# DEVELOPMENT OF PHENOTYPICALLY NORMAL TRANSGENIC TOBACCO PLANTS OVER EXPRESSING *ipt* : ASSESSMENT OF THE ROLE OF ENHANCED CYTOKININS ON GROWTH AND PHYSIOLOGICAL PARAMETERS

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## CERTIFICATE

This is to certify that the thesis entitled **"DEVELOPMENT OF PHENOTYPICALLY NORMAL TRANSGENIC TOBACCO PLANTS OVER EXPRESSING *ipt*: ASSESSMENT OF THE ROLE OF ENHANCED CYTOKININS ON GROWTH AND PHYSIOLOGICAL PARAMETERS "** submitted by **PURUSHOTHAMA, M.G.,** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **CROP PHYSIOLOGY** to the University of Agricultural Sciences, Bangalore, is a record research work carried out by him during the period of his study under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

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# *ABREVIATIONS*

| | |
|---|---|
| 'A'; | Assimilation rate |
| ABA: | Abscisic acid |
| bp: | base pair |
| BA : | Benzyl Adenine |
| BAP: | Benzyl Amino Purine |
| Ci: | Intercellular $CO_2$ concentration |
| CPM: | Counts per minute |
| 2,4-D: | 2,4-Dichloro Phenoxy Acetic Acid |
| DMSO: | Dimethyl sulfoxide |
| ELISA: | Enzyme linked immuno sorbent assay |
| GA: | Gibberellic Acid |
| gs: | Stomatal conductance |
| h: | Hours |
| IAA: | Indole Acetic Acid |
| Kb: | Kilo base |
| M: | Molar |
| min: | Minute |
| MCI: | Micro Curie |
| mg: | Milligram |
| mm: | Millimetre |
| NAA: | Napthalene Acetic Acid |
| RuBisCO: | Ribulose 1,5 Bis-Phosphate Carboxylase-Oxygenase |
| SOD: | Super oxide dismutase |
| SDS: | Sodium dodesyl sulphate |

# INTRODUCTION

# I INTRODUCTION

Growth and development is a result of genetic make up of an organism and its interaction with environment. Most of the plant processes are triggered on receiving an appropriate signal, which could be a developmentally regulated or environmentally perceived. These signals initiate complex signal transduction cascade leading to the expression of specific genes and the gene products ultimately bring about the requisite metabolic process and the ultimate growth.

Plant hormones are one of the most important groups of signal molecules, which have a key function in signal transduction and may be acting as initial triggers. Progress in the area of hormones is phenomenal and, in addition to the five major phytohormones, auxins, cytokinins ,gibberelins, ethylene and abscisic acid, a few others like brassinosteroids have been added to the growing list. Growth hormones bring about various formative effects through a cascade of complex molecular events resulting in biochemical and physiological changes. Auxins were the first hormones to be identified during 1926. Since then, the concerted efforts of several groups led to the isolation and characterization of other growth hormones like cytokinins, gibberelins, ethylene and abscisic acid. Since hormones bring about phenomenal changes in growth pattern, the interest has been to use them in regulating growth.

Most of the information on plant hormones has been obtained by taking clues from hormonal studies in animal system. Unlike animal system where each hormone has specific well-defined role, the plant hormones bring about formative effects, often due to interaction of different hormones. Much of this information has been acquired through either external application or the correlative studies, which are not very precise.

Cytokinins which are implicated in formative effects like growth of lateral buds, stomatal movement, delaying senescence and development of sinks, are one of the important groups of growth hormones. Synthetic cytokinins are widely used in tissue culture for morphogenesis and in fruit crops to increase the fruit set and growth. Even though cytokinins have been widely used and studied the metabolism and mode of action are still elusive. The involvement of cytokinins in certain *insitu* formative effects is still to be proven unequivocally.

More recently the molecular approaches substantially contributed in understanding the function and role of these hormones. The role and relevance of any hormone can be thoroughly understood if we have, (a) Isogenic lines differing in quantitative differences in particular hormone, (b) Mutants, for particular hormone or (C) Transgenic plants with enhanced or suppressed expression of genes coding for these hormones. The advantage of the transgenic approach is that expression of a specific gene can be manipulated and its relevance can be studied. Phenomenal progress has been made in recent years in understanding the relevance of several metabolic processes by transgenic approach (Stitt and Sonnewald., 1995). By altering the hormone levels agronomically superior genotypes have been developed by this approach. The major success of transgenic approach has been the postponement of fruit ripening in tomato, where the enzyme involved in ethylene biosynthesis was down regulated (Mattoo and White., 1991).

The transgenic approach to regulate any process will be possible only when the rate limiting steps are well characterized and are few. The proposed model for cytokinin biosynthesis involves conversion of isopentenyl pyrophosphate to isopentenyl adenosine, which is mediated by the rate-limiting enzyme ISOPENTENYL TRANSFERASE. Much of the information about isopentenyl transferase has been obtained from the studies on *Agrobacterium tumefaciens* induced tumor tissues (Chilton *et al*., 1977). Very limited studies have been done on plant *ipt* and the studies are not conclusive. *Agrobacterium ipt* is the only source available for genetic manipulation of plants with varied cytokinin levels. In a few studies transgenic *ipt* plants have been developed either expressing *ipt* on constitutive or inducible promoter. Constitutive expression with CaMV 35S promoter (Akiyoshi *et al*., 1989) resulted in severely deformed phenotypes and the induction treatments often bring about various pleotrophic changes in the plants (Medford *et al.*, 1989). The response of the crown gall tissues induced by *Agrobacterium* suggests that *ipt* on native promoter is quite relevant for optimum expression leading to substantial increase in endogenous cytokinin levels.

Yet another aspect still not understood is the level of expression that is likely to occur when transgenic plants are developed. The level of expression of the transgene may vary depending on several factors like copy number, site of integration, level of methylation etc. (Linn *et al.*, 1990). This aspect can be exploited to develop transgenic plants with varying levels of expression of the gene of interest (*ipt)* and the role and relevance of any gene product could be studied.

In the light of above information it was proposed to develop transgenic plants expressing *ipt* on native promoter, as it is known to give stable trasnformants. The following are the objectives of the present investigation.

1. To clone *ipt* with native promoter in to a suitable binary vector.
2. To develop phenotypically normal and distinct *ipt* transformants in FCV special cultivar of tobacco.
3. Characterisation of *ipt* transformants based on, a: endogenous cytokinin levels and b: phenotypic appearance
4. To study the relevance of cytokinins in a few *ipt* transformants differing in cytokinin levels.

# REVIEW OF LITERATURE

# II REVIEW OF LITERATURE

The overall plant growth and development is the sum effect of interaction between the environmental factors and a range of phytohormones like abscisic acid, auxins, cytokinins, ethylene and gibberelins, which are endogenously produced. Though each hormone has a unique mode of action, the formative effects are often due to interaction between the hormones. Cytokinins, which were shown to be involved in stimulation of cell division or cytokinesis, also bring about a number of other formative effects like, release of lateral buds from apical dominance (Sachs and Thimann, 1964), alteration of source and sink relationships (Leopald and Kawase, 1964), counteraction of effects of heat stress (Caers *et al.*,1985), stimulation of chlorophyll synthesis (Arnold and Fletcher, 1986) and delaying senescence (Richmond and Lang, 1957).

Except for substituted diphenyl ureas, all the naturally occurring cytokinins are adenine derivatives with an isopentenyl or hydroxyisopentenyl side chain at the N6 position (Skoog and Armstrong, 1970; Leonard *et al* ., 1968).

## Biosynthesis of Cytokinins

The biosynthetic pathway of cytokinins in plant system has not been clearly demonstrated. In *Agrobacterium tumefaciens* induced tumor tissues the cytokinin biosynthetic pathway has been conclusively proven. The proposed model for cytokinin biosynthetic pathway (Binns, 1994) in plants involves the addition of dimethyl allyl pyrophosphate (DMAPP) to the N6 position of Adenosine Mono Phosphate (AMP) by an isopentenyl transferase (IPT), yielding the cytokinin ribotide (Isopentenyl adenosine monophosphate). This is modified in a series of reactions to yield other types of cytokinin ribotides, ribosides and free bases. Although IPT activity is detected in many plant species attempts to purify the enzyme to homogeneity has not been successful. Besides this proposed model a denovo pathway exists which involves synthesis of less active cis Zeatin, found in certain transfer RNAs as the base next to the 3' end of the anticodon (Marechal-Drouard *et al.*, 1993). In *Agrobacterium* tumefaciens the pathway is similar to the one proposed for the plant tissues, and so far IPT has been clearly demonstrated only in this system. The enzymes involved in cytokinin biosynthesis and metabolism, have not been purified or characterized in the plant system. The only progress that has been made in this direction is the partial purification of IPT in cultured tobacco cells (Chen and Melitz, 1979). There seems to be very less homology between the native plant IPT and the IPT produced as a result of introduction of *Agrobacterium ipt*. Also in some of the studies where transgenic *ipt* plants were developed, the control non-transformed plants did not show hybridisation signal with the radiolabelled *ipt* probe prepared from *Agrobacterium* (Ainley *et al*.,1993; Beinsberger *et al* .,1991; Li *et al* .,1992; Martineu *et al*., 1994; Smigocki ,1991).

## Sites of synthesis

Cytokinin biosynthesis in developed plants takes place mainly in roots (Hanson and Wareing, 1976; Sossountzov *et al* ., 1988; Van Staden and Smith, 1978). Though smaller amounts are synthesized in shoot apex and other plant tissues, their contribution to the total cytokinin pool is negligible.

## Metabolism

Hormone mediated cellular response needs to be regulated when once the process is completed and the controlling mechanism needs to be highly precise. The active cytokinins are inactivated by degradation or conjugation to different metabolites like, sugars and aminoacids.

At present the only identified enzyme in plant that catalyzes the degradation of cytokinins to inactive products that lacks N6 side chain is cytokinin oxidase. This enzyme which converts isopentenyl adenosine to adenine, was first demonstrated in cell free system (Paces *et al* ., 1971). Since then Cytokinine oxidase activity has been reported in several systems like maize (Whitty and Hall, 1974 : Burch and Horgan, 1989), phaseolus (Chatfield and Armstrong, 1986) and in wheat (Laoue and Fox, 1989). N6 side chain containing cytokinins (Isopentenyl adenosines) are the preferred substrates for cytokinin oxidase. Among the predominant cytokinins, Zeatin and Zeatin ribosides are degraded by cytokinin oxidase *in vitro* (Chatfield and Armstrong, 1986), because of the presence of the double bond. O-glucosylation of the side chain protects cytokinins from cytokinin oxidase (McGaw and Horgan, 1983). On the other hand N-glucosylation or other ring substitutions may decrease the affinity of cytokinin oxidase for a given cytokinin but does not completely eliminate the activity (Lee *et al* ., 1985). From the data available from several studies it is clear that the cytokinin degradation varies in different tissues and in the same tissue during its development (Singh *et al* ., 1992). This type of spatial and temporal variation in degradation of cytokinins may have a significant role in controlling cytokinin levels during plant development.

Conjugation of cytokinin to various sugars and aminoacids is a common phenomenon. Cytokinins occur frequently as N-glucosides that are biologically inactive and active O-glucosides. They can be N-glucosylated on the purine ring and O-glucosylated on the N6 substituted side chain. The biological activity associated with O-glucosides is associated with release of free cytokinins, there fore, cytokinin O-glucosides are considered as intrinsically inactive forms of cytokinins which can be easily converted to free active cytokinins by the action of β glucosidases. Cytokinin O-glucosides are considered to be important in germination of seeds, bud development (Palmer *et al* ., 1981), breaking of bud dormancy and apical bud growth (Van Staden and Damilla, 1978).

## Mechanism of action of cytokinins

Though there have been many studies to understand the physiological and biochemical roles of cytokinins, the information on the mode of action of this hormone is very limited. Some of the studies indicate that the cytokinin action is related to gene expression in different systems (Lu *et al* ., 1990; 1992; Dominov *et al* ., 1992; Chen *et al* ., 1987). Cytokinin has been implicated in regulating the gene expression of Nitrate Reductase (Lu *et al.*,1990), NADP dependent glyceraldehyde phosphate dehydrogenase (Feirebend, 1970), RuBisco (Partheir *et al* ., 1980), hydroxypyruvate reductase (Chen and Leisner., 1985), RNA polymerase I (Ananiev *et al* ., 1987) and light harvesting chlorophyll a/b binding proteins (Flores and Tobin, 1986).

Much of the information on cytokinins has been generated with either *in vitro* studies by the addition of cytokinins to the medium or by studying the effect of externally applied cytokinins on a particular phenomenon. Most of these correlative studies suffer from lack of precision in entry and uptake of the externally added cytokinins. The *in vivo* effects of cytokinins could be studied either by enhancing the endogenous levels or down regulating the levels of this hormone by gene transfer technology. By employing genetic engineering methods significant insight has been developed in several aspects of plant metabolism.

### External application studies to understand the mechanism of action.

Kirichenko *et al.*(1995) studied the response of photosynthesis in field grown wheat and triticale in response to temperature and exogenous cytokinins. Ribulose-bisphosphate carboxylase (Rubisco) and phosphoenolpyruvate carboxylase (PEPC) activities in caryopses were higher and more heat resistant than activities in the flag leaf of triticale cv. AD 49. During short heat stress PEPC was more heat stable than RuBisco, while during longer periods of heat stress PEPC activity declined to lower levels than Rubisco activity. BAP [benzyladenine] and TDZ [thidiazuron] increased Rubisco activity in both the generative organs and the flag leaves of wheat but did not affect PEPC activity.

Catsky *et al.* (1996) studied the effects of external application of N6-(m-hydroxybenzyl) adenosine (HBA), one of the synthetic cytokinins, to the field-grown sugarbeet. Application of HBA maintained the high cytokinin content that normally decreases before harvesting. The total content of isoprenoid cytokinins at harvesting was 2.6-fold higher in HBA-treated plants than in the controls. Treatment had no significant effect on contents of chlorophylls a and b and carotenoids, nor on the rates of net photosynthesis or photorespiration, or on $CO_2$ compensation concentration measured throughout growth on detached leaves under optimum environmental conditions. Increased values in net photosynthesis, photorespiration and Chl a and b contents were found which are linked with a delay in leaf senescence before harvesting. Transpiration rate and stomatal conductances of adaxial and abaxial leaf surfaces were not significantly affected.

Palta and Ludwig. (1997) studied the effect of exogenously applied cytokinins on fruit set. Application of BAP to flowers had no effect on plant-water relations. The water deficit reduced the number of pods that reached maturity when no BAP was applied and increased seed yield in pods that filled seeds. Application of BAP increased the number of pods that reached maturity. However, the additional pods that reached maturity produced unfilled seeds. Seed yield and harvest index were reduced in the BAP treatments (both stressed and unstressed), mainly as a result of a reduction in seed number. An increase in seed abortion during seed filling probably caused the reduction in seed number. They concluded that the reduction in seed number and pod filling resulted from a shortage of assimilates to fill all the mature pods produced.

Badenoch *et al* . (1996) studied the effect of externally applied cytokinins on retardation of senescence and transpiration in oat and wheat. Concentrations of some naturally occurring cytokinins (zeatin, dihydrozeatin and cis-zeatin, their ribosides, the O- glucosides and nucleotides) were determined in the xylem exudates of oat and wheat seedlings by radioimmunoassay.. No significant differences were observed in the concentrations of individual cytokinins between the xylem saps of 6-d-old oat and wheat plants. Using the data collected on individual cytokinin levels, individual ribosides (and a base/riboside mixture), O-glucosides (and mixtures of the O-glucosides) and nucleotides were tested in transpiration and senescence bioassays in multiples of those concentrations determined in the xylem sap (to compensate for the differences in transpiration rate between oat and wheat seedlings). Oat seedlings demonstrated a significant increase in transpiration rate when a base/riboside mixture was applied at twice the endogenous concentration. In contrast, wheat exhibited the same increase in transpiration at 5 times the endogenous concentration of the mixture. Derooted oat seedlings were more sensitive to 6-benzylaminopurine than those of wheat, although no significant increase in transpiration was observed in response to increasing O-glucoside application. Zeatin, the cytokinin with the highest endogenous concentration, elicited the largest increase in transpiration. Nucleotides were present in the xylem sap at lower concentrations than most of the bases, ribosides and O-glucosides. They concluded that in oats, and possibly in wheat, naturally occurring cytokinins supplied to the leaf in xylem sap promoted transpiration when applied at concentrations which gave a cytokinin flux approaching that of the intact plant. O-glucoside cytokinins also retarded leaf senescence when supplied via the transpiration stream. Senescence retardation was less obvious in wheat seedlings, and it was less clear whether xylem cytokinins play a role in regulating leaf senescence *in vivo*.

## Manipulation of endogenous levels of cytokinins by transgenic appropach to study the role and relevence of cytokinins.

### Genetic transformation studies.

The progress in gene transfer technology has paved way for manipulation of the hitherto stable nucleus of prokaryotic bacterium to eukaryotic higher plants. The progress in plant tissue and organ culture has led to development of transgenic plants with desirable traits, altered metabolism, and altered qualitative and quantitative traits.

Though there are several methods like electroporation, liposome mediated transformation, microinjection and particle gun technology, the *Agrobacterium* mediated transformation is the widely used system. Using this system commercial transgenic products have been developed and a few products are already available for the end users.

*Agrobacterium* based transformation is the most widely used system for introducing genes into dicotyledons (such as tobacco, potato and *Arabidopsis*), whereas particle bombardment is the most frequently used for monocotyledons (such as maize, wheat and rice). All the techniques have both advantages and disadvantages.

The first transgenic plants were produced through *Agrobacterium* mediated transformation of *Nicotiana tabacum* leaf explants (Horsch *et al* ., 1985;DeBlock *et al* ., 1984). Since then many plant species have been genetically engineered by various techniques.

### *Agrobacterium* vectors for plant transformation

*Agrobacterium tumefaciens* is the causal agent for crown gall disease and produces tumorous crown galls on the infected species. Several studies related to the crown gall disease revealed that the crown galls were actually produced as a result of the transfer and integration of genes from the bacterium into the genome of the plant cells (Chilton *et al* ., 1977).

The virulent strains of *Agrobacterium* harbor a large (150-200 kb) Ti plasmids which are responsible for the DNA transfer and integration. Genetic and molecular analysis showed that Ti plasmids contain two sets of necessary sequences for gene transfer to plants. Ti plasmids contain one or more T-DNA (transferred DNA) regions that are transferred to the plant and the Vir (Virulence) genes that are not themselves transferred during infection. In addition, *Agrobacterium* has chromosomal virulence region in its nucleus for the successful activation of Vir genes. The T-DNA regions are flanked by border sequences called the right and left borders between which lies the T-DNA. The T-DNA contains eight to thirteen genes (Nester *et al* ., 1984) which includes a set of genes for the production of phytohormones, which are responsible for formation of the tumors, genes which codes for opines (sugar amino acid condensation products) and a gene which modulates the expression of the phytohormone coding genes.

The initial studies by several groups demonstrated that any foreign DNA inserted into the T-DNA could be transferred to plants along with the existing T-DNA genes. After this discovery, efficient plant transformation vectors have been constructed by removing the phytohormone biosynthetic genes from T-DNA region (Fraley *et al* ., 1985). The vectors thus constructed which required the homologous recombination between the T-DNA genes and the foreign gene were called the co-integrative vectors which were subsequently replaced by the binary vectors which are in wide usage. The binary vectors are capable of replicating in *Escherichia coli* and as well as in *Agrobacterium* and thus the name Binary vectors. There has been lot of modification of the binary vectors such as arrangement of genes of interest near to right border for better transfer and integration, introduction of multiple cloning sites for the introduction of the desired DNA in the right orientation, improved promoters, for higher and tissue specific expressions, introduction of newer reporter genes for easy confirmation and newer selectable marker for broad range selection of several plant species. Now, a number of binary vectors are commercially available and researchers have varieties of choices in choosing the right binary vectors for transformation studies.

The initial transformation studies were aimed at developing transgenic plants with traits related control of insects, weeds, and plant diseases. Transgenic approach has also been extensively employed to understand different metabolic processes either by over expressing or reducing the expression of target gene. There are studies in which the transgenic plants have been developed which produce ingredients of biodegradable plastics and production of edible vaccines.

