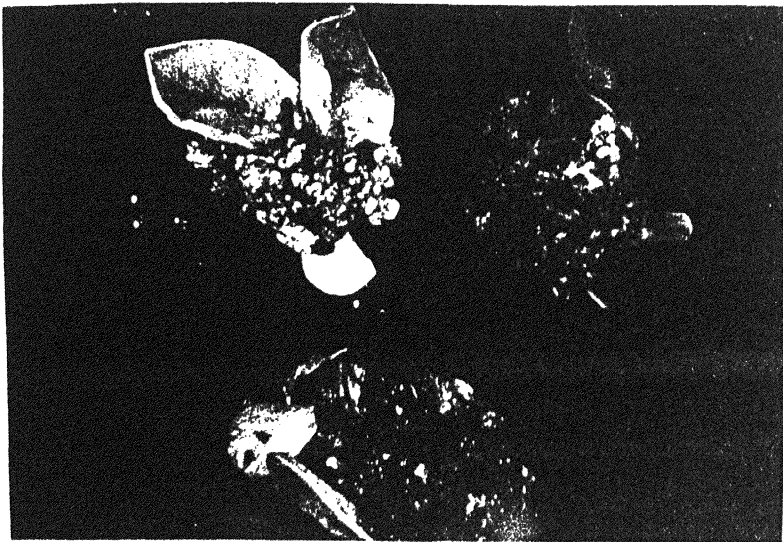


Fig. 16. Response of the d2 leaflet explant of groundnut to MS media with 4mg/L BAP+1mg/L NAA.

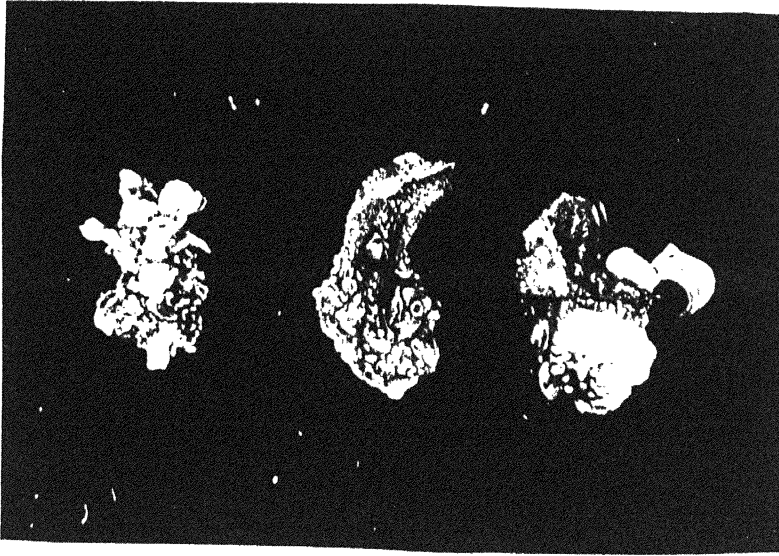


Fig. 17. Response of the d2 leaflet explant of groundnut to MS media with 4mg/L BAP+0.5mg/L NAA.