Genetic engineering has been successfully used to develop transgenic plants tolerant to different weedicides like glyphosate by overexpressing ESPS (5-enolpyruvylshikimate - 3 phosphate synthase, target enzyme for glyphosate, (Shah *et al* ., 1986) or glyphosate tolerant ESPS enzyme (Comai *et al* ., 1985).

## ISOPENTENYL TRANSFERASE over expression studies

Agrobacterium Gene 4(T-cyt, ipt, roi) which codes for **Isopentenyl Transferase** that catalyses the condensation of isopentenyl pyrophosphate and adenosine monophosphate giving isopentenyl adenosine monophosphate (iPMP)(McGaw., 1987) was cloned by Barry *et al* .(1984). Since then, *ipt* has been used extensively by number of researchers to express *ipt* both constitutively and under the control of different inducible, tissue specific promoters to study the effect of enhanced *ipt* expression on phenotype and physiological responses of different plant systems.

### Studies with constitutive promoters.

Smigocki and Owens (1988) placed *ipt* under the control of promoters of varying activities (the cauliflower mosaic virus 35,S promoter and nopaline synthase promoter) and generated transgenic plants in Tobacco and cucumber systems. They reported that there was shoot production in hormone free medium in all the explants although the number varied with the gene construct. Further, there was considerable variation in cytokinin content. In tobacco system cytokinin level varied from 36 pM/g fresh wt in control to 2416- 4009 pM/g in transformed lines. In cucumber it varied from 31 pM/g to 5340 pM/g. They also reported that the putative transformed shoots failed to root on medium containing varying levels of 1-napthaleneacetic acid and it was attributed to elevated cytokinin levels.

### Studies with native promoter.

Makarova *et al*. (1997) studied the capacities for regeneration, callus formation and organogenesis in tobacco, both wild-type plants and those transformed with the active isopentenyl transferase (ipt) gene. Transgenic plants carrying the *ipt* formed calluses only on medium supplemented with 2,4-D and kinetin. However, further callus growth did not depend on the presence of phytohormones. The regenerated transgenic plantlets had short stems with numerous leaves and the roots were initiated 3-5 days earlier than in wild-type regenerated plantlets. They concluded that the morphological traits of the transformed regenerants were probably conditioned by the hormonal levels.

Galis *et al.*(1995) studied the effect of both externally applied cytokinins and the endogenously produced cytokinins on tuberisation process in potato. The exogenous addition of 10 mg/L N6 benzyladenine into the tuberisation medium resulted in advanced tuber formation in intact plants and microtubers appeared 10-20 days earlier than the experiments in which no cytokinin was supplied. The ipt transformed potato plants produced endogenously elevated cytokinin levels (3-20 times). The onset of tuberization in intact ipt-transformed plants with low transgene expression was advanced in comparison with control material and exogenously applied BA further promoted the tuberization process. The tuberization was strongly inhibited in *ipt*-transformed nodes, and an external increase of the cytokinin levels caused complete inhibition of explant growth.

Though there were several studies related to ipt *there* are very few studies in which the detailed analysis of the native *ipt* promoter was carried out. Ma *et al.*(1996) studied the location of GUS [uidA] gene expression under control of the T-cyt gene promoter in transgenic tobacco and potato plants. Results showed that T-cyt was expressed in roots, stems, leaves and buds. Highest levels of GUS activity were found in tobacco stems during axillary bud initiation and in tuber buds. Levels of expression were also high in wounded leaves of transgenic potato. GUS expression was induced in transgenic tobacco stems by cytokinin treatment but not by auxin treatment, indicating that the T-cyt promoter might be selectively induced by exogenous plant hormones.

### Studies with heat inducible promoters.

Medford *et al.* (1989) genetically engineered ipt gene by placing it under the control of a heat-inducible promoter(maize hsp70). They developed hsp70-ipt plants in tobacco and Arabidopsis systems. Heat induction of transgenic plants increased the level of zeatin 52-fold (1450ng/g), Zeatin riboside 23-fold (2230ng/g), and zeatin riboside 5'-monophosphate two fold (1600ng/g) over un- induced transgenics. At control temperature 3-7 fold increase was seen over wild type plants. This uninduced cytokinins increase in transformants affected various aspects of development, which included characters like release of axillary buds, reduced stem and leaf area, and an underdeveloped root system.

Veselov *et al.* (1995) studied the effect of enhanced endogenous cytokinin levels under heat stress condition on the metabolism of the hormone. The pattern of the endogenous cytokinin content was monitored during the day in the shoots of transgenic tobacco plants containing a heat-inducible ipt gene. Heating transgenic plants at $40^0$C for 1 h yielded an increase in endogenous cytokinins, as compared to the normal level in the plants kept at $24^0$C for the whole period. However, the increase was not permanent, as after 5 h following heat-shock treatment, there was essentially no difference in cytokinin content between heated and untreated plants. In the shoots of wild-type tobacco, heat shock activated the processes diminishing cytokinin concentration, which is the typical plant response to heat shock.

### Studies with RubiSco small sub unit promoter.

Synkova *et al* . (1995) made an extensive analysis of the tobacco cv. Petit Havana SR1 transformed plants overexpressing ipt under the control of Pssu promoter. Compared to wild type plants, transgenic plants exhibited: (1) an approximately 6-fold increase in endogenous cytokinin content but the same or lower abscisic acid content; (2) severely affected electron transport around photosystem (PS) I but not PSII; (3) low stomatal conductance and water potential mainly in mature and older leaves, probably the result of a poor root system; (4) lower photosynthesis and higher photorespiration due possibly to closed stomata and permanent water and $CO_2$ deficits; and (5) no marked disturbances in PSII functioning, indicating the presence of very efficient water stress defense mechanisms.

Van Loven *et al.*(1995) coupled *ipt* to the promoter sequence from *Pssu*, the gene encoding the ribulose-bisphosphate carboxylase small subunit in *Pisum sativum*, and transformed into tobacco. The transgenic plants were used to study the effect of endogenous changes in cytokinin content on photosynthetic activity. As roots could not be induced on transgenic shoots derived from callus, grafting was introduced. Primary roots did develop in transgenic progenies, although seedling development was arrested at the cotyledon stage. Non-photochemical quenching was reduced in all transgenic plants, but particularly so in grafts. Starch content of transgenic plants exceeded that of the wild type by a factor of 3.5 on average. Cytokinin content was increased in transgenic plants by 10-fold in young plants and up to 100-fold in old grafts. It was concluded that the increased cytokinin content of Pssu-ipt plants affected photophosphorylation activity so that starch accumulation in the chloroplasts was promoted and, hence, sugar translocation was reduced, thus altering the sink-source relationship within the plant. Differences in photosynthetic parameters between young leaves and older tissues supported the idea that an optimal cytokinin concentration exists for stimulation of photosynthetic activity.

There are indications that the cytokinin content in transgenic tissues expressing the ipt gene is under metabolic control, which prevents the accumulation of cytokinins to lethal levels. Redig *et al.* (1997) investigated the relationships between the content of endogenous cytokinins and the activity of cytokinin oxidase in ipt transgenic tobacco callus. In addition, the effect of exogenously applied N6-benzyladenine on this relationship was examined. Endogenous cytokinin concentrations were measured in callus of *Nicotiana tabacum* cv. Petit Havana SR1 transformed with the *ipt* of *Agrobacterium tumefaciens* under the control of a light- inducible promoter and in non-transformed tissue using LC-tandem mass spectrometry. The transformed callus had a 25-fold higher content of cytokinins than the non-transformed tissue. Mainly Zeatin- and dihydrozeatin-types of cytokinins (free bases, ribosides, nucleotides and O-glucosides) accumulated in the *ipt* transgenic tissue. The cytokinin pool of both *ipt*-transformed and non-transformed tissues consisted predominantly of cytokinins that are either resistant to cytokinin oxidase attack (nucleotides and O-glucosides of cytokinins and cytokinins bearing an N6-saturated side chain) or have a low affinity for the enzyme (Zeatin and its riboside). The former represented 71.6 and 74.8% and the latter 27.7 and 24.4% of the pool of endogenous cytokinins in *ipt*-transformed and non-transformed tissues, respectively. Enzyme preparations from *ipt*-transformed tissue exhibited 1.5-fold higher cytokinin oxidase activity compared with that observed in control tissues. Application of exogenous BA affected the total levels of cytokinins of the two tissue lines in different ways. The cytokinin content increased 1.7- and 1.5-fold in ipt-transformed tissues 6 and 12 h after BA application, respectively, while it declined in the non- transformed control to an extent 1.6- to 2.0-fold between 3 and 12 h after BA application. The increase in cytokinin content in the ipt callus was due to an increase of Zeatin- and dihydrozeatin-type cytokinins (nucleotides, ribosides and free bases) leading to an enhanced accumulation of O-glucosides after 12 h. Following BA treatment, the cytokinin oxidase activity increased up to 1.8- fold in ipt-transformed and 1.6-fold in non-transformed tissues.

Geuns *et al.*(1997) determined the free sterol and polyamine contents in the apex and leaves of *Nicotiana tabacum* cv. Petit Havana SR1 transformed with Pssu-ipt. Older leaves of ipt-transformed plants contained much higher putrescine content than those of control plants, whereas no significant differences in spermidine or spermine content were found. Transformed plants had much higher cytokinin content than control plants, and putrescine content was correlated with endogenous cytokinin (free-bases) content and with ornithine- and ornithine-decarboxylase activities. Plants transformed with ipt were characterized by a higher leaf sterol content and by a delay in the increase in the stigmasterol/sitosterol ratio that occurred from the upper to lower leaves.

## Tissue specific expression studies.

Roeckel *et al.*(1997) placed the *ipt* under the control of 1.9 kb of promoter sequence from the 2S albumin AT2S1 gene isolated from an Arabidopsis thaliana library. The construct was introduced into canola [rape] (Brassica napus) and tobacco (Nicotiana tabacum) and ipt transcripts were followed during embryo development of transgenic plants by northern hybridization. The phenotype of transformed plants from the T1 generation was analysed, and increased branching of inflorescence in tobacco and canola plants expressing ipt was observed. Compared with controls, the average number of capsules and siliques in AT2S1-ipt plants was 82.6 and 24.8% higher, respectively. This result was correlated with an increase in cytokinin levels in transgenic plants, as revealed by RIA [radioimmunoassay]. Indeed, cytokinin contents of T1 AT2S1-ipt B. napus seeds were 2.2-fold higher than cytokinin contents of control seeds, and T1 AT2S1-ipt N. tabacum capsules contained 2.6-fold more cytokinins than control capsules. In tobacco, average seed weight/capsule was lower in AT2S1-ipt plants, while the seed number/siliqua and average seed weight were not modified in canola carrying this construct. Average seed yield/plant was not significantly increased in AT2S1-ipt tobacco or canola plants.

### Developmental stage specific expression of isopentenyl transferase.

Gan and Amasino (1995) fused the *ipt* to senescence inducible autoregulatory promoter. Controlling the expression of *ipt* with a senescence-specific promoter from Arabidopsis thaliana (SAG12 promoter) resulted in the suppression of leaf senescence. Transgenic tobacco plants expressing this chimaeric gene did not exhibit the developmental abnormalities usually associated with IPT expression because the system was autoregulatory. Because sufficient cytokinin was produced to retard senescence, the activity of the senescence- specific promoter was attenuated. Senescence-retarded leaves exhibited a prolonged, photosynthetically active life-span. This result demonstrates that endogenously produced cytokinin can regulate senescence and provide a system to specifically manipulate the senescence program.

### Studies with non-specific expression of *ipt.*

Eklof *et al.* (1996) used a novel approach to study the levels enhanced endogenous cytokinins in transgenic plants with normal appearance. A plant expression vector carrying a promoterless isopentenyl transferase (ipt) gene was constructed and was used to transform *Nicotiana tabacum* cv. Wisconsin 38. Several primary transformants were obtained that displayed a range of phenotypes characteristic of cytokinin overproduction. Two of the transformants with moderately altered phenotypes, both of which produced viable offspring and expressed the ipt gene at a low level, were selected for use in studies of the regulation of cytokinin metabolism. Both lines were found to contain high concentrations of *zeatin-7-glucoside (Z7G), indicating that Z7G can accumulate in plants even when the* rate of endogenous overproduction of cytokinins is low. This supports the hypothesis that 7-glucosidation is an important step in the regulation of zeatin levels. Very sharp gradients in concentration of cytokinin riboside and ribotides, related to age of tissue and distance from the apex, were found in both wild-type and transformed plants, which could be important in developmental regulation and could also account for some of the discrepancies between reported cytokinin levels in various plants.

### Effect of other hormones on *ipt* expression.

Eklof *et al.* (1997) investigated the interactions between auxins and cytokinins in tobacco by manipulation of levels of the growth regulator followed by analysis of the induced changes in the metabolism of the other growth regulator. Cytokinin-overproducing plants had lower than wild-type levels of free IAA and reduced rates of IAA synthesis and turnover, but there were no differences in the profiles of metabolites they produced from fed IAA. Similarly, auxin- overproducing plants (expressing the *A. tumefaciens* iaaM and iaaH genes), had lower levels of the major cytokinins than wild-type plants and lower cytokinin oxidase activity, but there were no differences in the profiles of metabolites they produced from fed cytokinins. The data demonstrate that cytokinin or auxin overproduction decreases the content of the other growth regulator, apparently by decreasing its rate of synthesis and/or transport, rather than by increasing rates of turnover or conjugation.

### Effect of enhanced cytokinins on other hormones.

Makarova *et al.*(1996) studied the levels of different types of cytokinins and different phytohormones in transgenic plants carrying ipt under different promoters. Total cytokinin (zeatin, zeatin riboside, isopentenyladenine and isopentenyladenosine) level and free IAA content were always higher in shoots regenerated from transgenic cultures although the concentrations were lower in roots. In transgenic plants, IAA-oxidase activity was lower and the concentration of its protectant chlorogenic acid was increased. Transgenic plants also contained lower concentrations of abscisic acid.

Strabala *et al.* (1996) studied the effects of the auxin transport inhibitors 1-naphthylphthalamic acid (NPA) and 2,3,5- triodobenzoic acid (TIBA) on leaf morphogenesis of transgenic *Nicotiana tabacum* (cv. Xanthi) plants expressing the Agrobacterium tumefaciens cytokinin biosynthetic gene, ipt,. The formation of saucer-shaped leaf-like organs at the shoot apex and at lateral buds was observed. The formation of apical saucer- shaped leaf-like organs could be duplicated by the application of exogenous NPA and cytokinin to wild-type tobacco seedlings. Adventitious leaf-like organs with altered petiole and blade morphology were also observed in the transgenic plants treated with auxin transport inhibitors. These authors concluded that the combination of diminished auxin transport and elevated cytokinin lead to alterations in leaf development in tobacco.

**Effect of enhanced cytokinins on metabolism.**

Motyka *et al.* (1996) studied the effect of enhanced endogenous cytokinin levels on the cytokinin catabolic enzyme cytokinin oxidase. Metabolic control of cytokinin oxidase by its substrate was investigated in plant using wild-type and conditionally ipt gene-expressing transgenic tobacco callus cultures and plants. The derepression of the tetracycline (Tc)-dependent ipt gene transcription was followed by a progressive, more than 100-fold increase in total cytokinin content in IPT calluses. The activity of cytokinin oxidase extracted from these calluses began to increase 16-20 h after gene derepression, and after 13 d it was 10-fold higher than from Tc-treated wild type calluses. An increase in cytokinin oxidase activity, as a consequence of elevated cytokinin levels, was found in detached leaves (8-fold after 4 d) and in roots of intact plants (4-fold after 3 d). The partially purified cytokinin oxidase from wild type, repressed IPT, and Tc-derepressed IPT tobacco calluses exhibited similar characteristics. The increase in cytokinin oxidase activity in cytokinin-overproducing tissue was associated with the accumulation of a glycosylated form of the enzyme. These data indicate the substrate induction of cytokinin oxidase activity in different tobacco tissues, which may contribute to hormone homeostasis.

## Studies on enhancing the endogenous cytokinins by transfer of genes involved in conversion of stored cytokinins

Pospisilova *et al.* (1995) studied transgenic tobacco plants carrying gene zpm60, which encodes a protein capable of binding IAA and displays beta-glucosidase activity. Growth and morphology of transgenic plants did not differ from controls. IAA content of all leaves and internodes was higher than in the controls. Contents of free and conjugated cytokinins were dependent on leaf age, being lower in upper leaves and higher in lower leaves of transgenic plants compared to controls. Higher cytokinin content of lower leaves in transgenic plants was reflected in their higher total stomatal conductance. Contents of chlorophylls a and b, and carotenoids were higher in transgenics than in controls. These data plus data on chlorophyll a fluorescence and photosynthetic rate showed that increased endogenous cytokinin and IAA levels had no dramatic effects on photosynthetic parameters.

Joersbo and Okkels (1996) worked out a novel principle for selection of transgenic plant cells. In contrast to traditional selection where the transgenic cells acquire the ability to survive on selective media while the non-transgenic cells are killed (negative selection), this selection method actively favours regeneration and growth of the transgenic cells while the non- transgenic cells are starved but not killed (positive selection). The *Escherichia coli* beta-glucuronidase (GUS) gene [uidA] was used as selectable (as well as screenable) gene and a glucuronide derivative of the cytokinin benzyladenine as a selective agent which is inactive as cytokinin but, upon hydrolysis by GUS, active cytokinin is released stimulating the transformed cells to regenerate. Selection of *Agrobacterium tumefaciens* inoculated tobacco leaf discs on benzyladenine N-3-glucuronide (7.5-15.0 mg/L) resulted in 1.7 to 2.9-fold higher transformation frequencies compared to kanamycin selection. A significant advantage of this selection procedure is the elimination of the need for herbicide and antibiotic resistance genes.

From the above discussion it could be inferred that, *ipt* from *Agrobacterium* was transformed into tobacco and other systems and stable transformants were obtained. The expression of *ipt* varied depending on the promoter, plant type and prevailing environmental conditions. Same promoter resulted in differential expression of *ipt* in different species of tobacco. Variability was noticed in different *ipt* clones irrespective of the constructs used. Not many studies have been carried out on understanding the nature of *ipt* native promoter in detail. Thorough studies are very much needed to understand the expression of *ipt* with the native promoter. In depth physiological studies need to be done to explore the stability of different clones of transformants. Spatial and temporal regulation of hormone mediated physiological and biochemical processes have not been analysed fully. There is a necessity to develop transgenic cytokinin plants with relatively low level of constitutive expression. *Agrobacterium* native *ipt* seems to be better for this kind of study.

# MATERIAL AND METHODS

# III MATERIALS AND METHODS

## 3.1.1. STANDARDISATION OF EFFICIENT REGENERATION PROTOCOL FOR FCV- SPECIAL TOBACCO.

Main objective of the present study was to develop transgenic plants overexpressing *ipt* under the control of native promoter in tobacco system. Initially, the regeneration protocol for variety special FCV was developed which is a pre-requisite for any transformation protocol development.

Healthy plant material was raised *in-vitro* by germinating FCV-Special tobacco seeds. The seeds were sterilized with 0.1% mercuric chloride for 3 min. and were washed with sterile double distilled water repeatedly for five times to remove the traces of mercuric chloride. The seeds were blotted dry on sterile filter paper and were placed on to 1/2 MS medium solidified with 0.8% agar. The germinated seedlings were again transferred to 1/2 MS medium after 21 days and were allowed to grow for six weeks. Healthy leaves were harvested and were cut into small pieces of approximately $1.0cm^2$ size Four leaf pieces were inoculated into each culture bottle with different media combinations. The media compositions tried were as follows.

1. MS +0.0 NAA+0.0 Cytokinin
2. MS +0.2 mg/L NAA + 0.5 mg/L BA
3. MS +0.2 mg/L NAA + 1.0 mg/L BA
4. MS +0.2 mg/L NAA + 1.5 mg/L BA
5. MS +0.2 mg/L NAA + 2.0 mg/L BA
6. MS +0.2 mg/L NAA + 0.5 mg/L Kinetin
7. MS +0.2 mg/L NAA + 1.0 mg/L Kinetin
8. MS +0.2 mg/L NAA + 1.5 mg/L Kinetin
9. MS +0.2 mg/L NAA + 2.0 mg/L Kinetin

Four weeks after inoculation observations were recorded on number of shootlets developed per explant in each treatment.

### 3.1.2 KANAMYCIN SENSITIVITY TEST ON FCV-SPECIAL TOBACCO.

The leaf piece explants of size 1.0 cm$^2$ were taken from six weeks old *in-vitro* grown plant and were inoculated on to MS morphogenic medium with kanamycin of following concentrations.

1. MS medium with 0.0 μg/mL kanamycin
2. MS medium with 50 μg/mL kanamycin
3. MS medium with 100 μg/mL kanamycin
4. MS medium with 150 μg/mL kanamycin
5. MS medium with 200 μg/mL kanamycin.

About 250 mL each MS morphogenic medium with 0.8% agar was autoclaved and appropriate volume of filter sterilized kanamycin from a stock of 100 μg/mL was added to achieve a final concentration of 50, 100, 150 and 200 μg/mL, when the medium was about to solidify. After 21 days after inoculation, observations were recorded on number of shootlets per explant. The experiment was repeated three times to confirm the sensitivity of the explants.

### 3.1.3 *Agrobacterium* mediated transformation of FCV special tobacco leaf discs with EHA105(*p*KIWI105).

Leaf disc method of *Agrobacterium* mediated transformation was followed in the current study. Leaf pieces of size approximately 1.0cm$^2$ were cut with leaf punch from an aseptically grown 6 weeks old tobacco plant. Approximately 12 leaf explants were inoculated onto tobacco morphogenesis media for pre-incubation for 2 days. On the day of incubation of the leaf discs on to morphogenesis media, here after referred to as day one, individual colonies picked using a sterile bacterial loop were transferred to 5 mL AB minimal medium with 50μg/mL kanamycin and were grown at 28$^0$C for 16-18 h. On day two evening , 0.5, 1.0, 1.5 and 2.0 mL of the overnight grown *Agrobacterium* culture was inoculated to 25 mL each AB minimal medium and was kept for shaking at 28$^0$C for overnight. On day three morning the growth of *Agrobacterium* was monitored by measuring the OD at 595nm. The *Agrobacterium* (EHA105(*p*KIWI105), Fig.1) culture,

Fig.1: Binary vector pKiwi105 (21.6Kb)

which measured OD of 0.6-0.8, was selected for infecting the explants. The explants that were pre-incubated for 48 h. were carefully transferred to a sterile petriplate that contained *Agrobacterium* culture. The explants were treated for 4 min and the explants were blotted on sterile filter paper to remove the excess culture adhering. The explants were transferred back to their respective petriplates and were incubated for 48 h. to allow *Agrobacterium* infection to occur. After 48 h. of co-cultivation the explants were removed from the petriplates and washed thoroughly with MS medium. The explants were again blotted and transferred to culture bottles containing selection media(MS medium + 2.0mg/L. BA + 100 µg/mL kanamycin + 300 µg/mL cefotaxime). The regeneration of shootlets on selection media was observed and observations were recorded after 21 days. The putative transformants developed were, further allowed to grow by transferring them to fresh medium with antibiotics. Once the putative transformants putforth appreciable growth, they were transferred to half strength MS medium for root initiation.

## 3.2 Subcloning of *ipt* into *p*BI101

### 3.2.1 Preparation of competent cells

*E.coli* strain DH5α was used to prepare competent cells for routine transformation experiments to multiply different plasmids used in the current study. From a freshly streaked LB plate a single colony was inoculated to 5.0ml LB broth and was incubated overnight at 37°C on a rotary shaker with vigorous shaking. Overnight grown culture was inoculated to a sterile 1L flask containing 250 mL of tryptone yeast extract (TYM) medium and was grown for 3-4 h. The growth of the culture was monitored every 30 min. by measuring the OD in a spectrophotometer at 600nm. When the OD reached to around 0.6-0.8, the flask was removed from the shaker and was suddenly cooled on ice bath for 30 min. to arrest the growth. The cells were pelleted by centrifuging at 4000rpm for 15 min. at 4°C. The pellet was re-suspended in 20mL pre-chilled TFB1(30mM $MnCl_2$, 100mM KCl, 10mM $CaCl_2$ and 15% V/V glycerol) and was kept on ice for 45 min. After the cells were spun at 4000 rpm at 4°C for 10 min. the pellet was re-suspended in 10 mL of TFB2(10 mM Na MOPS pH 7.0, 15mM $CaCl_2$, 10 mM KCl and 15% V/V glycerol), and was kept on ice for 30 min. The cells were aliquoted into prechilled sterile microfuges(1.5mL) and were frozen in liquid nitrogen before transferring to -80$^{0}$C.

### 3.2.2 BACTERIAL TRANSFORMATION

Sufficient amount of plasmid DNA(pRZ1), encoding ipt gene (gift from Dr. Narayana Upadhya, CSIRO Australia) was obtained from transformed DH5α cells, for further subcloning into the binary vector pBI101(CLONETECH, USA)

### Description of pRZ1

Here a 1538 bp *Ssp* I fragment containing the entire 723 bp of *ipt* coding region, 533 bp of 5' flanking region and 282 bp of 3' flanking region has been excised from a cloned 12.5 kb Ach5 T- DNA fragment and further subcloned into pUC118 at the *Sma*I site, to generate pRZ1(Fig.2). *Eco*RI-*Hind* III digestion releases a fragment of 1570 bp size. The 1538 *Ssp*I fragment has an internal *BamHI* site at around 830 bp and a *PstI* site at around 918 bp from the 5' end.

### Description of the fusion plasmid *p*BI101.

pBI101 (Fig.3) has been derived from the *Agrobacterium tumefaciens* binary plasmid vector pBIN 19. A 'promoter-less' GUS gene cassette from pRAJ260 has been ligated into the *Asp I-Kpn I* site of the pBIN19 polylinker. A 260 bp *Sst I-Eco RI* fragment containing the terminator from the nopaline synthase gene of the *Agrobacterium* Ti plasmid has been inserted downstream of the GUS gene. Thus, plant promoters can be easily cloned upstream of GUS gene and transferred to plants. pBI101 has a low-copy RK2 origin of replication, and confers kanamycin resistance in both bacteria and plants. *Eco*RI-*Hind* III digestion releases the entire MCS-GUS cassette.

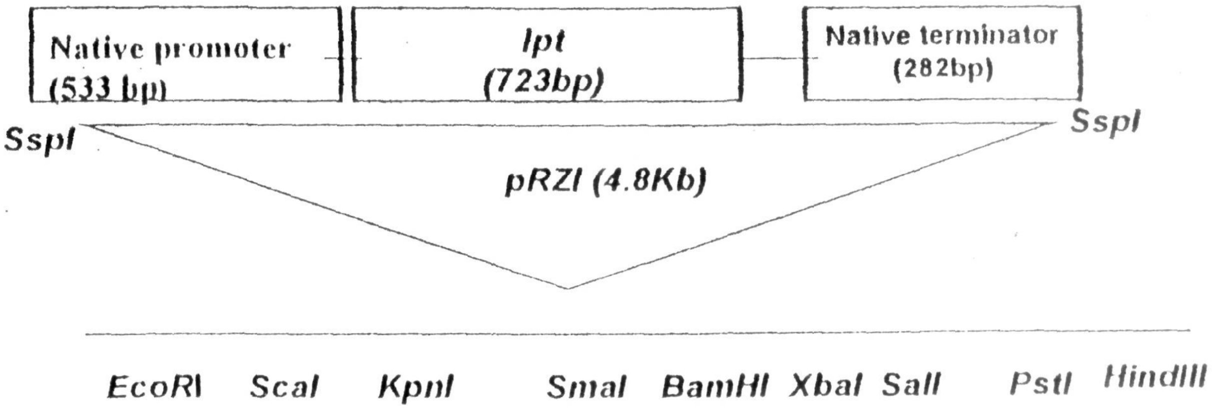

**Fig.2: 1538bp*SspI* ftragment cloned into *SmaI* site of pUC118 generating pRZI**

**pBI101**

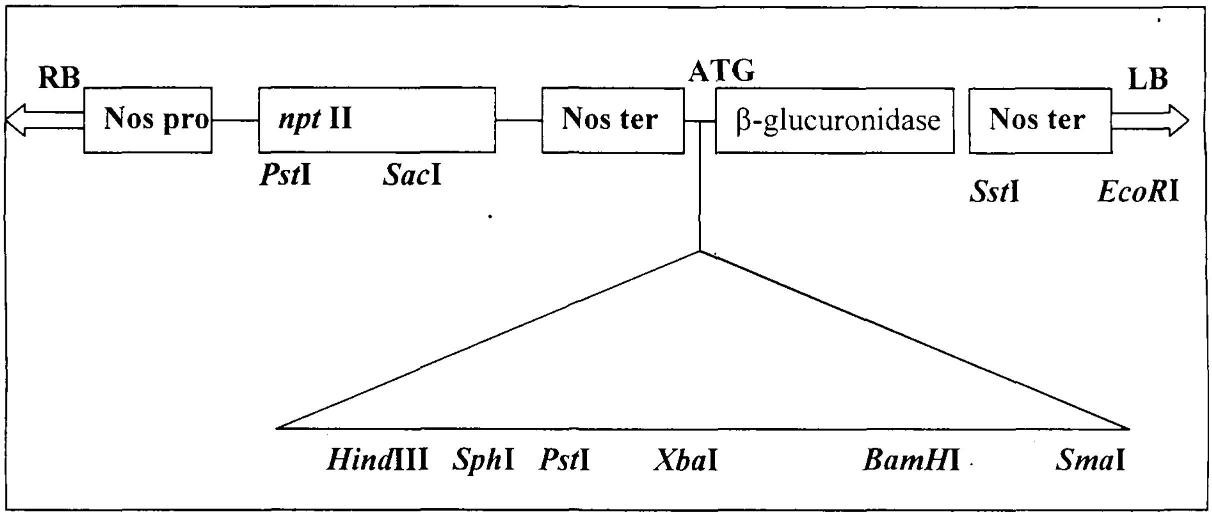

**Fig 3: The promoterless GUS cassette in pBI101**

### Transformation of pRZ1 and pBI101 into *E.coli* DH5α

About 500ng of plasmid DNA (pRZ1 and pBI101) was added to 100μL of competent cells taken in microfuge tubes. These tubes were placed on ice for 1h. The cells were subjected to heat shock by placing the tubes in a drybath heated to $42^0$C for 90 sec. The cells were then chilled on ice for 15 min. and to this 900 μL LB media was added and incubated at $37^0$ C for 1h. From this 100μL was spread on LB agar plates with 100μg/mL ampicillin for pRZ1 and 50μg/mL kanamycin for pBI101, and incubated at $37^o$ C for overnight to select transformants.

### 3.2.3 Plasmid DNA isolation

Plasmid DNA was isolated by Alkaline lysis method (Sambrook et.al .1989). A single transformed colony containing either pRZ1 or pBI101, was transferred to 5.0mL LB medium with 50μg/mL ampicillin for pRZ1 and 50μg/mL kanamycin for pBI101 and was incubated overnight at $37^0$C on a rotary shaker at 200 rpm. The grown culture was dispensed equally into 1.5 ml microfuge tubes and was pelleted by centrifuging at 12000 g for 10 min. The clear medium was poured off and the microfuge tubes were inverted to get rid off the adhering medium. The bacterial pellet was suspended in 100 μL ice cold solution I(TEG buffer; 50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA).

To the above suspension 200 μL of freshly prepared solution II (0.2 N. NaOH and 1% SDS) was added and the contents of the tubes were mixed thoroughly by inverting the microfuge several times and were kept in icebath for 5 min.

To the above mixture 150 μL of ice cold solution III ( mixture of 60 mL 5 M potassium acetate, 11.5 mL glacial acetic acid and 28.5 mL double distilled water) was added and mixed thoroughly and stored on ice for 10 min. After 10 min it was centrifuged at 12,000 g for 15 min and the supernatant was transferred to fresh tubes. To this equal volume of phenol:chloroform (1:1) was added and vortexed for few seconds. After spinning at 12,000 g for 5 min the aqueous phase was carefully transferred to fresh tubes without disturbing the interphase.

DNA in the upper aqueous phase was precipitated by adding one-tenth volume of 3M Sodium acetate (pH 5.3) and 2 volumes of double distilled cold ethanol and was kept at -20$^0$C for 1 h. Further it was centrifuged at 12,000 g for 15 min at 4 $^0$C in a microfuge. After removing the supernatant the DNA pellet was washed with 70% ethanol and was vacuum dried . The pellet was then re-dissolved in 25 µL of TE (pH 8.0) and was stored at -20$^0$C.

**Agarose Gel Electrophoresis.**

To check the purity and the quality of the plasmid DNA, gel electrophoresis was carried out.. Agarose Gel electrophoresis is a simple and highly efficient method routinely employed for separating, identifying, and purifying 0.5 to 25 kb DNA fragments.

Agarose Gel was prepared by adding the required amount of electrophoresis-grade agarose to a known volume of electrophoresis buffer(TAE) and was melted in microwave oven. The melted agarose was cooled to 55$^0$ C in a waterbath and ethidium bromide(4µL/100 mL from a stock of 10mg/mL) was added before pouring onto the gel platform. The open end of the gel platform was sealed with adhesive tape and the molten agarose was poured with the gel comb inserted so that the thickness of the gel was about 8mm. After the gel was formed the tape and the comb was removed carefully and the gel casting platform containing the set gel was placed in the electrophoresis tank. Sufficient volume of electrophoresis buffer was added to cover the gel to a depth of 1 mm.

DNA samples prepared in 10X gel loading buffer were loaded into the wells with a micropipette. The leads were attached to a DC powerpack so that the DNA would migrate into he gel towards the anode. Generally the voltage used was 8 V/cm of the gel. Gel was removed when the bromophenol blue tracking dye had migrated to a distance sufficient for separation of the DNA fragments . DNA was visualised under UV-transilluminator.

### 3.2.4 Experimental procedure for sub- cloning *ipt* into binary vector pBI101.

About 1.0µg of pBI101 or pRZ1 DNA was digested with the restriction enzymes, *EcoR*I and *Hind*III (Amersham, UK) in 20 µL reaction volume at $37^{0}$C . DNA samples that were digested to completion were electrophoresed on 0.8% low melting point agarose gel. The gel was stained with ethidium bromide(4µL/100 mL from a stock of 10mg/mL) and the desired bands (1.5 kb fragment from pRZ1 or 13 kb fragment from pBI101) were cut with a scalpel. To isolate the DNA fragments of interest the gel slice was melted at $65^{0}$C for 10 min and sufficient quantity of TE buffer was added to decrease the agarose percentage to less than 0.4%. To this equal volume of phenol was added and vigorously mixed and was centrifuged at 10,000 rpm for 10 min at room temperature. The aqueous phase was kept aside and the phenol phase was re extracted with equal volume of TE . The second aqueous phase and the first aqueous phase were pooled together and were mixed with equal volume of chloroform and the DNA was precipitated from the aqueous phase by addition of 1/10th volume of 3M sodium acetate and double the volume of ethanol. The precipitate was dissolved in sterile double distilled water and was used for ligation.

**The ligation reaction was setup in the following way**

**Component DNAs (0.05 to 0.5 µg) 8µL**
**pRZ1( 50, 100, 150,200,250ng)**
**pBI101(500ng) in 1:1, 10:1, 100:1, 150:1, 200:1 and 250:1 proportion.**

| | |
|---|---|
| **2x ligase buffer** | **10µL** |
| **10 mM ATP** | **1µL** |
| **T4 DNA ligase(5u/µL)** | **1µL** |

. The 20 µl reaction mixture was incubated overnight at $15^{0}$C. The ligase was inactivated by heating the reaction mixture to $75^{0}$C for 15 min. The ligated products were transformed into competent *E.coli* cells and the transformants were selected on LB media containing kanamycin(50µg/mL). From the individual *E.coli* transformants, plasmids were purified by miniprep procedure. The presence of the insert(ipt gene) was confirmed by restriction digestion and Southern analysis.

### 3.2.5 Agrobacterium transformation: Triparental mating.

For tri-parental mating(Ditta et al., 1980) the binary plasmid from *E.coli* was transferred in to *Agrobacterium* by a conjugation process. Conjugation is facilitated by a conjugative plasmid(pRK2013) which can transfer plasmids between these two species. The procedure involves mixing the three bacterial strains; two E.coli strains, one containing the binary vector, the other pRK2013 and the *Agrobacterium* strain. The bacteria are allowed to grow on a plate at $28^0$C for 16-48 h.. during which time conjugal transfer took place.

**Protocol**

**<u>Bacterial strains:</u>**

| | |
|---|---|
| *Agrobacterium* host | LBA4404, EHA105 and C58C1 (Rif$^R$) |
| *E.coli* donor | DH 5α: pBI.-*ipt* (Kan$^R$) |
| *E.coli* helper: | DH 5α: pRK2013 (Kan$^R$) |

DAY ONE

Respective *Agrobacterium* strains were streaked on AB minimal plates supplemented with 20μg/mL Rifampicin and grown for 48 h. at $28^0$ C.

DAY TWO

The two *E.coli* strains were streaked on LB plates with 50μg/mL kanamycin

DAY THREE

On an LB plate a loop full of each bacterial strain was mixed thoroughly and incubated at $30^0$C for 24 h.

DAY FOUR

A loopfull of bacteria from the above plate was streaked on AB minimal media with 20μg/mL rifampicin and 50μg/mL and kanamycin and was incubated for 48 h. at $28^0$C.

DAY SIX

Isolated *Agrobacterium* colonies were picked, grown and the DNA isolated was re-transformed into *E.Coli.* After isolating the DNAs from *E.coli* it was analysed by restriction digestion and Agarose Gel Electrophoresis.

### 3.2.6 Southern blotting and Hybridisation

**Solutions required**

20X SSC 175.3g NaCl , 88.2g Na Citrate pH 7.0 Volume made upto 1L.

**Solution No.1**

0.25M HCl
20.825 mL Concentrated HCl in 1L

**Solution No.2**

0.5 M NaOH
20g NaOH in 1L

**Solution No.3**

0.5 M NaOH,1.5 M NaCl
20g NaOH
87.66g NaCl Volume made upto 1L

**Solution No.4**
1M Tris HCl,
3M NaCl
Tris base 121.1g
NaCl 175.32g Volume made upto 1L, pH 6.5

## Composition of Prehybridisation solution

| STOCK SOLUTION | VOLUME TAKEN | FINAL CONCENTRATION. |
|---|---|---|
| 20X SSC | 5.0mL | 5X |
| 10% SDS | 0.2mL | 0.1% |
| Formamide | 10.0mL | 50% |
| 10mg/mL Salman sperm DNA | 0.2mL | 100µg (denatured at 100$^0$C for 10min and chilled.) |
| 50X Denhards solution | 2.0mL | |
| $H_2O$ | 2.6mL | |
| Final Volume | 20.0mL | |

### Development of blot.

### Procedure

1. Approximately 10µg each of pBI101, pBI-*ipt* and pRZ1 DNA was digested to completion in a final volume of 20µL with either *Eco RI* or *Hind III* alone or *EcoRI-Hind III*, double digestion

2. 0.8% Agarose gel was prepared to resolve the expected restriction fragments.

3. The DNA samples were electrophoresed for 3 h at 5 V/cm.

4. After visualising the gel under UV light, the gel was transferred to a tray containing 500mL of 0.2N HCl(solution 1) on a rocker arm shaker for 30 min.

5. After decanting the acid solution the gel was rinsed several times in double distilled water. The treatment was repeated until the bromophenol blue tracking dye turned yellow from blue.

6. About 500mL of denaturation solution (solution 2)was again added to the tray and was rocked for 15 min until the bromophenol blue tracking dye turned blue from yellow.

7. After decanting the denaturation solution about 500 mL neutralization solution (solution 3) was added to the tray and rocked gently for 30 min.

8. Nitrocellulose membrane filter was cut in such a way that it was 3mm smaller in both dimensions than the gel. The membrane was wetted with distilled water for 1min and was placed into a tray with 20X SSC for 10min.