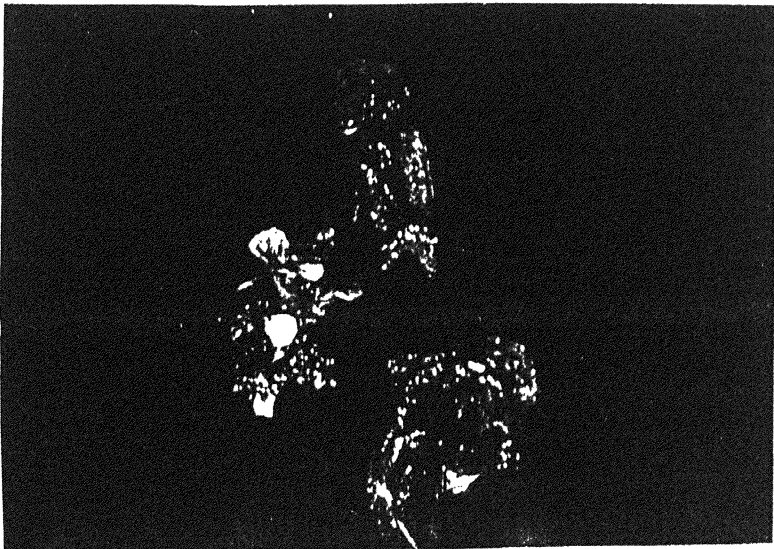


Fig. 18. Response of the d2 leaflet explant of groundnut to MS media with 5mg/L BAP+0.1mg/L NAA.

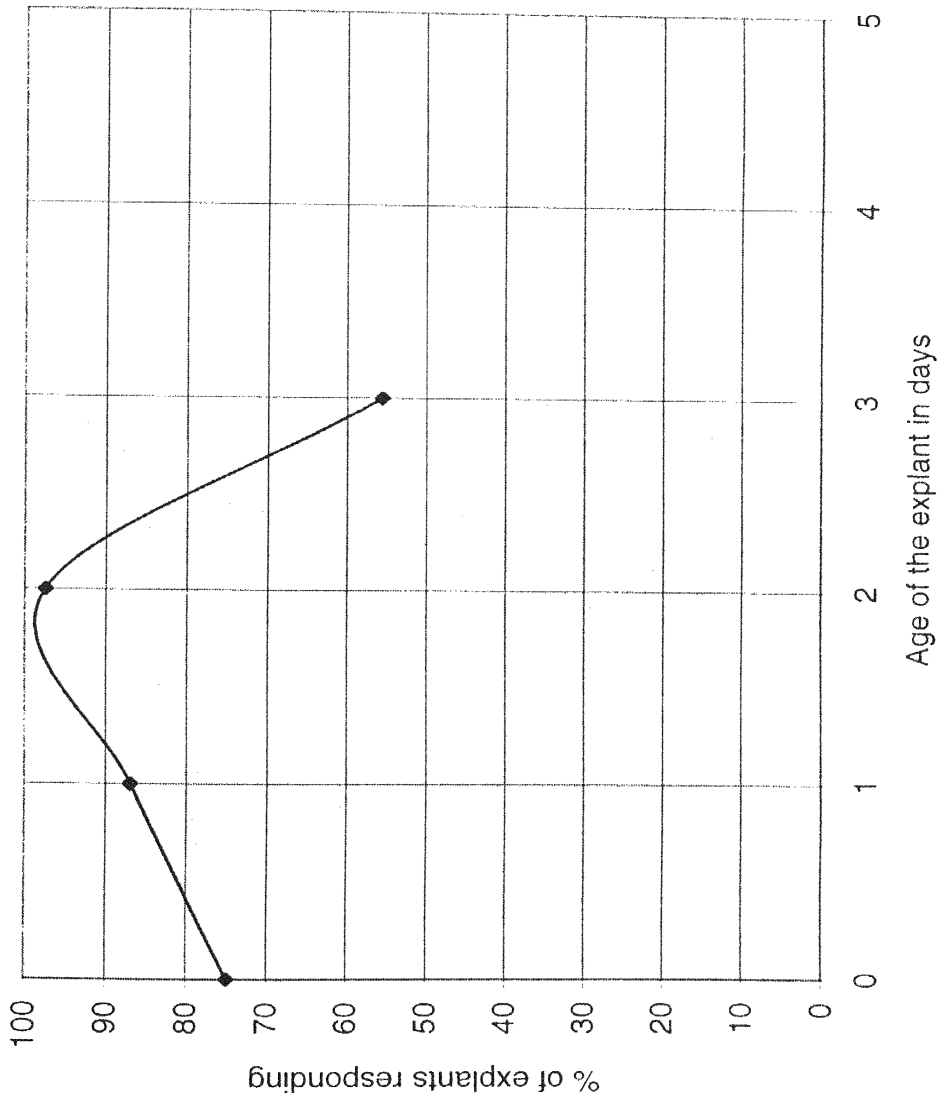


Fig. 19 Regeneration frequency of the different aged explants of groundnut responding to MS media with 3mg/L BAP+1mg/L NAA.