9. Three sheets of Whatman 3 mm paper was cut into pieces that were 7mm smaller in both dimensions than the nitocellulose membrane.

10. A wick was made by cutting one piece of Whatman 3MM paper, 2cm wider than the width of the gel and 30-40cm long. Around 300 mL 20XSSC was poured into the tray. The wick was thoroughly wetted.

11. A glass plate was placed over the tray and the wick was put on the plate with both ends of the wick hanging over the plate into the 20XSSC. Care was taken to remove the air bubbles trapped between the wick and the glass plate by rolling a 10mL pipette back and forth over the wick.

12. The gel was removed from the neutralization solution and excess liquid was allowed to drain off the gel and was placed on top of the Whatman 3MM wick. Air bubbles trapped between gel and the wick was removed by rolling a pipette as described earlier. The wetted nitrocellulose filter was placed on the top of the gel.

13. About 10 pieces of Whatman 3MM paper were placed on the membrane taking care not to allow the pieces to hang over the nitrocellulose. About 2-3cm thick stack of paper towels was put on 3MM paper. A glass plate was placed on top of the filter paper over which a weight of 0.3 Kg was placed.

14. The open ends of the tray were covered with saran wrap to minimize evaporation during the transfer. The transfer was done overnight for 18 h..

15. After the transfer was complete the stack of wet papers was removed without disturbing the membrane. Using a pencil a mark was made on the filter towards the upper right hand corner of the gel. Using blunt forceps the nitrocellulose filter was removed and was placed in a tray with 2X SSC for 5min to wash away the debris. Excess liquid was allowed to drain by touching one end of the filter to a Whatman filter paper. The filter was then subjected to UV crosslinking. The transfer of the DNA fragments was checked by visualising the gel on UV transilluminator. absence of ethidiumbromide fluorescence indicated complete transfer.

**Prehybridisation, hybridisation and Autoradiography**

16. The UV-crosslinked filter was placed in big glass tube supplied by the manufacturer (Amersham ). Prehybridisation solution(10 mL) was added to the tube and prehybridisation was carried out for 3 h at $42^0$C.

17. The digoxigenin labeled probe was prepared by using random primer labeling kit supplied by Amersham. the reaction mixture contained approximately 500ng ( 1.5 Kb *ipt* ) DNA dissolved in 4μL TE, Hexanucleotide mix 2μL, dig labeled nucleotide 2μL, Klenow 1μL and water 11μL making up the total volume to 20 μL. DNA was denatured at $100^0$C for 10 min (before adding to the mixture) and was kept at $37^0$C overnight. A mixture of 2 μL 200mM EDTA, 2μL 2M Licl and 50 μL double distilled ethanol was added to the labeling mixture and was kept at $-70^0$C for 2 h. After centrifugation the precipitate obtained was resuspended in 250 μL TE.

18. 5ml of the prehybridisation solution was removed and the labeled probe that was heated to $100^0$C for 10 min and chilled was added and the hybridisation was done for overnight at $42^0$C.

19. The filter was washed twice with 2XSSC, 0.1% SDS at room temperature and once at 50$^0$C for one hour.

20. The filter was washed in washing buffer for 20 min and the blot was transferred to 1% blocking buffer for 1h.

21. The filter was incubated for 30min in 20ml of diluted antibody conjugate solution.

22. Membrane was washed with washing buffer on ice for 20min.

23. The membrane was equilibrated with buffer 3 for 5min.

24. The lumigen PPD (10mg/mL) was diluted to 1:200 in buffer 3 and the membrane was incubated for 10 min in the substrate solution.

25. The membrane was removed from the substrate and the excess liquid was allowed to drop, off.
26. The damp membrane was sealed in a kitchen hybridisation bag.
27. The membrane was incubated at 37$^0$C for one hr.
28. The membrane was exposed to X-Ray film for 1h and was developed.

## 3.3 Plant transformation with pBI-*ipt* mobilised to different *Agrobacterium* strains.

### 3.3.1 *Agrobacterium* mediated leaf disc method of transformation.

Leaf disc method of *Agrobacterium* mediated transformation was used in the current study and the procedure followed was similar to the one described in 3.1.3. Here three different *Agrobacterium* strains were used and transformation was effected by using all the three and strain that elicited higher transformation frequency was selected for subsequent experiments. The putative *ipt* transformants were selected on kanamycin media and were further multiplied.

### 3.3.2 Multiplication of putative transformants.

The putative *ipt* transformants were separated from the cocultivated leaf discs and the pooled shootlets were inoculated on to MS medium without any growth regulators and were maintained in the same media for further experiments.

### 3.3.3 Root Initiation studies

The putative transformants were initially inoculated on to 1/2 strength MS media without any hormones. For *ipt* transformants that failed to root on 1/2 strength MS medium a series of experiments varying the auxin concentrations were carried out as follows. For preliminary experiments putative *ipt* transformants were inoculated onto MS media with 0.0,2.5, 5.0, 10.0 and 20.0 mg/L IBA. In the next set of experiments MS medium supplemented with 10.0mg/L either IBA or NAA or IAA or 2,4-D was tried for root initiation response of the *ipt* transformants. After 3 weeks observations were recorded on visual root initiation response and healthy growth of the explants. About 3 shootlets were inoculated onto 20mL MS medium in culture tubes, with the following media compositions. MS and 1/2 MS medium with 0, 2,4,6 and 8 mg/L. IBA was used with 3 replications. MS and 1/2 MS medium with 0, 0.2,0.4,0.6,0.8 and 1.0 mg/L. IBA was used with 3 replications.

#### 3.3.3.1 Shoot elongation studies

Putative transformants were inoculated on to MS medium with 0.0, 2.5, 5.0, 10.0, and 20.0 GA mg/L and the observations were recorded after 30 days.

### 3.3. 4 Hardening and planting of the *in vitro* grown plantlets.

The ***in vitro*** rooted shootlets that were four weeks old were removed from the culture bottles without disturbing the roots . The plantlets were washed with sterile water with cefotaxime(250μg /μL), until most of the adhering media was washed off. The washed shootlets were transferred to perforated plastic pots(25mm, dm) with 1:1 mixture of soilrite and sand. The small pots with the shootlets were kept in 0.5 Kg polyethylene bags and the mouth of the bag was tied with rubber band. Small holes were made on the polythene to avoid build up of humidity. The bags were kept under tissue culture incubation room for 2 weeks. After 2 weeks the polyethylene bags were kept open for 2 h daily for two days , subsequently the bags were kept opened for 4-6 h. The plants that did not show any wilting symptoms were transferred to 10 cm ,dm pots with red soil and sand mixture(75:25). The pots were then transferred to mist chamber , which is covered on the top with 50% shade net and with 40% mesh on all the four sides. The mist chamber is fitted with foggers connected to centrifugal pump with an electronic timer , which is set at 20 min off time and 30sec on time, so that the temperature is maintained around $30^0$C and RH 85%. After 2-3 weeks healthy looking hardened plants were transferred to big battery containers and were kept in an enclosure that was insect proof and the plants were watered daily in the evening. The growth of the plants was measured in terms of stem girth, plant height , number of leaves, number of inflorescence, number of flowers, and number of capsules.

## 3.4 Molecular analysis of transgenic plants.

### 3.4.1 Extraction of plant DNA

1. One gram fresh weight of young healthy leaf tissue from either in-vitro grown or pot grown plant was ground in liquid nitrogen until it was powdered.

2. To the leaf powder 10 ml of extraction buffer was added and the tissue was ground until it was thawed. The homogenate was transferred to 50 mL centrifuge tube.

3. The mortar was rinsed with additional 5 mL extraction buffer and the solution was added to the homogenate to make the volume to 15 mL.

4. To the above homogenate 1 mL of 20% SDS was added and mixed thoroughly by swirling. The mouth of the centrifuge tube was covered with parafilm with holes made into it and the tube was incubated at $65^0$C for 10 min with occasional stirring.

5. After removing the centrifuge tube from the water bath 5mL of 5M potassium acetate was added and mixed thoroughly until a large precipitate was visible. The tube was incubated at $0^o$C for 20 min.

6. After centrifuging the mixture at 15,000 rpm the supernatant was poured through the sterile cheese cloth in to fresh centrifuge tube and about 7mL cold isopropanal was added and was incubated at $-20^o$C for 45 min.

7. The mixture was centrifuged at 15,000 rpm for 15 min and after pouring the supernatant the pellet was transferred to 1.5 mL eppendorf with the addition of 1mL 70% ethanol. Eppendorf containing the pellet in ethanol was spun in a microfuge briefly for 1 min. The supernatant was removed and the pellet was washed with 100% cold ethanol and was spun for 1 minute, the supernatant was allowed to drain slowly and the pellet was vacuum dried.

8. The vacuum dried pellet was resuspended in 0.5 mL TE for overnight at $4^0$C with gentle periodic finger vortexing

9. The undissolved debris was spun down for 2 min. and the supernatant was pipetted into new eppendorf. To this 55 µL 20X SSC was added along with 10 µg RNase and was incubated at $37^0$C for 30 min..

10. The above solution was extracted once with an equal volume of Phenol:Chloroform:Isoamylalcohol(25:24:1) , after mixing gently it was spun for 2 minutes in a microfuge and the upper phase was transferred to a new eppendorf tube.

11. The residual phenol was removed by 2 sequential extraction with equal volume of chloroform Isoamylalcohol (24:1). The chloroform Isoamyl alcohol was removed after centrifugation. Only the lower organic phase was removed avoiding the transfer of upper aqueous phase and thereby loss of DNA.

12. The resulting aqueous phase was centrifuged for 2 min to pellet the insoluble debris. The supernatant was transferred to the fresh eppendorf tube and 0.9 mL cold ethanol and 25 µL 4M sodium acetate were added and mixed thoroughly The eppendorf was incubated at -20$^0$C for 10 min and was spun for 1 minute in a microfuge.

13. The pellet was washed in 70% and 100% cold ethanol and then vacuum dried. The pellet was re-suspended in 0.2mL TE.

14. The eppendorf was spun in a microfuge for 1.5 min and the upper 0.19 mL was transferred to new eppendorf and the DNA was quantified spectrophotometrically.

### 3.4.2 PCR analysis of putative ipt transgenic plants.

DNA was extracted from transformed and untransformed plants by the above described method. PCR amplification was carried out in a thermal cycler using primers for *nptII*. PCR was performed with Taq DNA polymerase and 25 ng of template DNA. The two primers used were 5'- GAG GCT ATT CGGCTA TGA CTG-3' and 5'- ATC GGG AGG GGC GAT ACC GTA-3'. On amplification the expected product would be around 700 bp.

**protocol.**

1. The following ingredients were added and mixed before starting the reaction. 5 μL 10X PCR reaction buffer

5μl 88 mM neutralised dNTP mix solution
5 μl of "upstream" PCR primer( .25 μg)
5 μl of "downstream" PCR primer(.25 μg)
29 μl template DNA (25 ng)
1μl Taq polymerase (1 unit)
Total volume was 50 μL

2. The reaction mixture was overlaid with 100 μL mineral oil to prevent evaporation.

3. The template DNA samples were denatured for 5 min at $94^0$C

4. Annealing step was carried out at $58^0$C for 45 sec. and the synthesis was carried out at $72^0$C for 1.5 min in each cycle.

5. The above steps were repeated for 25 cycles.

6. The reaction products were examined on a 1.5% agarose gel and was photographed.

### 3.4.3 Southern blotting and Hybridisation

**Protocol:**

1. Approximately 40 μg of the plant DNA was digested to completion with *EcoR*I in a final volume of 50μl .

2. Agarose gel was prepared as required to resolve the expected restriction fragments. 20X20 cm gel with slots nearly 10mm wide and 1mm thick was used for plant DNA

3. After adding required amount of 10X gel loading buffer the samples were loaded on to the gel and electrophoresed for 5 h. at 3 V/cm.

4. After electrophoresing the gel was removed from the box and placed in ethidium bromide staining solution (20μL of 10mg/mL ethidium bromide in 500 mL water) for 30

min. The gel was viewed on trans-illuminator and the mobility of the marker DNA was recorded in comparison with the sample DNA.

5. The gel was placed in a tray containing 500mL of 0.2NHCl on a rocker arm shaker for 30 min.

6. After decanting the acid solution the gel was rinsed several times in double distilled water. The acid treatment was repeated until the bromophenol blue tracking dye turned yellow from blue.

7. About 500mL of denaturation solution was added to the tray and was rocked for 30 min until the bromophenol blue tracking dye turned blue from yellow.

8. Nylon filter was cut in such a way that it was 3mm smaller in both dimensions than the gel. The cut filter was placed directly on the gel.

9. Three sheets of Whatman 3MM paper was cut into pieces that were 7mm smaller in both dimensions than the piece of nylon membrane.

10. A wick was made by cutting one piece of Whatman 3MM paper, 2cm wider than the width of the gel and 30-40cm long. Around 300 mL 20XSSC was poured into the tray. The wick was thoroughly wetted.

11. A glass plate was placed over the tray and the wick was put on the plate with both ends of the wick hanging over the plate into the 20XSSC. Care was taken to remove the air bubbles trapped between the wick and the glass plate by rolling a 10mL pipette back and forth over the wick.

12. The gel was removed from the denaturing solution and excess liquid was allowed to drain off the gel and was placed on top of the Whatman No. 3 wick. Air bubbles trapped

between gel and the wick was removed by rolling a pipette as described earlier. The dry nylon filter was placed on the top of the gel.

13. About 3 pieces of Whatman No. 3 paper was placed on the membrane taking care not to allow the pieces to overhang the nylon. As a precaution saran wrap was put on all the four sides of the filter. About 2-3cm thick stack of paper towels was put on 3MM paper. A glass plate was placed on top of the filter paper over which a weight of 300g was placed.

14. The open ends of the tray were covered with saran wrap to minimize evaporation during the transfer and the transfer was allowed to continue for overnight.

15. After the transfer was complete the stack of wet papers was removed so that the nylon membrane was still lying on the gel. Using a pencil a mark was made on the filter towards the upper right hand corner of the gel. Using blunt forceps the nylon membrane was removed and was baked at $80^0$C for 2 h.. The transfer of the DNA fragments was checked by Visualising the gel on UV transilluminator. Absence of ethidiumbromide fluorescence on the gel indicated complete transfer.

### Prehybridisation, hybridisation and Autoradiography

16. The baked filter was placed in a big glass tube supplied by the manufacturer(Amersham ). either 5or10 mL prehybridisation solution was added to the tube. Pre-hybridisation was carried out for 3 hr. at $55^0$C.

17. The $P^{32}$ labelled probe was prepared by using random primer labeling kit supplied by Amersham. The probe that would give around 10,00,000 cpm /mL in the hybridisation solution , was boiled for 5 min. and chilled on ice.

18. The prehybridisation solution was removed from the glass tube and later the hybridisation solution was added and kept for hybridisation at $55^0$C overnight.

19 . After overnight hybridisation the filter was removed and the filter was washed once with 1X SSC, 0.1% SDS at room temperature and once with 0.1XSSC, 0.1% SDS at $65^0$C as the probe used was homologous one. The filter was allowed to air dry and was wrapped in thin polythene.

20. The filter was exposed to X -ray film by placing it in a cassette with an intensifying screen for 1-2 days .

## 3.5 Analysis of transgenic *ipt* plants for physiological traits.

### 3.5.1 QUANTIFICATION OF CYTOKININS IN TOBACCO PLANTS BY ELISA

**Extraction:**

The procedure of Hansen *et al.*(1984) was used to extract different cytokinins. Five gram of leaf material was taken and ground using pestle and mortar under cold condition. While grinding Beta hydroxy toluene(BHT) was added to prevent oxidation of cytokinins. The ground tissue was transferred to 5 mL of chilled ethanol and extracted for 24 h. at $-20^0$C. The extract was filtered using Whattman No.1 filter paper lined Buchner funnel under mild suction. The residue was re-extracted twice with 5mL of ethanol and the pooled extract was evaporated using a rotary evaporator at room temperature. The residue was dissolved in distilled water.

**Butanol Partitioning:**

Water saturated n-Butanol that has high partitioning co- efficient of the cytokinins was used at alkaline pH. The pH of the crude extract was adjusted to 8-8.555 with 1M KOH and partitioned 5 to 6 times against water saturated n_Butanol(1:1 v/v). Partitioning was done in 15 ml centrifuge tubes that were spun at 3000 rpm for 5 min in a refrigerated centrifuge to separate the layers. The butanol layer was carefully pipetted out each time. Combined butanol extract was taken in siliconised beakers and evaporated in a vacuum evaporator at $30^0$ C. The residue was dissolved in 1ml of 0.5 M TBS pH 7.5.

**Cytokinins were estimated by Indirect ELISA**

**Materials required:**

1. Hapten - Protein conjugate(CK-BSA)
2. Primary antibodies
3. Secondary antibody: Goat anti-rabbit IgG conjugated with alkaline phosphatase.
4. Buffers

a. Coating buffer (Carbonate buffer/ bicarbonate buffer, 0.05M pH 9.5

| | | | |
|---|---|---|---|
| $Na_2Co_3$ | 1.59g | $NaHCO_3$ | 2.93g |
| $NaN_3$ | 0.20g | $H_2o$ | 1000mL |

stored at $4^oC$ for 2 weeks

b. Washing buffer

| | |
|---|---|
| NaCl | 8.0g |
| KCl | 0.20g |
| $KH_2P0_4$ | 0.20g |
| $Na_2HPO_4.2H_2O$ | 1.15g |
| Tween-20 | 0.550mL |
| $H_2O$ | 1000mL |

stored at $4^OC$

c. Dilution buffer(blocking buffer)

Casein 1.0g

Washing buffer 100mL was prepared just before use.

**Procedure:**

**A. Coating:**

1. Conjugate was diluted using coating buffer and 200 µL of it was added to each well
2. The plates were incubated overnight at $4^OC$. The contents of the well were discarded and the plate was washed three times with washing buffer, each washing being retained for 3 min..

### B. Blocking

Blocking buffer (300μL) was added to each well and the plate incubated for 2h at $37^0$C. Then the wells were washed three times with washing buffer as mentioned above.

### C. Addition of standards/ samples and primary antibody

1. 100 μL of samples or standards were added to different wells to which 100μL of diluted IgG was added to the wells

2. The plate was incubated at $37^0$C for two h.
3. The wells were washed three times with washing buffer.

### D. Addition of Secondary antibody:

1. 200μL of secondary antibody (Goat anti-rabbit antibody) diluted 1:2000 times with dilution buffer was added to each well.
2. The plate was incubated at $37^o$c for 1.5 hour
3. Plate was washed thrice with washing buffer.

### E. Detection

1. The substrate p-nitrophenol phosphate (PNPP) was prepared in substrate buffer (1mg/mL) and 200 uL of it was added to each well.
2. The plate was incubated at $37^0$C for 30 min
3. Reaction was stopped by adding 50 μL of 5N KOH
4. The absorbence was read at 405 nm using ELISA reader.

### Construction of standard curve:

### Materials required:

1. Cytokinin-BSA conjugate
2. Primary antibody
3. Cytokinin standards ( 0 - 100 p M)

**Procedure:**

The procedure followed in constructing standard curve was similar to the ELISA procedure described before except that in step C, only 100 uL primary antibody was mixed with 100 uL standard Cytokinins and was added in duplicate to the micro titer plate wells already coated with respective CK- Protein conjugate.

To determine 100 per cent binding, 100 µL of primary antibody +100 µL of TBS was added to the wells without coating. The graphs were plotted using logit transformation of values.

Percentage binding of each standard was calculated using the formula,

$$\text{percent binding} = \frac{\text{Standard OD - NSB OD}}{B_0 \text{ OD - NSB OD}} \quad X\ 100$$

$B_0$ = 100% binding
NSB = Non specific Binding (0% binding)

Percent binding was converted to a Logit $B/B_0$ using the formula Logit $B/B_0$ = Ln $(B/B_0\%)/\ 100 - B/B_0$

Cytokinin contents of the different extracts were computed by plotting Logit$B/B_0$ Vs ln of Cytokinin concentrations of the standards.

### 3.5.2 Gas exchange measurements in transgenic *ipt* and wild type tobacco plants.

With an objective to study the effect of enhanced endogenous cytokinin levels on Photosynthetic characteristics, gas exchange parameters were recorded in the transgenic cytokinin lines and wild type plants. Gas exchange parameters like assimilation rate ('A', µmol $CO_2/m^2$ leaf area/sec), stomatal conductance (gs, mmol $/m^2$/sec) and intercellular C02 concentration (Ci in ppm) was recorded on leaves of top, middle and bottom canopy positions. All the observations were recorded between 9 and 11 am, on a bright sunny day using a portable Photosynthesis system (IRGA, ADC- model LCA4, UK).

### 3.6 Growth and yield measurements in wild type and transgenic cytokinin lines.

The growth parameters like, plant height (cm), Total leaf number per plant, stem girth and total leaf area per plant( $cm^2$), were recorded in 120 day old wild type and transgenic plants. The seed yield components like total pod number per plant and average pod weight(mg) were recorded when the plants reached maturity (approximately 150 days after planting).

### 3.7 Analysis of R1 population

#### 3.7.1 Analysis of R1 population: Assessement of segregation pattern of kanamycin resistance trait in different transgenic lines.

The R1 seeds of different transgenic lines were obtained from the selfed flowers of R0 plants. The seeds were soaked in 250 ppm GA for 48 h. at room temperature and subsequently incubated at $37^0$ C for over night period. The pre incubated seeds of wild type and transgenic lines were spread on filter paper cut to the size of the petri plate. About 10ml of either water or kanamycin solution (400 and 800 µg/mL) was added to each petriplate. The seeds after transfer were incubated at $37^0$C. After 4 days of incubation observations were recorded on the number of seeds germinated. The ratio of germinated to ungerminated seeds was calculated to study the segregation pattern.

#### 3.7.2 Growth studies in R1 seedlings of wild type and transgenic cytokinin seeds.

To compare the growth pattern of the R1 germinated seeds, seeds that were imbibed with 250ppm GA grown in petriplates. After 7 days observations were recorded on the root length (mm) and shoot length (mm) and the shoot to root ratio was calculated.

# RESULTS

# IV RESULTS

## 4.1 Development of protocol for transformation and regeneration of FCV-special variety of Tobacco.

### 4.1.1 Regeneration studies in FCV-special tobacco

The regenerability of FCV-special was tested on different media with varying levels of auxins and cytokinins to arrive at an efficient regeneration protocol for regular transformation studies. At the end of fourth week after inoculation, at zero level of auxin and cytokinin there was senescence of the leaf explants. Maximum number of shootlets per explant(132.5) was observed at 0.2mg/L NAA and 2.0mg/L BA (Table-1). There was an increasing trend in terms of number of shootlets per explant as the level of BA was increased from 1.0 to 2.0 mg/L, but the shootlets obtained at 2.0mg/L were very small and tiny compared to those obtained at 1.0 and 1.5 mg/L BA (Plate-1). Callusing and rooting response was observed at 0.2mg/L NAA and 0.5mg/L BA. Kinetin at all concentrations tested produced shooting response unlike that of BA, with an increasing trend, but the number of shootlets per explant was less compared to that of BA. Even at highest level of cytokinins tested the number of shootlets produced with kinetin was 50% of the maximum number of shootlets produced with BA.

The shootlets when transferred to half strength MS media showed rooting response after one week. After an incubation period of 3 weeks on rooting media the rooted shootlets were transferred to small pots with 50:50 mixture of soilrite and sand. The micropots containing tray was covered with polythene covers and was kept for hardening in culture room for one week, subsequently the pots were transferred to mist chamber for further hardening before they were transferred to pots kept in green house.

**Plate1: Effect of different hormones on morphogenic response of FCV special tobacco leaf explants.**

Leaf explants from *in vitro* grown plants were incubated on different concentrations of NAA and BA. Four leaf explants per bottle were maintained with five replications. Observations on number of shoots developed per explant was recorded on 30 days after inoculation.

**Table-1: Effect of different hormones on morphogenic response of FCV-Special tobacco leaf explants 4 weeks after inoculation.**

| Hormonal concentration mg/L MS media | Average number of shootlets per explant. |
|---|---|
| NAA(0)+BA/Kinetin(0) | dead |
| NAA(0.2) + BA(0.5) | Callus |
| NAA(0.2) + BA(1.0) | 48.75 |
| NAA(0.2) + BA(1.5) | 86.25 |
| NAA(0.2) + BA(2.0) | 132.25 |
| NAA(0.2) + Kinetin(0.5) | 1.75 |
| NAA(0.2) + Kinetin(1.0) | 10.75 |
| NAA(0.2) + Kinetin(1.5) | 29.75 |
| NAA(0.2) + Kinetin(2.0) | 73.75 |
| CD. at 5% | 7.53 |

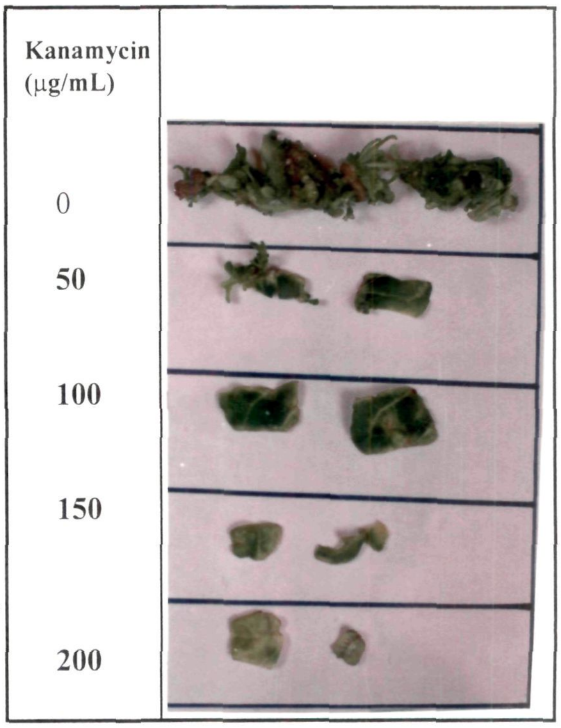

**Plate 2: Effect of different levels of kanamycin on morphogenic response of leaf explants in tobacco.**
Six leaf explants obtained from *in vitro* grown plants were incubated on MS morphogenic medium with different concentrations of kanamycin. Observations were recorded three weeks after incubation.

### 4.1.2 Kanamycin sensitivity test in FCV-Special tobacco.

The leaf explants were inoculated on to different concentrations (50-200μg/mL) of kanamycin added to MS morphogenic media. The observations that were recorded 21 days after inoculation suggests that kanamycin at a concentration of 50μg/mL was sufficient to cause nearly 100% inhibition of morphogenic response when compared to control (Plate-2). In the treatment with 100μg/mL kanamycin the leaf explants remained green but, at 150μg/mL the explants turned yellow and at the highest level of kanamycin there was necrosis of the explants. However, there was development of 2-3 shoots in one or two explants at 50μg/mL kanamycin (Plate-2). Considering the above observations, for further transformation experiments 100μg/mL kanamycin was used.

### 4.1.3 Standardisation of protocol for FCV-special Tobacco transformation with EHA105 (pKIWI105)

The binary vector pKIWI 105(Fig-1) has *npt*II driven by Nopaline synthase (*nos*) promoter and terminator sequences and *E.coli uid*A (*gus*A) coding for reporter enzyme β-glucuronidase under Cauliflower mosaic 35S promoter and *nos* terminator. Initially the transformation studies were done by following the leaf disc method of co-cultivation (Horsch et al., 1985). In this experiment the leaf explants that were co-cultivated for two days were thoroughly washed and were selected on MS morphogenic medium containing 100μg/mL kanamycin and 500μg/mL cefotaxime. After four weeks of selection observations were recorded on the number of kanamycin resistant putative transformants developed per explant. The average number of putative kanamycin resistant transformants was ten. On the other hand the number of shootletss produced on media without kanamycin was 91 (Table- 2), here the shootlets appeared throughout the surface of the explant. The putative transformants appeared only around the cut surface. The non-transformed leaf explants turned brown and showed necrosis.

**Table-2: Regeneration frequency(number of shootlets / explant) of the putative transformants on MS morphogenic medium with 100 μg/mL kanamycin.**

| Kanamycin μg/mL | Cefotaxime μg/mL | Control leaf disc No. of shootlets/explant | Co-cultivated leaf disc No. of shootlets/explant. |
|---|---|---|---|
| 0 | 0 | **91** | Senescence |
| 100 | 500 | **0** | **10** |

The putative transformants were transferred to half strength MS medium for rooting, here all the shootlets produced roots. The well-rooted shootlets were transferred to small pots with soilrite. The survival rate of the potted plants was approximately 90% . The well-rooted potted plants were further transferred to green house for one week during which period they were covered with polythene cover that was subsequently removed after 5 days. Of the 12 plants transferred to big pots only 8 plants survived. The leaf discs taken from these eight plants readily survived on kanamycin, without any yellowing symptoms whereas the untransformed leaf discs showed yellow coloration. Subsequently the leaf pieces taken from these plants were examined by histochemical staining with X-Gluc (Plate-3). Out of these eight plants only two plants were GUS positive. Further, the seeds collected from these selfed $T_0$ plants were tested on 800µg/mL kanamycin showed 3:1 segregation pattern for kanamycin resistance trait (Table- 3) and stable- GUS expression (Plate- 4).

### 4.2 Construction of fusion plasmid pBI-*ipt*

#### 4.2.1 Sub cloning of *ipt* in to binary vector pBI101

A 1500 bp *EcoR*I-*Hind* III fragment from the *ipt* clone pRZ1 (Fig.2) was sub cloned into pBI101 binary vector and the resultant plasmid was named pBI-*ipt*. pRZ1 (gift from CSIRO Australia) has been constructed by sub cloning a 1538bp *Ssp*I fragment containing the entire 723 bp of *ipt* coding region, 533 bp of 5'flanking region and 282 bp of 3' flanking region, excised from a cloned 12.5 kb Ach5 T-DNA fragment, into pUC118 at the *Sma* I site. The resultant plasmid was named pRZ1 (Fig-2.) The binary vector pBI101 (Fig.3) has *npt*II under Nopaline Synthase promoter and terminator sequences and a "promoter-less" GUS cassette with *nos* terminator. Initially the authenticity of pBI101 plasmid was checked by digesting with different enzymes. *EcoR*I -*Hind* III digestion of pBI101 released 2.5 kb fragment (promoter-less GUS cassette), *BamH*I digestion linearised the plasmid, *Pst* I digestion resulted in release of a 2.0kb, 8kb and 5kb (Plate-5) fragments as against the two fragments expected according to the map indicating

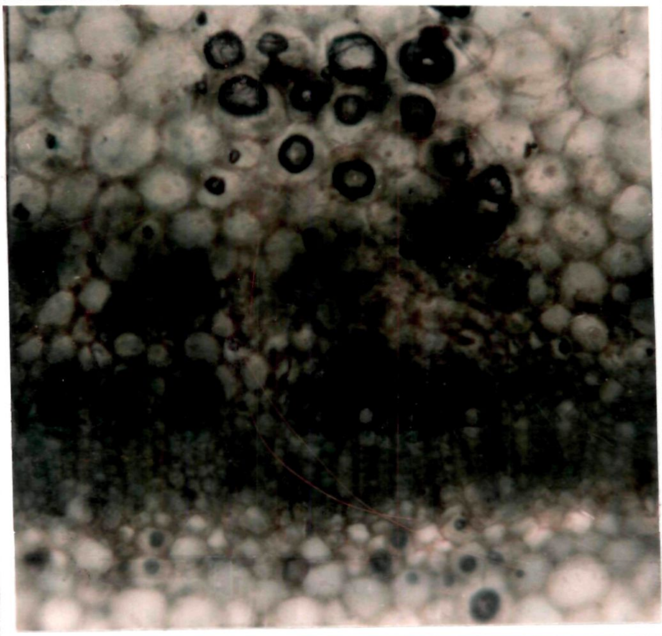

**Plate-3. Histochemical staining for β-glucuronidase activity in *npt*II-*gus* transformed tobacco leaf sections.**

The leaf sections taken from the green house grown *npt*II-*gus* transformed tobacco were stained for GUS activity. The GUS activity in these plants was assayed by taking transverse sections of second fully expanded leaves from 45 day old plant.

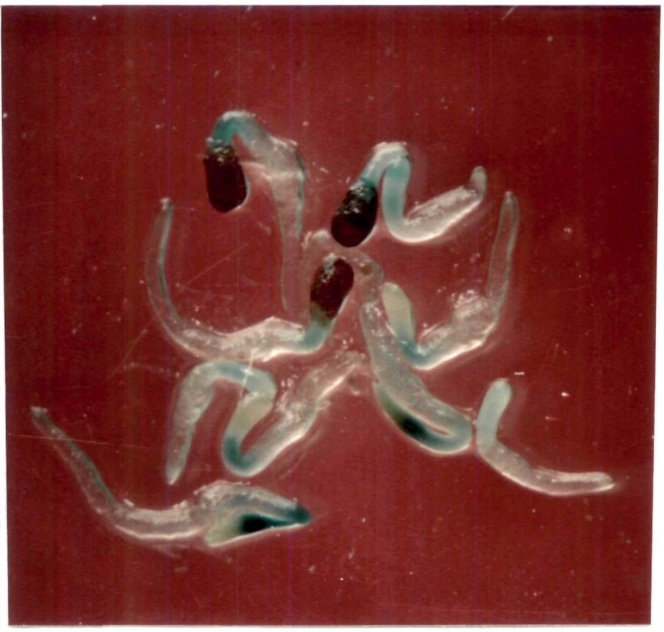

**Plate- 4: β-glucuronidase activity in *nptII-gus* transformed transgenic $T_1$seedlings of tobacco.**

*npt*II-*gus* transformed plants were grown to maturity under green house conditions and $T_1$ seeds from these plants were germinated and were tested for GUS activity.

**Table-3: Segregation pattern of kanamycin resistance trait in $T_1$ seedlings of *npt-gus(uidA)* transformed tobacco .**

| **Kanamycin concentration (μg/mL)** | **Wild type FCV-Spl seeds** | | | ***npt-gus* (*uid*A)transformed seeds** | | |
|---|---|---|---|---|---|---|
| | **Germinated seeds (A)** | **Ungerminated seeds (B)** | **Ratio (A/B)** | **Germinated seeds (A)** | **Ungerminated seeds (B)** | **Ratio (A/B)** |
| **200** | 198 | 25 | 7.92 | 158 | 36 | 4.3 |
| **400** | 172 | 76 | 2.26 | 161 | 42 | 3.83 |
| **800** | 21 | 161 | 0.13 | 184 | 56 | 3.18 |

The germination percentage was recorded 7 days after transfer to the petriplates. Only the seedlings showing complete expansion of the cotyledons were considered as germinated.

the presence of additional *Pst*I site out side the T-DNA borders. *BamH*I-*Pst*I double digestion also produced the same pattern as the *BamH*I and *Pst*I sites are next to each other (Fig.2 and Plate- 5)

The *ipt* clone pRZ1 upon digestion with *EcoR*I-*Hind*III produced a 1.5kb and a 3.2-kb fragment. *BamH* I, *Pst*I *and Bam H*I-*Pst*I digestion released 0.7kb, 0.6kb and 0.6kb fragments respectively (Plate- 5).

### 4.2.2 Restriction pattern of pBI-*ipt*

*EcoR*I-*Hind*III double digestion released a large (12.5kb) and a small (1.5kb) fragment. *Pst*I and *Pst*I-*Bam H*I *digestion* released four fragments (6.0,5.5, 2.0 and 0.6kb). The pattern of release of *ipt* specific fragments from pBI,-*ipt* (Fig.4) was similar to that of pRZ1 (Plate- 5).

### Southern analysis of pBI-*ipt* clones.

DNA isolated from five individual pBI-*ipt* clones (1-5), pBI101 and clone pRZ1 was digested with *EcoR*I-*Hind* III and was blotted onto nitrocellulose membrane. The hybridisation pattern of the digested DNA to the Digoxigenin labeled *ipt* probe was analysed. There was uniform hybridisation of the probe to the 1.5 kb DNA fragments in the lanes of five pBI-*ipt* clones and pRZ1. There was no visible signal in the pBI101 DNA lane. The signal at 5kb region in the pRZ1 lane corresponds to un-digested DNA. The hybridisation pattern was specific to the *ipt* sequences and there was no signal with the non *ipt* sequences even at low stringency, and thus confirming the successful cloning of the *ipt* to binary vector pBI101 (Plate-6).

After southern analysis the individual pBI-*ipt* plasmid was mobilized to *Agrobacterium* strains LBA4404, C58C1 and EHA105 by Tri-parental mating procedure and the pooled pBI- *ipt* DNA samples of respective strains were digested with *EcoR*I-*Hind*III and there was release of *ipt* specific 1.5kb fragment (Plate- 7).

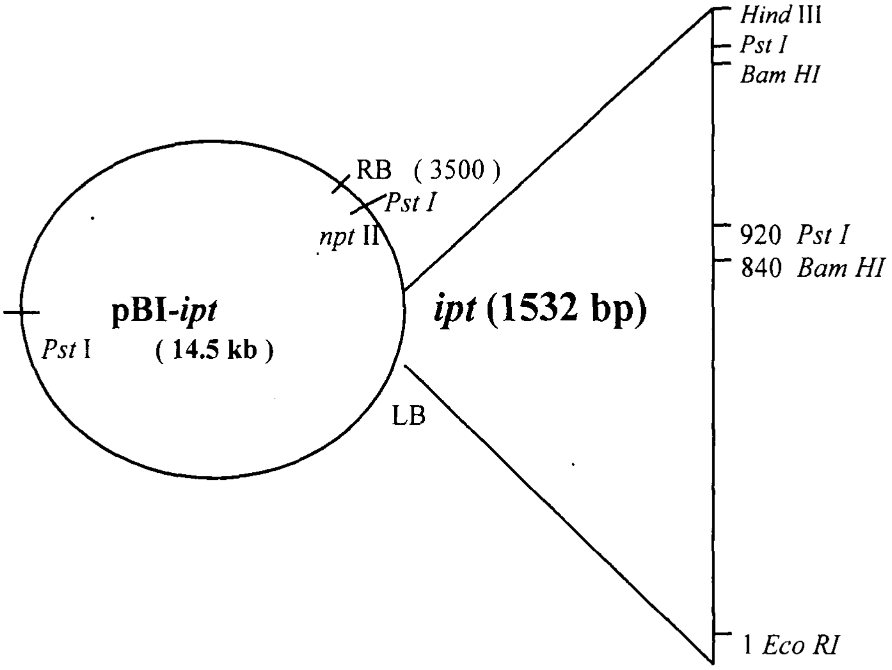

**Fig 4: pBI-*ipt* fusion plasmid.**

pBI- *ipt* was derived from pBI 101. A 1.5 kb *EcoR*I - *Hind* III fragment of pRZ 1 was cloned into *EcoR*I - *Hind* III site of pBI 101, and the resultant plasmid was named as pBI-*ipt*.

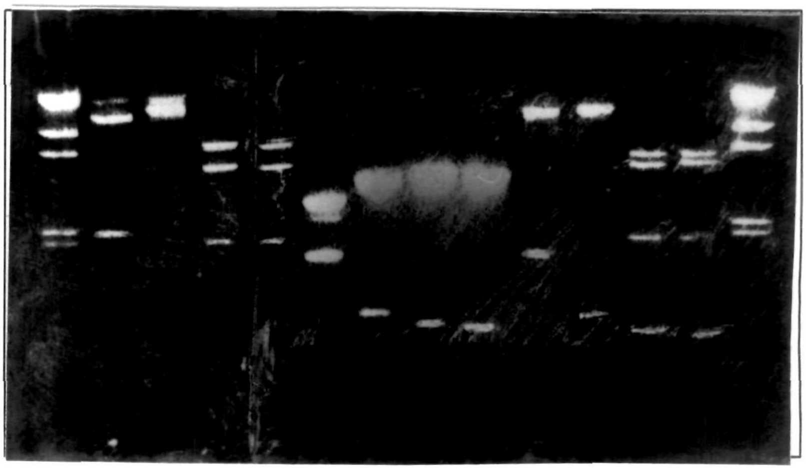

Plate- 5. 0.8 % Agarose gel showing the release of different *ipt* fragments from the DNA isolated from pBI-*ipt* clones.