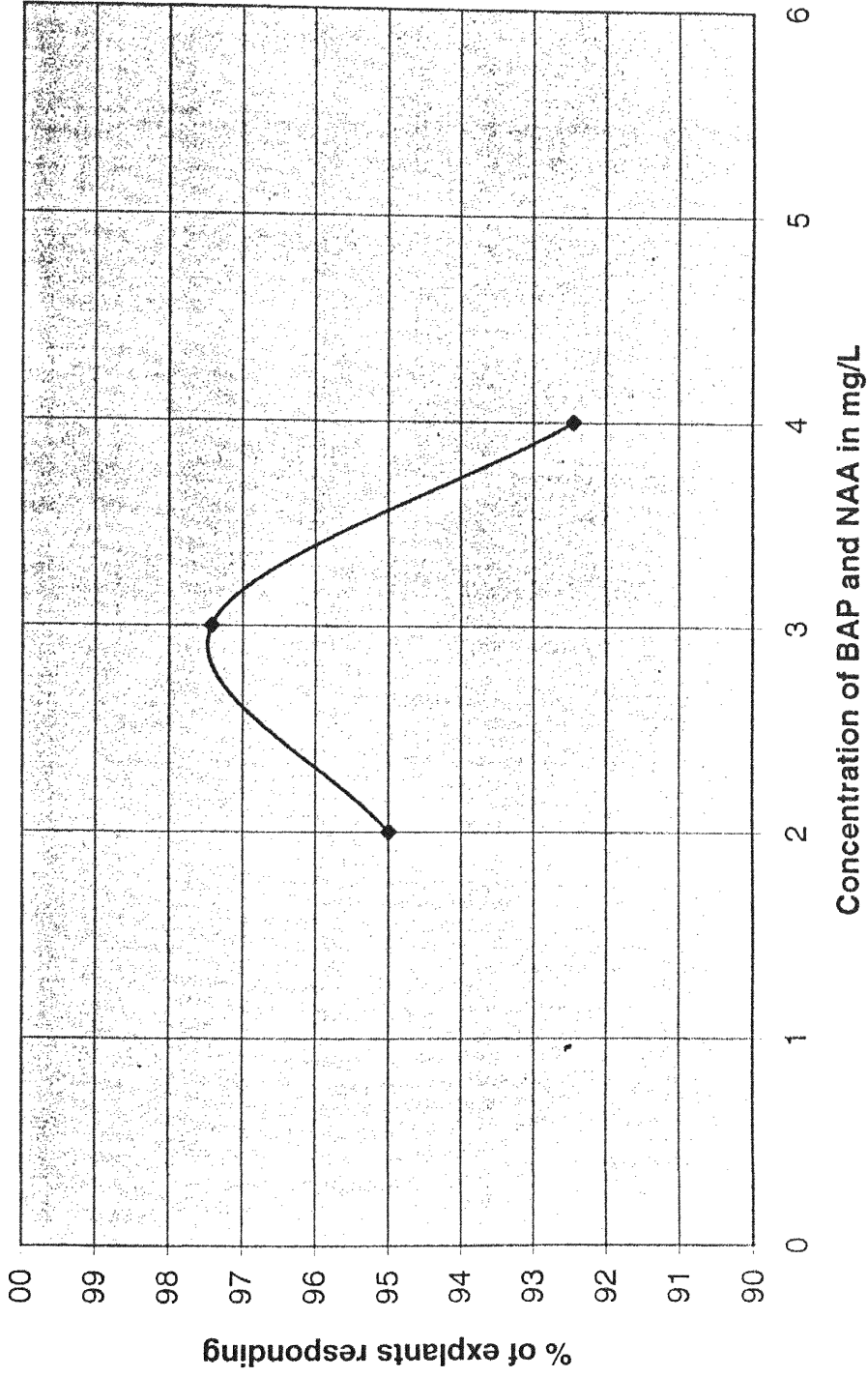


Fig. 20. Regeneration frequency of d2 leaflet explants of groundnut with different concentrations of BAP and NAA.