Lane 1 : λ-DNA/*HindIII* diges
Lane 2 : *pBI101 /EcoR*I-*Hind* III digest
Lane 3 : *pBI101 /BamH*I digest
Lane 4 : *pBI01/PstI* digest.
Lane 5 : *pBI101 /BamH*I- *PstI* digest
Lane 6 : *pRZ1/EcoRI-HindIII* digest
Lane 7 : *pRZ1 /BamH*I digest
Lane 8 : *pRZ1/PstI* digest.
Lane 9 : *pRZ1 /BamH*I- *PstI* digest
Lane 10 : *pBI-ipt/EcoRI-HindIII* digest
Lanes 11 : *pBI-ipt/BamHI* digest
Lane 12 : *pBI-ipt/PstI* digest
Lane 13 : *pBI-ipt/BamHI-PstI* digest
Lane 14 : : λ-DNA/*HindIII* digest

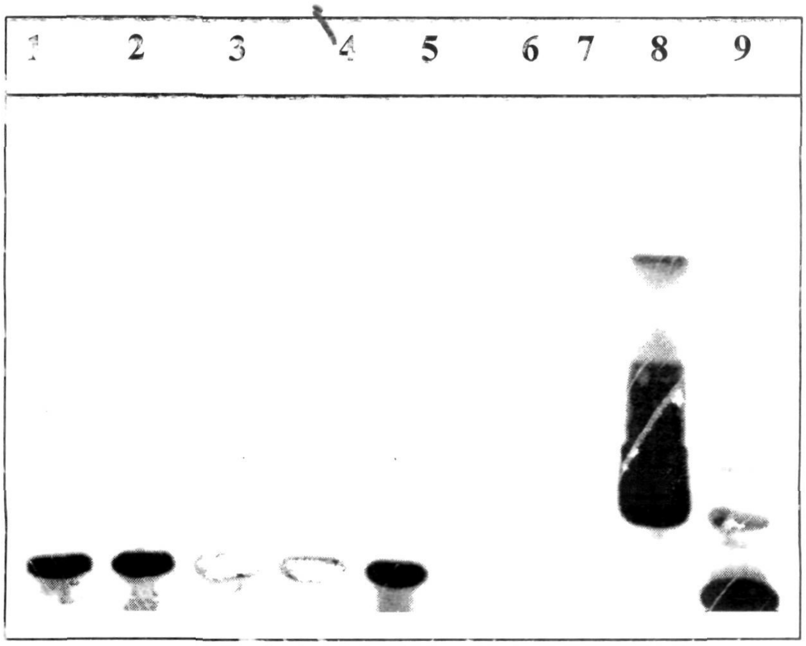

Plate 6. Southern blot showing hybridization pattern of digoxygenin labelled *ipt* to DNA isolated from pBI-ipt clones 1-5.

Lane 1 to 5 : *pBI-ipt/EcoRI-HindIII* digest of clones 1-5
Lane 6 : Blank
Lane 7 : *pBI/EcoRI-HindIII* digest.
Lane8 : *pRZ1* undigested
Lane 9 : *pRZ1/EcoRI-HindIII* digest

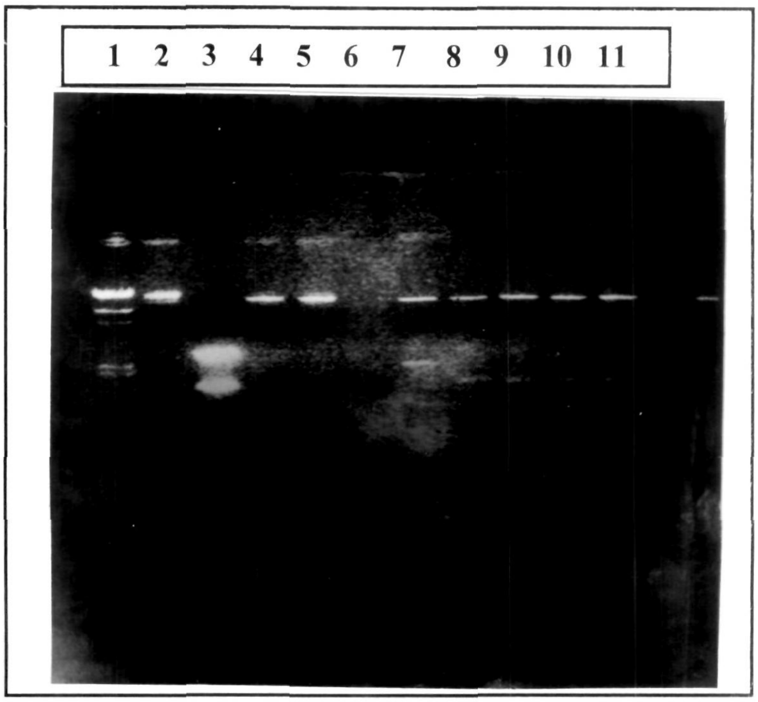

Plate 7. 0.8 % Agarose gel showing the release of 1.5 kb *EcoRI -Hind III ipt* fragment from the DNA samples of EHA 105 (*pBI-ipt*) clones.

Lane 1 : λ-DNA/*HindIII* digest
Lane 2 : *pBI101* undigested
Lane 3 : *pRZ1/EcoRI-HindIII* digest
Lane 4 : *pBI-ipt/EcoRI* digest.
Lane 5 : *pBI-ipt/Hind III* digest
Lane 6 : Blank
Lane 7 : *pBI-101/EcoRI-HindIII* digest
Lanes 8-11: EHA 105 *(pBI-ipt)/EcoRI-HindIII* digest

## 4.3 Plant Transformation studies using pBI-*ipt*

### 4.3.1.1 Experiments with LBA4404 (pBI-*ipt*)

Initially experiments were conducted with pBI-*ipt* mobilized in to LBA4404. In this experiment attempts to obtain transgenic plants were not successful. In one of the experiments, two explants from one of the Plate-s, out of six Plate-s showed regeneration of few shoots. Attempts to grow them further resulted in callus production.

### 4.3.1.2 Experiments with C58C1 (pBI-*ipt*).

In the next set of experiments the *Agrobacterium* strain C58C1 (pBI- *ipt*) was used. Here, in these experiments the explants after co-cultivation for two days were subjected to selection on medium containing 100µg/mL kanamycin and 500µg/mL cefotaxime. Till eighth day on selection medium explants were healthy and on the ninth day *Agrobacterium* appeared around the explants and the attempts to wash them with liquid medium containing cefotaxime (500-1500µg/mL) were unsuccessful. To control the *Agrobacterium* growth, several antibiotics like, augmentin, chloremphenical, combination of cefotaxime and augmentin, were used. The *Agrobacterium* growth was observed in all the treatments. Therefore, for further experiments EHA105 (pBI- *ipt*) was used.

### 4.3.1.3 Transformation studies with EHA105 (pBI- *ipt)*

### Antibiotic Sensitivity studies in EHA105.

*Agrobacterium* strain EHA105 was tested for its sensitivity to different antibiotics. EHA105 was found to be susceptible to all the antibiotics tested. Sulbactum was found to be effective even at 100µg/mL, cefotaxime, cefadtidizyme and carbenicilin were found to be effective at a concentration of 300µg/mL and above. Though in few plates bacterial growth was observed after four weeks at 300µg/mL ,at 400 and 500µg/mL there was no growth. For further experiments cefotaxime at a concentration of 400µg/mL was used. (Table- 4).

**Table 4. Effect of different antibiotics on growth of *Agrobacterium* strain EHA-105 at the end of 21 days of incubation at 28$^0$C .**

| Concentration (μg/mL) | Antibiotics | | | |
|---|---|---|---|---|
| | Carbenicilin | Sulbactum | Cefotaxime | Cefatidizyme |
| 100 | + | - | + | + |
| 200 | + | - | + | - |
| 300 | - | - | - | - |
| 400 | - | - | - | - |
| 500 | - | - | - | - |

### Transformation efficiency studies with five pBI-*ipt* constructs mobilized to EHA105 *Agrobacterium* strain.

Five pBI-*ipt* constructs after southern confirmation (Plate- 6) were mobilized individually to EHA105 *Agrobacterium* strain and were maintained separately after the confirmation of the release of 1.5kb *EcoR*I-*Hind*III fragment with the DNA isolated from respective *Agrobacterium* clone.

A transformation experiment was conducted in FCV-special cultivar using all the five clones of EHA105 (pBI-*ipt*). On selection medium the putative kanamycin resistant plants emerged only from clones 1,3,4 and 5 but not with clone 2. On an average 5.0 shootlets per explant were obtained with clone 3, 2.5 with clone 4 and 1.8 with clone 1.0 and 1.6 with clone 5 (Table- 5)

### Development of transgenic *ipt* plants

Using the clones 1,3,4 and 5 transformation experiments were carried out to obtain putative transformants. The transformants showing regeneration on kanamycin were multiplied and were further analysed.

### 4.3.2 Multiplication and maintenance of putative transformants

The putative transgenic shootlets obtained were separated from the senescent leaf discs and were further allowed to grow on MS medium without any hormone. Many putative transformants showed abnormal growth. The observed growth of the shootlets was very poor and there was production of numerous side shoots (Plates- 8a and 8b), the leaves had prominent trichomes on leaf lamina, there was production of shootlets on the leaf lamina or at the base of the stem which was in contact with the media. Some of the shoots, whose leaf was in contact with media, produced shootlets from the leaf tip (Plate-9), on hormone-free MS medium. Of the many putative transgenics obtained none of them showed root initiation.

**Plate- 9: Cytokinin specific phenotypic variations in transgenic *ipt* shoots.** The PCR confirmed transgenic *ipt* shoots (refer plate 8(a and b) showing distinct cytokinin specific phenotypic characters are given in this plate.

**Plate- 10: Rooting response of wild type and *ipt* transformants to different concentrations of IBA.**
**C- Wild type T- Transgenic**

**1. MS+ 0.0 mg/L IBA 2.MS+2.5mg/L IBA 3. MS+5.0mg/L IBA**
**4. MS+ 10.0mg/L IBA5. MS+ 20.0 mg/L IBA**

The PCR confirmed transgenic *ipt* plants were used for rooting experiments. Few lines showing normal phenotypic characters were transferred to rooting media containing different concentrations of IBA and the observations on rooting response was recorded after 30 days..

Plate 8(a): Note enhanced cytokinin associated characters like dark green coloration, wrinkled leaves, enhanced side branches and appearance of numerous small leaves.

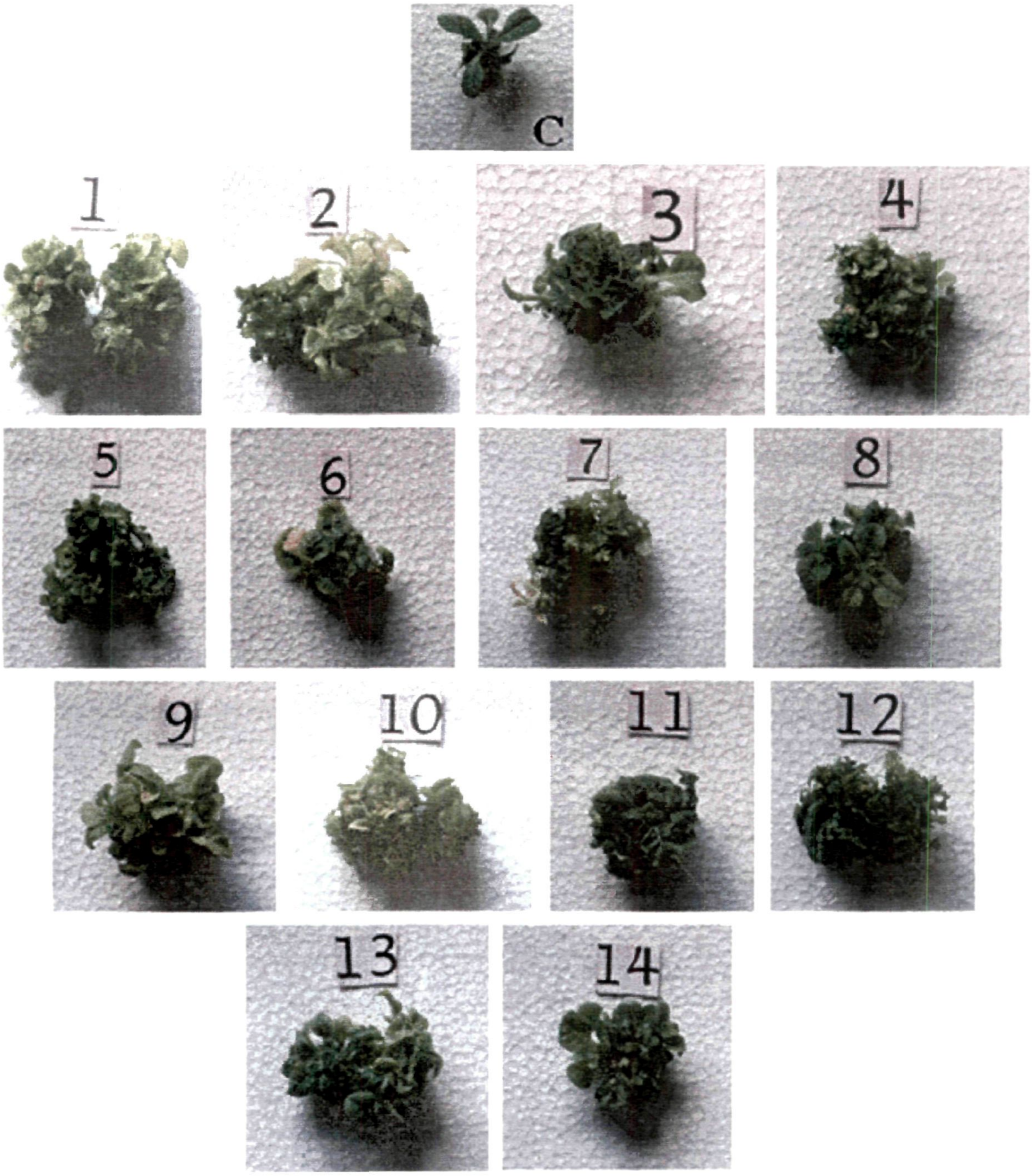

**Plate 8 (a) : Phenotypic variations in wild type (C) and different transgenic Cyt lines (1-14)**

Plate 8(b): Note enhanced cytokinin associated characters like dark green coloration, wrinkled leaves, enhanced side branches and appearance of numerous small leaves.

**Plate 8 (b) : Phenotypic variations in wild type (C) and different transgenic Cyt lines (15-27)**

**Table- 5:Transformation frequency (no. of Kanamycin resistant shootlets/explant) in FCV spl. tobacco leaf explants cocultivated with different pBI-*ipt* clones mobili to EHA 105 *Agrobacterium.***

| **EHA 105 (pBI-*ipt*) clone** | **No. of putative shootlets/explant** |
|---|---|
| 1 | 1.81 |
| 2 | 0 |
| 3 | 5.06 |
| 4 | 2.56 |
| 5 | 1.68 |
| CD. at 5% | 2.2 |

As the putative *ipt* shoots lacked rooting ability and had very high shoot multiplication potential than the transgenic and non-transgenic control plants, few root initiation and shoot elongation experiments were tried by altering hormone combinations in the media.

#### 4.3.3.1 Rooting experiments with putative *ipt* transformants.

#### Experiment 1:Effect of IBA on root initiation in transgenic *ipt* plants.

Initially, based on the assumption that rooting response could be elicited by maintaining a high auxin to cytokinin ratio, explants were subjected to high levels of generally used rooting hormone IBA (0,2.5, 5.0, 10.0 and 20.0 mg/L).

There was distinct difference in the response of the wild type and the transgenic *ipt* shoots. The wild type plants produced roots and normal looking shoots at 0.0 and 2.5mg/L IBA on the contrary the *ipt* shootlets produced small sized leaves at 0.0 level of IBA and shoot with expanded leaves and a big callus clump at the base at 2.5mg/L IBA. With 5.0, 10.0 and 20.0 mg/L IBA there was progressive reduction in the leaf size and stem length. In case of wild type shoots there was accelerated senescence of lower leaves, more conspicuous at 10.0 and 20.0 mg/L IBA (Plate- 10). The *ipt* shootlets showed increase in shoot number with increase in IBA levels and there was gradual reduction in leaf size, at 20.0 mg/L IBA there was vitrification and callus production at the base of shootlets. As opposed to wild type shootlets none of the *ipt* shootlets showed senescence symptoms.

#### Experiment-2. Effect of different types of auxins on root initiation response of transgenic *ipt* shoots.

As there was no favorable response with enhanced levels of IBA in terms of rooting response and shoot elongation, an experiment was conducted with different sources of auxins.

The shootlets were cultured on MS media with 10mg/L IAA, IBA, NAA and 2,4-D. The observations were recorded at the end of three weeks on the appearance of root initials. There was appearance of prominent callus at the base of the shoots with reduced leaf size. This effect was more pronounced in treatments with 10.0mg/L NAA and 2,4-D (Plate-11).

### Experiment 3: Effect of low levels of IBA on root initiation response.

### Experiment 3(a).

In this set of experiments IBA at 2.0, 4.0, 6.0 and 8.0 mg/L was tried in combination with full strength and half strength MS media. Here, rooting was observed in all the treatments in case of wild type FCV-special plants. However, at 8.0mg/L IBA there was callus production at the base along with root initiation. In general shoot length in MS medium was more compared to that of half strength medium. With increase in IBA concentration a decrease in shoot length was noticed. The leaf shape and size was also affected by IBA in the media. It was more pronounced at half strength MS media, where, with an increase in IBA concentration reduction in leaf size was observed. At very high concentration of IBA (6&8 mg/L) the lower leaves showed browning (Plate-12).

The transgenic *ipt* plants never exhibited any root initiation response in any of the treatments tried. Tiny shootlets with small leaves emerged in all the treatments. Visually the number of shootlets was less with half strength MS media. On half strength MS media there was profused callusing at 4.0, 6.0 and 8.0mg/L IBA. None of the explants showed callusing response on MS media with different levels of IBA (Plate- 12)

**Plate - 11: Response of transgenic *ipt* shoots to different auxins.**
**1. MS+ 10.0 mg/L IBA**
**2. MS+ 10.0 mg/L IAA**
**3. MS+ 10.0 mg/L NAA**
**4. MS+ 10.0 mg/L 2,4-D**

The PCR confirmed transformed *ipt* plants (refer plate 8(a&b) were transferred to rooting media containing different auxins and the observations were recorded after 30 days.