Isolation and restriction of plasmid pHS 737:SBTI : The plasmid thus isolated and the fragments separated by electrophoresis is shown in the fig.21. The restriction analysis of the plasmid PHS 737:SBTI gave different bands is shown in the fig. 22.

Genetic transformation and gus assay

Transformation of the plasmid pHS737:SBTI was done through *Agrobacterium* and gave positive results by GUS assay in tobacco as shown in the figs.23, 24, and 25. The transformation frequency was found to be around 50% .

GUS assays were conducted for the putative transformants at 24 hrs after the biolistic transformation and gave positive results for both the plasmids i.e.pHS737:SBTI and pRT99GUS, for groundnut leaflets which is shown in the figs.26 and 27 and the transformation frequency was found to be 55%.

The transformants obtained through Biolistics and *Agrobacterium* were found to be responding in the selection media which are shown as follows in figs.28 and 29. Since the gene transfer through biolistics involves direct transfer more number of putative transformants were found than for the *Agrobacterium*-mediated gene transfer. The number of blue spots formed in this assay were scattered, in biolistic method some of them being concentrated at the region of regeneration where as the transformation with *Agrobacterium* gave the blue spots concentrated at the cut end region of the explants. This distribution of the blue spots in the explants has also been observed for the floral stem segments of monocot tulip when transformed by both *Agrobacterium* and biolistics.

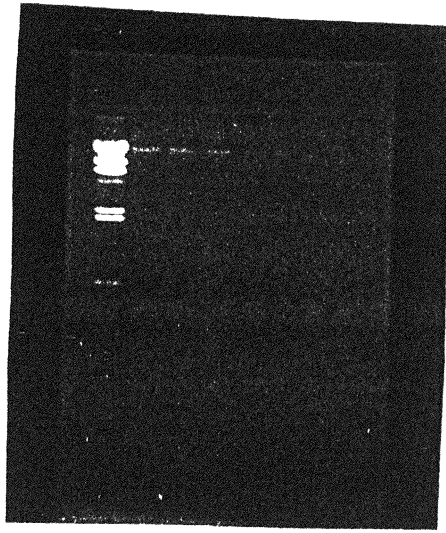


Fig. 21. Agarose gel electrophoresis of the plasmid pHS737:SBTI on 0.6% gel and run at 50V.

- Lane 1----- λ Hind III marker.
- Lane 2-----pHS737: SBTI.
- Lane 3-----pHS737: SBTI.
- Lane 4-----pHS737: SBTI.
- Lane 5-----pHS737: SBTI.
- Lane 6-----pHS737: SBTI.

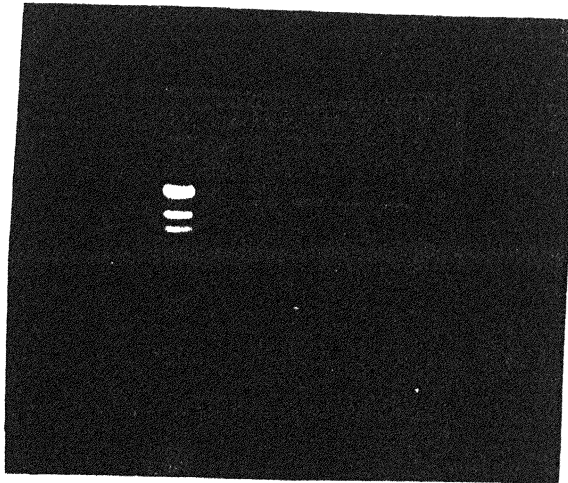


Fig. 22. Restriction analysis of the plasmid pHS737:SBTI separated on 0.8% agarose gel run at 50V.

- Lane 1----- λ Hind III marker.
- Lane 2-----Uncut pHS737:SBTI.
- Lane 3-----Hind III digested pHS737:SBTI.
- Lane 4-----Bam HI digested pHS737:SBTI.
- Lane 5-----Apa I digested pHS737:SBTI.

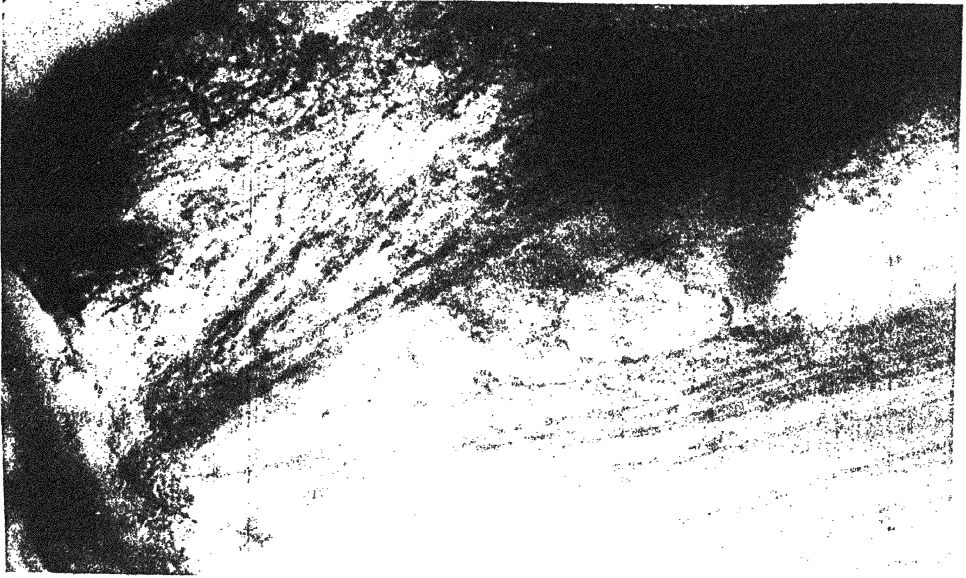


Fig 23. Region of the putatively transformed explants transformed through Agrobacterium with SBTI gene showing GUS expression.

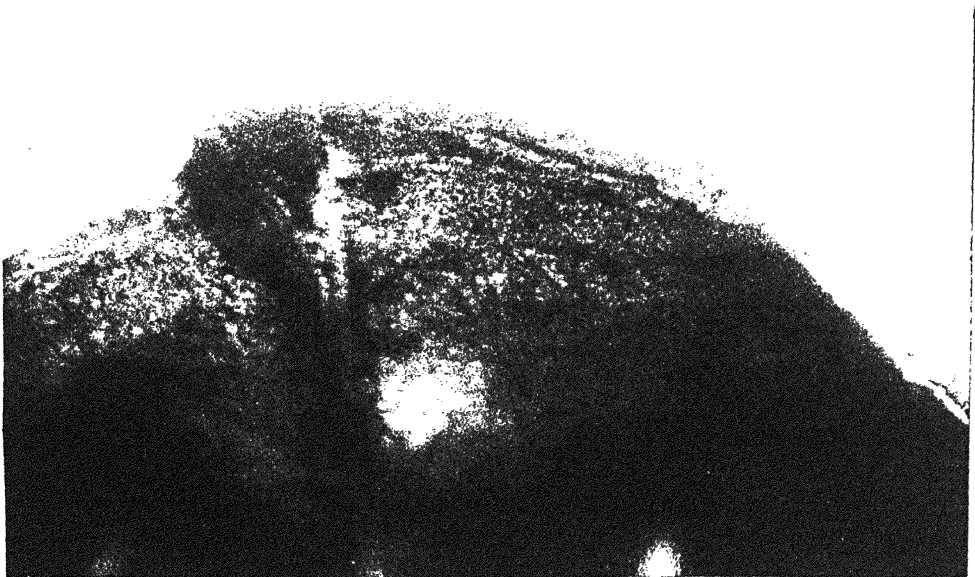


Fig 24. Region of the putatively transformed explants transformed through Agrobacterium with SBTI gene showing GUS expression.