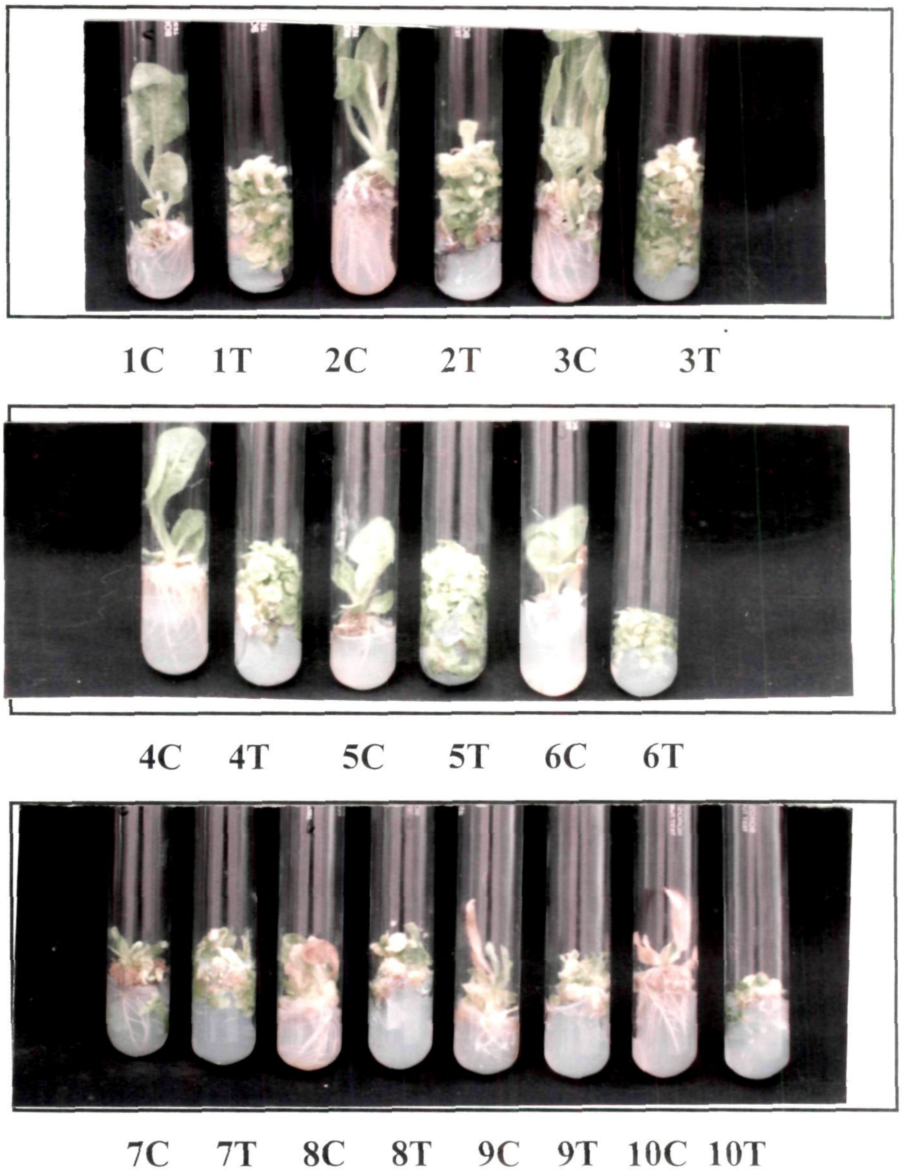

**Plate -12. Rooting response of wild type and transgenic *ipt* shoots to to low levels of IBA.**

**1. MS+ 0.0 mg/L IBA 2. MS+ 2.0 mg/L IBA 3. MS+ 4.0 mg/L IBA**
**4. MS+ 6.0 mg/L IBA 5. MS+ 8.0 mg/L IBA 6.1/2 MS + 0.0 mg/L IBA**
**7. 1/2 MS+ 2.0 mg/L IBA 8. 1/2 MS+ 4.0 mg/L IBA**
**9. 1/2 MS + 6.0 mg/L IBA 10. 1/2MS+ 8.0mg/L IBA**

**C- wild type T- Transgenic**

As described in plate 11, in this experiment the shootlets were transferred to lower concentrations of IBA and the observations were recorded after 30 days.Untransformed wild type plant served as check.

### Experiment 3 (b)

In this experiment still lower concentrations of IBA was tried for rooting response. The concentrations tried were MS media with 0.2, 0.4, 0.6, 0.8 and 1.0 mg/L IBA. In this study 25 individual visibly uniform shoot explants were inoculated in each of the treatments. Even in this experiment there was no definite trend. Only 2 explants out of 25 explants showed rooting response at 0.6 mg/L IBA (Plate-13 and 14). Only one explant showed rooting at 0.8mg/L IBA.

### Development of rooted transgenic *ipt* plants

To obtain transformed *ipt* rooted plants a large number of explants were inoculated on to MS media with 0.6mg/L IBA. Out of nearly five hundred explants inoculated on to rooting media only twelve plants showed rooting response

### Root characteristics of transgenic *ipt* plants.

The root characteristics of the transgenic *ipt* lines were different from that of the control wild type plants. The wild type plant had long un-branched roots, while the trangenic plants developed roots which had less apical dominance and highly branched main root (Plate-14).

### 4.3.3.2 Effect of GA on stem elongation of transgenic *ipt* shootlets

For shoot elongation experiment different levels of GA (0.0, 2.5, 5.0, 10.0 and 20.0 mg/L), were tried to enhance shoot growth. In this experiment wild type explants showed altered leaf morphology, along with decrease in green pigmentation. The leaves had elongated petiole, leaf blade and reduced stem. The leaves were yellow and there was production of callus at the base of all the wild type explants at 10.0 and 20.0 mg/L GA (Plate-15). Though there was increased number of small and tiny shoots with very narrow leaves, there was no callus production at the base at

**Plate -13: Rooting response of wild type and transgenic *ipt* shoots on MS medium supplemented with 0.6 mg/L IBA.**

**T- Transgenic** **C- Wild type**

In this experiment lower concentrations of IBA(0.6 &0.8 mg/L) were used with MS media for induction of rooting.

**Plate- 14: Altered root characters in transgenic *ipt* plant.**

Rooting response was observed in transformed ipt shoots(plate 13) at o.6 mg/L IBA. Significant alterations in root characters were observed in these rooted plants.

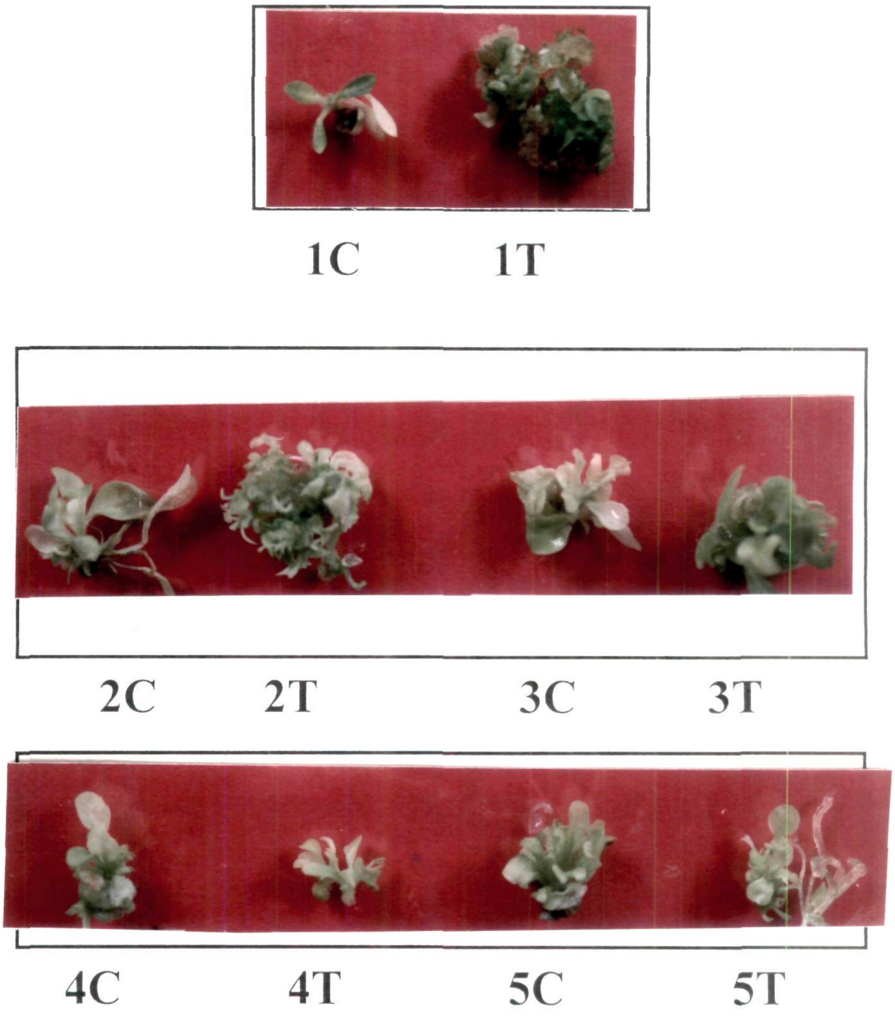

Plate 15: Shoot elongation response of wild type and transgenic ipt shootlets on MS media with different concentrations of $GA_3$.

1. MS+ 0.0 mg/L GA 2. MS+ 2.5 mg/L GA 3. MS+ 5.0 mg/L GA

4. MS+ 10.0 mg/L GA 5. MS+ 20.0 mg/L GA

The transformed ipt shoots(one of the lines showing relatively low internodal elongation) were transferred to different concentrations of $GA_3$. The shoot characters, interms of leaf expansion and internodal elongation were recorded.

higher GA levels in case of *ipt* shoots. At 20.0 mg/L GA there was vitrification of the explants that appeared very lanky. On the whole there was no improve improvement either in terms of stem growth or leaf expansion in response to enhanced GA concentration in the media.

### 4.3.4 Hardening of rooted *ipt* plants.

The rooted plants were initially transferred to 50:50 mixture of soilrite and sand and were pre-hardened under tissue culture conditions. Here, all the twelve plants survived. The pre-hardened plants were transferred to big plastic pots and were kept in mist chamber for further hardening. Of the twelve hardened plants that were transferred only four plants survived when transferred to green house. These plants were further multiplied *in vitro* and physiological and molecular analysis were made in the multiplied plants of different lines.

## 4.4 Molecular analysis of transgenic plants

Many putative transformants characterised based on their survivability on kanamycin media, were further confirmed by PCR analysis and these PCR confirmed plants were used for describing the variations in morphology( Plates-8(a) and 8(b) ) and also for rooting experiments. The results of the few of PCR analysed plants are described here.

### 4.4.1Testing for enhanced kanamycin resistance of transgenic *ipt* plants.

To test the stable- integration and expression of the genes transferred, the phenotypically differing different transformants were sub cultured onto kanamycin media in the alternate subculture. After the eighth sub culture leaves from one of the cytokinin over producing plants were inoculated onto MS media with 0.5mg/L BAP. Incase of wild type plants the regeneration potential ceased at 50µg/mL kanamycin and none of the leaf explants showed regeneration beyond 50µg/mL. At 200µg/mL the explants turned yellow. The *ipt* explants showed regeneration even upto 200µg/mL.and there was no senescence symptom in any of the explants (Plate- 16).

### 4.4.2 PCR analysis for confirmation of successful transformation

Integration of the T-DNA region in the putative transformed plants was done by PCR analysis using a pair of primers for *npt*II. Here the *npt*II specific forward and reverse primers were added to the DNA samples extracted from different transgenic lines and allowed for extension of the strands with Taq polymerase. The PCR product was analysed on 1.5-% agarose gel. PCR analysis of the different lines showed the presence of 700 bp *npt*II fragment. Signal was not observed incase of wild type plant and was present incase of transgenic *npt-gus(uid*A) plant and transgenic *npt-ipt* lines (Plate-17).

### 4.4.3 Southern analysis of transgenic *ipt* plants

Southern analysis was done in five transformed plants established in pots. For confirmation of the integration of the transgene the undigested and *EcoR*I digested DNA blotted on to nylon membrane was probed with radiolabelled *ipt*. Prominent signal was observed at high molecular range, both in case of digested and undigested DNA isolated from different pot grown *ipt* plants. There was signal in the range of 8kb and 5kb incase of lines 2 and 4 respectively in the undigested DNA samples. However, there was a week signal in 9.4-kb and around 8 kb region in line 2 and 3 respectively, in the digested samples. The intensity of the hybridisation signal was more with line 4, which could have been due to multiple integration (Plate-18).

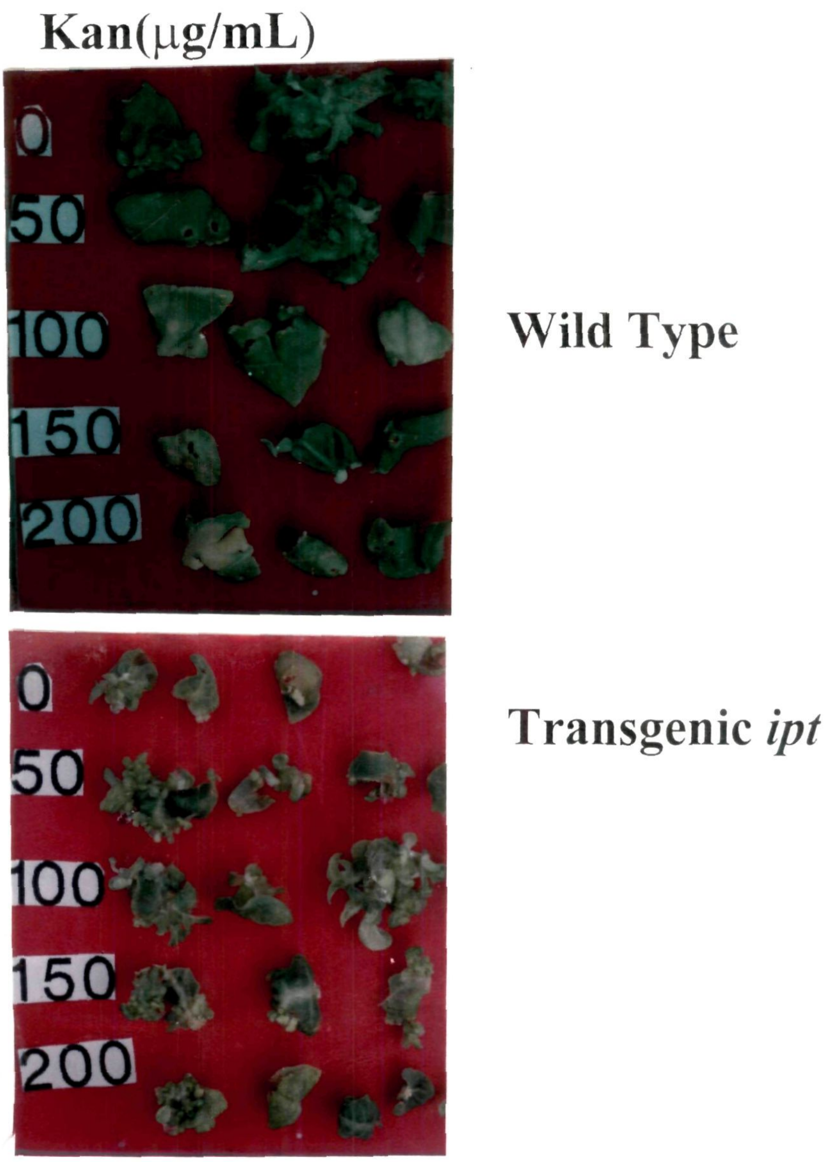

**Plate- 16: Regeneration response of wild type and transgenic *ipt* leaf discs cultured on different levels of kanamycin added to MS morphogenesis media.**

The rooted transformed *ipt* plants were maintained under *in vitro* condition. Kanamycin sensitivity of these lines was further examined to rule out the possibility of escapes. The leaf explants from these plants were transferred to MS morphogenic media and sensitivity was examined based on regeneration and survival of the explants.

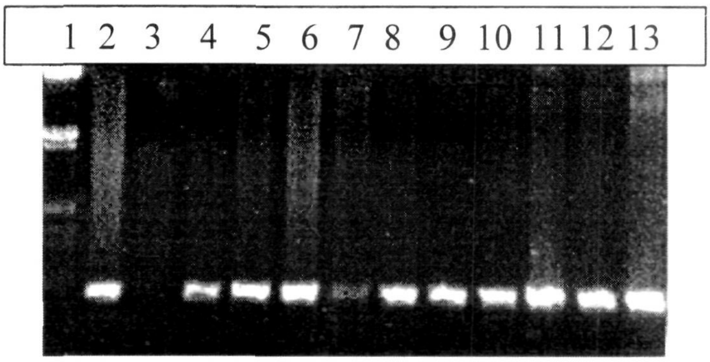

Plate 17: PCR analysis of kanamycin resistant transgenic plants showing presence of an expected 700 bp npt II fragment.

Lane 1: λ DNA /*EcoR*I-*Hind*III double digest
Lane 2: nptII-gus tobacco
Lane 3: Wild type tobacco
Lane 5: Positive plasmid control
Lanes 6-13: Transgenic *ipt* lines

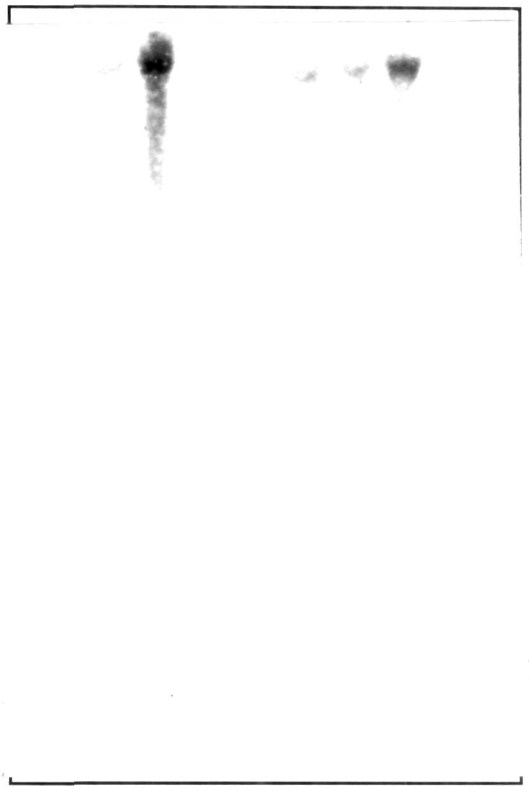

Plate- 18: Southern blot analysis of DNA isolated from *ipt* transformed tobacco plants.

Lanes 1-5: Undigested genomic DNA of transgenic *ipt* lines

Lanes 6-10: *EcoR*I digested genomic DNA of transgenic *ipt* lines.

## 4.5 Quantification of endogenous cytokinin levels and physiological characterisation of *ipt* transformants.

The Physiological analysis of the potted plants was done only in three lines, which showed distinct phenotypic variations. Of the five transgenic lines, (cyt1, cyt2, cyt3, cyt4, and cyt5) the cyt2 line showed moderate wilting symptoms, was named SLIGHTLY **WILTY (SLW)**, cyt3 never showed wilting symptoms hence named **NEVER WILTY (NW)** and cyt4 showed relatively more pronounced wilting symptoms under adequate moisture conditions and thus named **WILTY, (WL)**. Therefore gas exchange measurements were made in these plants. Though cyt5 line also showed higher wilting symptoms it was not used for gas exchange studies because of few other phenotypic abnormalities.

### 4.5.1.1 Cytokinin levels in a few transformed plants maintained *in vitro.*

The cytokinin (*trans* zeatin riboside) levels of few plants were analysed by indirect ELISA the data for five different *ipt* transformants distinctly differing in cytokinin levels was analysed. The cytokinin content of the five transgenic cytokinin lines varied from 190.2 pM/g fresh weight to 3756.82 pM/g fresh weight.

The cytokinin content in case of plants with normal appearance was nearly two hundred times less (190.2 pM/g fresh weight) than the abnormal looking plants (3756.82). However,the endogenous cytokinin content of the wild type plant (11.84 pM) was nearly twenty times less than the normal looking plants (Table-6) grown *in vitro.*

### 4.5.1.2 Cytokinin content of the rooted plants grown in pots

The cytokinin content of the transgenic plants grown in the green house conditions varied significantly. The values ranged from 14.1pmols to 270.1 pM/g fresh weight (Table- 7). The cytokinin content in the leaf samples of Wilty, Never wilty and Slightly wilty was 118.78, 8.22, and 60.79 pM/g fresh weight respectively. The cytokinin content of the cyt1 plant was found to be 14.1 pM/g fresh weight. The cytokinin content of the wild type plant (4.81pM) differed significantly with that of the transgenic cytokinin plants.

**Table- 6: *trans*-Zeatin Riboside content of wild type and transgenic *ipt* lines maintained *in-vitro***

| Plant type | pM $g^{-1}$ fresh wt. of leaf |
|---|---|
| Wild type | 11.84 |
| Cyt-1 | 190.20 |
| Cyt-2 | 264.03 |
| Cyt-3 | 800.80 |
| Cyt-4 | 1282.14 |
| Cyt-5 | 3756.82 |
| CD at 5% | 107.03 |

**Table-7: *trans*-Zeatin Riboside content of pot grown wild type and transgenic *ipt* lines.**

| Plant type | pM $g^{-1}$ fresh wt. of leaf |
|---|---|
| Wild type | 4.81 |
| Cyt-1 | 14.10 |
| Never Wilty | 8.22 |
| Slightly Wilty | 60.79 |
| Wilty | 118.78 |
| Cyt-5 | 270.10 |
| CD at 5% | 7.55 |

### 4.5.2. Gas exchange parameters in selected transgenic *ipt* and wild type plants.

With an objective to study the effect of enhanced endogenous cytokinin levels on photosynthetic characteristics, gas exchange parameters were recorded in the transgenic *ipt* and wild type plants.