Fig 25. Region of the putatively transformed explants transformed through *Agrobacterium* with SBT1 gene showing GUS expression.

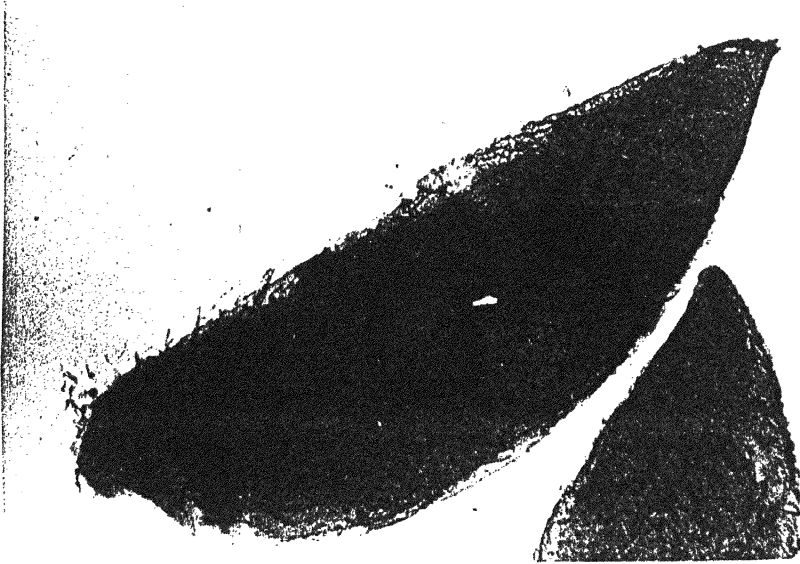


Fig 26. Putatively transformed leaflet explants of groundnut transformed with SBTI gene through Biolistics showing GUS expression.



Fig 27. Putatively transformed leaflet explants of groundnut transformed with SBTI gene through Biolistics showing GUS expression.

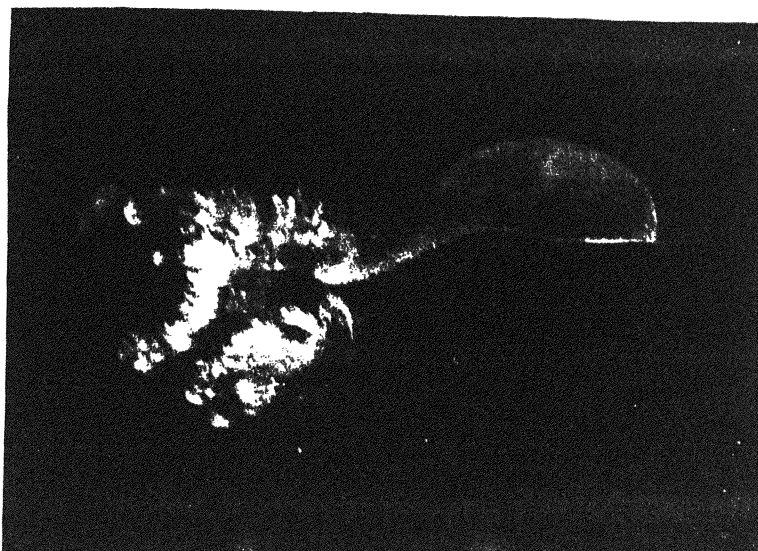


Fig 28. Response of the putatively transformed d2 leaflet explant of groundnut with SBT1 gene through Biolistics on MS media with 3mg/L BAP+1mg/L NAA containing kanamycin.



Fig 29. Response of the putatively transformed d2 leaflet explant of groundnut with SBT1 gene through Agrobacterium on MS media with 3mg/L BAP+1mg/L NAA containing kanamycin.

V. Conclusions

V. CONCLUSIONS

1. By using various concentrations of auxins and cytokinins it was found that 3 mg/L BAP+1mg/L NAA is ideal for regeneration of the leaflet explants of groundnut.
2. Transformation of the leaflet explants of groundnut with soybean trypsin inhibitor (SBTI) gene had been done through *Agrobacterium*.
3. The leaflet explants of groundnut had also been transformed with SBTI gene through Biolistics.
4. The putatively transformed leaflet explants were assayed histochemically for the presence of this gene in the transformants.

VI. References

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VII. Appendices

APPENDIX - A

1. MS MEDIA COMPOSITION

Major nutrients:		mg/L
CaCl ₂	-	440.
NH ₄ NO ₃	-	1650.
KNO ₃	-	950.
MgSO ₄ 7H ₂ O	-	370.
Na ₂ EDTA	-	37.3.
Fe(SO ₄) ₃	-	27.8.

Minor nutrients :		mg/L
KI	-	0.83.
H ₃ BO ₃	-	6.2.
MnSO ₄	-	22.3.
ZnSO ₄ 7H ₂ O	-	8.6.
Na ₂ MoO ₄	-	0.25.
CuSO ₄ 5H ₂ O	-	0.02.
CoCl ₂	-	0.02.

Vitamins

MS Organics:		mg/L
Nicotinic acid	-	0.5.
Pyridoxine Hcl	-	0.5.
Thiamine Hcl	-	0.1.
Glycine	-	2.0.

B5 Organics :		mg/l
Nicotinic acid	-	0.5.

Pyridoxine Hcl	-	0.5.
Thiamine Hcl	-	5.0.
Myo-inositol	-	0.1.
Sucrose	-	30.0.
Agar	-	8.0.
pH	-	5.8.

2. MS – Basal media

MS media without phytohormones.

3. Phytohormones: mg/100ml

1) IAA - 175_r

Dissolve it in 100% ethanol or in 1N NaOH and make up the volume to 100ml with distilled water and store it at 0°C.

2) BAP: - 225_r

Dissolve it in 1N NaOH and make up the volume to 100ml with distilled water.

3) NAA - 186_r

Dissolve it in 100% ethanol and make up the volume to 100ml with distilled water.

APPENDIX – B

1) YEB media:		gm/L
Bacto peptone	-	5.0.
Yeast extract	-	1.0.
Beef extract	-	5.0.
MgSO ₄	-	0.5.
Sucrose	-	5.0.
Agar	-	15.0.
pH	-	7.0.

2) GTE (Glucose/ Tris/ EDTA)

Glucose	-	50 mM .
Tris-cl (pH 8.0)	-	25mM .
EDTA (pH 8.0)	-	10mM .

3) NaOH / SDS (Lysis buffer)

NaOH (freshly prepared)	-	0.2N.
SDS	-	1% (w/v).

4) Potassium acetate (pH 4.8)

5M Potassium acetate	-	60ml.
Glacial acetic acid	-	11.5ml.
Distilled water	-	28.5ml.

The resulting solution is 3M with respect to potassium and 5M with respect to acetate.

5) T.E (pH 8.0)

Tris-cl	-	10mM .
EDTA (pH 8.0).	-	1mM .

6) T A E (Gel running buffer) - 50X stock.

Tris base	-	242gm.
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Glacial acetic acid - 57.1ml.

EDTA (Ph 8.0) - 100ml.

7) RNase free of DNase

Dissolve pancreatic RNase (RNase) at a concentration of 10mg/ml in 10Mm Tris cl (pH 7.5), 15Mm Nacl. Heat to 100°C for 15min. Allow to cool slowly to room temperature.

Dispense into aliquots and store at -20°C.