Gas exchange parameters like Assimilation rate ('A'), Stomatal conductance ($g_s$) and intercellular $CO_2$ concentration (Ci) were recorded in leaves of top, middle and bottom canopy positions. All observations were recorded between 9 and 11 am, on a bright Sunny day using a portable- Photosynthesis system (IRGA, ADC- model LCA4, UK).

The gas exchange parameters like, stomatal conductance, internal carbon dioxide concentration and assimilation rate were measured in 110 days old pot grown plants to understand the relationship between the enhanced endogenous cytokinin concentrations and the gas exchange parameters. These observations were made in grown up plants (110 days old) since the main objective was to asses 'A' in older leaves, which may remain non senescent in *ipt* plants.

#### *Assimilation rate ('A')*

There was a significant variability in 'A' among the transgenic plants compared to the wild type (Table- 8). A maximum assimilation rate of 13.79 ($\mu$M $CO_2/m^2/s$) was recorded on the top canopy leaves of the transgenic WILTY plant type. This was followed by never wilty and slightly wilty transgenic and lowest 'A' of 10.84 was noticed in the wild type plant. These results clearly suggest an increase of 27% in 'A' in the wilty transgenic compared to the wild type (Table- 8).

## Table 8. Gas exchange parameters in wild type and transgenic FCV-Spl. Tobacco.

| **Plant type** | **Leaf position** | **A** | $g_s$ | **Ci** | $A/g_s$ | $Ci/g_s$ |
|---|---|---|---|---|---|---|
| **Wild type** | 15 | 10.84 | 400 | 324 | 27.10 | 810 |
| | 13 | 11.69 | 304 | 322 | 38.45 | 1059 |
| | 10 | 6.04 | 250 | 337 | 24.16 | 1348 |
| | | | | | | |
| **Wilty** | 15 | 13.79 | 914 | 309 | 15.09 | 338 |
| | 13 | 13.70 | 632 | 308 | 21.67 | 487 |
| | 10 | 9.58 | 320 | 323 | 29.94 | 1009 |
| | | | | | | |
| **Slightly Wilty** | 15 | 12.43 | 1164 | 304 | 10.68 | 261 |
| | 13 | - | - | - | - | - |
| | 10 | 8.14 | 890 | 317 | 9.15 | 356 |
| | | | | | | |
| **Never Wilty** | 15 | 13.15 | 474 | 323 | 27.74 | 681 |
| | 13 | 12.02 | 380 | 328 | 31.63 | 863 |
| | 10 | 6.56 | 334 | 338 | 19.64 | 1012 |
| **CD at 5%** | | 0.762 | 164.0 | 3.26 | - | - |

Further 'A' decreased significantly with increase in age of the leaf (Fig-5) in all the transgenic plants and also in wild type. The wild type plants recorded a maximum reduction of 58% in 'A' in the bottom canopy leaves). But, the percent reduction in 'A' was less in transgenic *ipt* plants. On an average the highest 'A' values in the older leaves was recorded in the wilty transgenic plant.

## Stomatal conductance (gs)

Corresponding to the changes in the assimilation rate, stomatal conductance has also showed a considerable variability in both between the transgenic plants and the wild type, as well as with the age of the leaves (Table- 8).

The wild type and transgenic (Never wilty) plant have recorded lowest **gs** of 400 and 474 $mM/m^2/s$, on the other hand a maximum gs of 1164 was recorded in fully expanded leaves of slightly wilty, followed by the wilty transgenics. A significant reduction in gs was noticed in bottom canopy positions of all the transgenic plants. The slightly wilty and never wilty transgenic (Fig-6) showed a relatively lower reduction in $g_s$ with age when compared to the wild type and the wilty transgenic. However a maximum decrease in **gs** was noticed in the wilty transgenic plants.

## Intercellular $CO_2$ concentration (Ci) and the ratio of A/gs and Ci/gs

The Ci recorded in both transgenic and wild type plants showed a significant variability. The wild type plants and the never wilty transgenic plants showed a higher Ci while wilty and slightly wilty transgenics recorded lower Ci content (Table- 8).

Although gs decreased significantly with the age of the plant the Ci levels increased in all the plant types, indicating a possible non stomatal regulation of Photosynthesis with ontogeny.

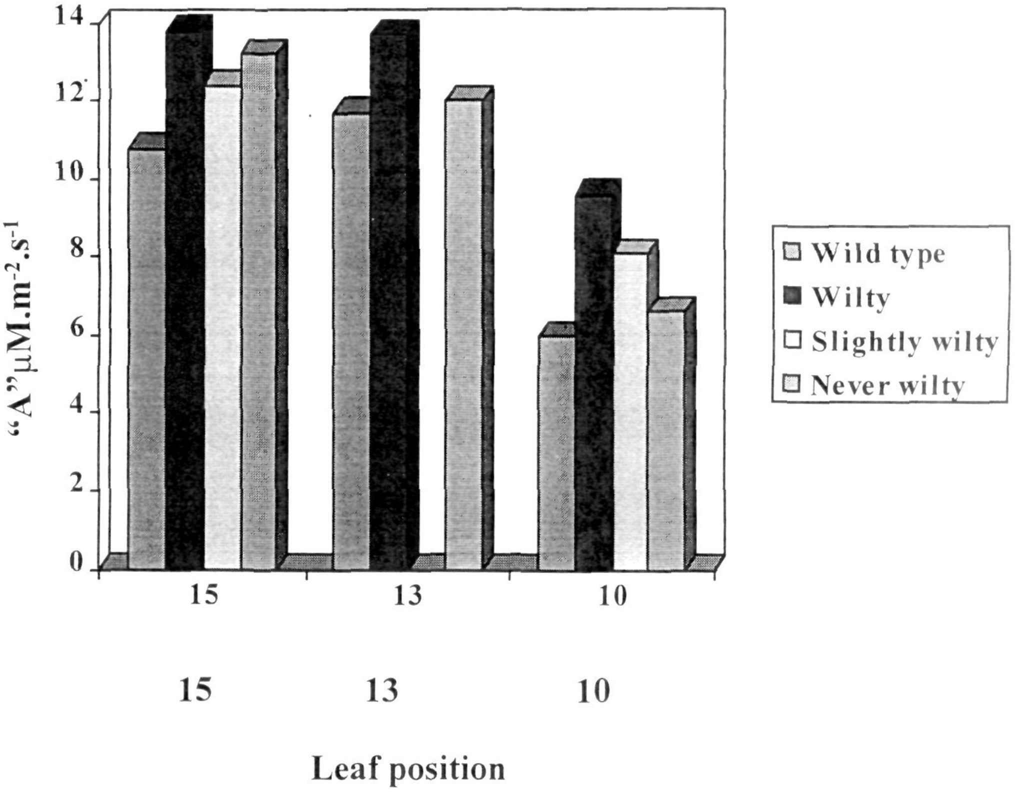

**Fig 5:** Photosynthetic rate ('A') measured in leaves of wild type and transgenic *ipt* tobacco

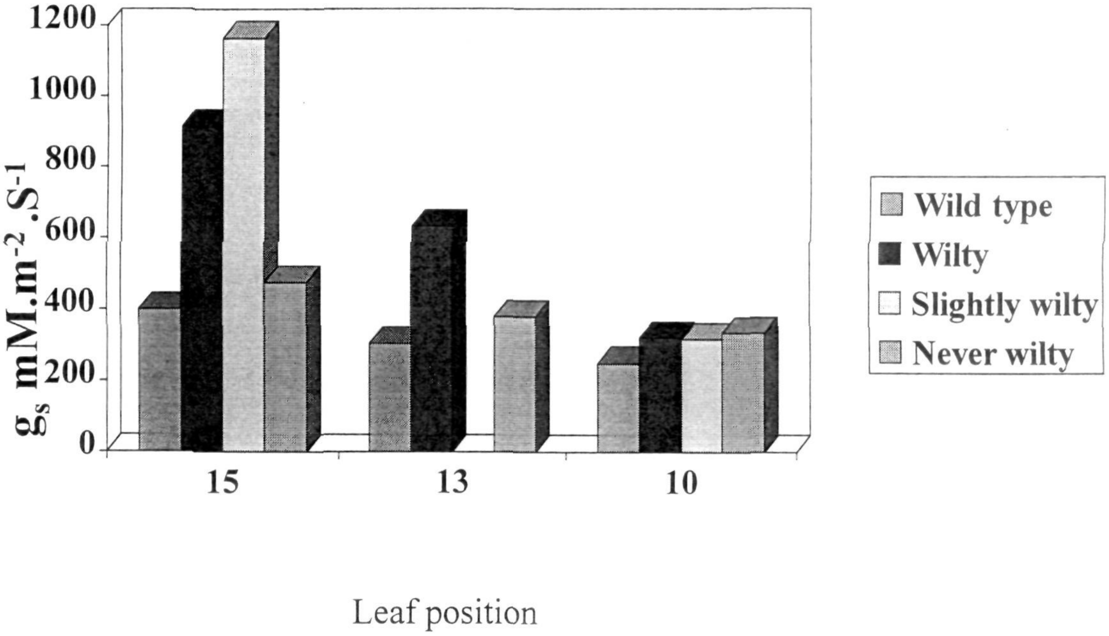

**Fig. 6: Stomatal conductance(gs) measured in leaves of Wild type and transgenic *ipt* tobacco.**

The Ci at a given **gs** (Ci/**gs**), is generally considered as an indirect estimate of mesophyll efficiency. An increase in Ci/**gs** ratio with age in all the transgenic plants offers further proof for reduction in 'A' due to reduced mesophyll efficiency, that resulted in an increased Ci. The mesophyll efficiency of slightly wilty and wilty transgenics was relatively superior compared to never wilty and wild type plants ( Table- 8).

## 4.6 Growth and yield analysis of transgenic and wild type plant

Two separate experiments were carried out for comparative growth studies between the transformed and wild type plant. In the first experiment wild type was compared with one of the *ipt* transgenic cytl plant, where as in the second experiment growth rates were assessed in three transformed plants Wilty, Slightly wilty and never wilty plants.

### 4.6.1 Growth and yield measurements in cytl and wild type tobacco.

The growth parameters like plant height (cm), total leaf area/plant (cm2), number of leaves and stem diameter (cm) was measured in 120 days old, wild type and cytl line of *ipt* transgenic plant. In the transgenic cytl plant considerable variations were observed. The total leaf area per plant was comparatively more in cytl(Table- 9) plants and the number of leaves was nearly 30% more in cyt 1 plant than the wild type plants. Cytl plant was 46.2 cm taller than the wild type plant (115.8cm). The cytl plants were relatively dark green (Plate-19) and showed reduced lower leaf senescence (Plate-20) than the wild type plant. The total chlorophyll content that was estimated in different leaves of wild type and cytl plant showed considerable variations. The chlorophyll content measured in top most leaf of the canopy did not differ between wild type and transgenic plant. It was true with the leaves subtending the inflorescence. There was considerable difference in the chlorophyll content of the lower leaves, 7th and 10th (Table-10). The lower leaves of the cytl plants had nearly 3 times more chlorophyll (0.97mg/g fresh weight) than the wild type plant (0.36 mg/g fresh weight). The wild type plants had more senesced leaves than the Cytl plants (Plate-19)

**Plate- 19: Enhanced growth of Cyt1 transgenic plant**
**C- Wild type T- Cyt1 transgenic tobacco**
One of the confirmed ipt transformants with normal phenotype was transferred to green house. This line was designated as Cyt1. The growth rate of this line was compared with the wild type plant. The photograph was taken at flowering.

**Plate 20: Pattern of senescence in wild type(C) and transgenic Cyt1(T) plants.**
The Cyt1 (refer plate- 19 for details) was compared with wild type fordifferences in senescence of lower leaves at the time of capsule formation stage.

**Table 9. Growth parameters of 120 days- old wild type and transgenic (Cyt-1) plants(average of two plants).**

| Plant type | Total leaf area (cm$^2$) per plant | No. of leaves per plant | Plant height (cm) | Stem girth (cm) |
|---|---|---|---|---|
| **Wild type** | 7412 ± 495 | 20 ± 1 | 115.8 ± 6.8 | 5.38 ±0.21 |
| **Cyt-1** | 10269 ± 652 | 27 ± 2 | 162 ± 10.2 | 7.9 ± 0.42 |

**Table 10: Chlorophyll content (mg/g fresh weight) of Cyt 1 and Wild type plants at different leaf positions**

| Leaf position from top | Wild type | Cytl |
|---|---|---|
| **Below inflorescence** | 1.04 | 1.09 |
| **1** | 2.67 | 2.51 |
| **3** | 1.68 | 1.66 |
| **2** | 1.06 | 1.30 |
| **7** | 0.36 | 0.97 |
| **10** | 0.08 | 0.14 |
| **CD at 5%** | 0.18 | |

The Cytl plants which produced more flowers(Plate-22) produced 126 pods with average weight of 400.7 mg, while the wild type plant produced 79 pods with an average weight of 340.6-mg (Table-11).

### 4.6.2 Growth and yield measurements in Wilty, Slightly wilty, Never wilty and wild type plants.

The observations that were taken on 115 days after transplanting indicated there were considerable differences (Plates-21, a, b, and c) in phenotype and several of the growth parameters, between the transgenic *ipt* lines and the wild type.

The transgenic wilty plant had the maximum leaf area per plant (8508 $cm^2$) as compared to the wild type (4539 $cm^2$) and the transgenic *npt- gus* plants (5829 $cm^2$). Among transgenic cytokinin plants the never wilty had the least total leaf area (6799 $cm^2$, Table-12). The leaf number also varied between different lines and the wilty type had the maximum (24) and the transgenic *npt-uid* A(*gus*) plants had the least (Table-12). There was not much difference in plant height among different lines though the cytokinin plants were slightly taller (Table-12). The transgenic cytokinin plants had increased stem girth, with the wilty and slightly wilty types showing relatively greater stem girth (Table-12). There was not much difference between the transgenic *npt uidA* plant and the wild type plant.

The transgenic cytokinin plants flowered late by one week when compared to the wild type plants. The transgenic cytokinin plants produced dark pink colored flowers compared to the light pink colored flowers (Plate-23) of the wild type plant that also had less shelf life.

The capsule number was maximum in wilty type (163) and was least in case of never wilty type (68). There was considerable difference in average pod weight and the never wilty types weighed maximum of 270.2mg while the wilty pods weighed 238.8mg. The wild type and the transgenic npt-*uid*A(*gus*) plants that produced 83 and 94 capsules respectively weighed only 203.6 and 197.3 mg per capsule respectively (Table-13).

**Plate- 21(a): Variations in leaf morphology of transgenic *ipt* plants.**
**NWL- Never Wilty, WL- Wilty , SLW- Slightly Wilty.**
A few PCR and Southern confirmed ipt transformed lines were transferred to green house and based on phenotype they were designated as Never Wilty, Wilty and Slightly Wilty. The variations in these characters are shown in this photograph.

**Wilty** **Never Wilty**

**Plate- 21(b): Close up view of morphological variations between Wilty and Never Wilty transformants.**

**Wilty** **Never wilty**

**Plate- 21(C): Phenotypic variations in 30 day old transgenic *ipt* plants.** Variations in morphological characters of two 30 day old *ipt* transformants(fig 21(a) are shown in this photograph.

**Wild type** **Transgenic Cyt 1**

**Plate- 22: Variations in inflorescence characters of wild type and transgenic Cyt1 plants.**

The flower and capsule number was more in transgenic plant.

Wild type Transgenic *ipt*

**Plate- 23: Difference in flower colour between Wildtype and Transgenic *ipt* plants.**

**Table 11. Yield parameters in fully matured wild type and Cyt-1 transgenic plants**

| Plant type | Total pod number per plant | Average pod wt (mg) |
|---|---|---|
| Wild type | 79 ± 12 | 340.6 ± 14.5 |
| Cyt-1 | 126 ± 16 | 400.7 ± 12.8 |

**Table 12. Growth parameters of different transgenic plants (120 days-old. Average of two plants)**

| Plant type | Total leaf area ($cm^2$) per plant | No. of leaves per plant | Plant height (cm) | Stem girth (cm) |
|---|---|---|---|---|
| Wild type | 4539 ± 295 | 15±1 | 110.6±5.1 | 6.1±0.2 |
| Transgenic *npt-gus* | 5829 ± 342 | 13±2 | 107.1±6.4 | 6.8±.15 |
| Slightly wilty | 7954 ± 196 | 19±2 | 115.2±3.9 | 7.1±0.1 |
| Never wilty | 6799 ± 652 | 18±1 | 119.0±2.9 | 5.8±0.6 |
| Wilty | 8508 ± 437 | 24±2 | 114.7±3.6 | 7.6±0.3 |

**Table 13. Yield parameters of different transgenic plants at maturity.**

| Plant type | Total pod number per plant | Average pod wt (mg) |
|---|---|---|
| Wild type | 83±14 | 203.6±14.6 |
| Transgenic *npt*-uidA | 94±9 | 197.3±6.9 |
| Slightly wilty | 79±6 | 244.8±18.4 |
| Never wilty | 68±11 | 270.2±14.2 |
| Wilty | 163±19 | 238.8±16.1 |

### 4.7 Genetic and physiological analysis of T1 population.

#### 4.7.1 The segregation analysis of Kanamycin Resistance trait in T1 seedlings.

The Wilty type showed segregation ratio of resistant too susceptible 2.95:1 at 800µg/mL, slightly wilty 4.3:1, and never wilty 2.88:1. The corresponding segregating ratios at 400µg/mL kanamycin were not as expected and the ratios were 4.5:1, 4.15:1 and 3.2:1 for wilty, slightly wilty and never wilty respectively (Table-14).

#### 4.7.2 Seedling vigor analysis of $T_1$ seedlings.

In a separate experiment the $T_1$ seeds germinated in petriplates, were grown for 10 days and the shoot and root length was measured. The wild type seedlings showed a better root growth than the different cytokinin over producing lines (Table-15). Seedlings from transformed *ipt* wilty showed least root growth. There was not much variation in shoot length of different wild type and cytokinin lines though the cytokinin over producers showed slightly increased shoot growth and the Never wilty seedlings had the maximum shoot length (Table- 15 ). The shoot to root ratio was maximum in transformed *ipt* wilty and the least was in wild type plants. The slightly wilty and never wilty types showed a ratio of 2.32 and 2.73 respectively (Table- 15).

**Table 14. Segregation pattern of Kanamycin resistance trait in $T_1$ seedlings of transgenic *ipt* tobacco**

| **Seedling type** | **400 μg/mL Kanamycin** | | | **800 μg/mL Kanamycin** | | |
|---|---|---|---|---|---|---|
| | **Sensitive** | **Resistant** | **R/S ratio** | **Sensitive** | **Resistant** | **R/S ratio** |
| **Wilty** | 55 | 248 | 4.50 | 70 | 207 | 2.95 |
| **Slightly wilty** | 69 | 287 | 4.15 | 36 | 255 | 4.30 |
| **Never Wilty** | 73 | 235 | 3.20 | 57 | 131 | 2.88 |

**Table 15. Growth measurements in 12 day-old seedlings of Wild type and $T_1$ seedlings from the transgenic *ipt* plants**

| Seedling type | Average Root length (mm) | Average shoot length (mm) | shoot:root ratio |
|---|---|---|---|
| Wild type | 6.1 | 13.1 | 2.15 |
| Wilty | 2.8 | 14.0 | 5.00 |
| Slightly wilty | 5.9 | 13.7 | 2.32 |
| Never Wilty | 5.4 | 14.9 | 2.73 |
| CD at 5% | 0.19 | 0.43 | |

# DISCUSSION

# V DISCUSSION

Transgenic plants have been developed to study the process of Photosynthesis, respiration, hormone metabolism, fatty acid metabolism etc. In all these cases the gene in question was either over expressed, either by using, the constitutive or inducible promoters, or, down regulated by the antisense approach.

The classical examples include studying the role and relevance of RuBisCO by down regulation of *rbcs* (Hudson *et al* ., 1992). Down regulation of genes coding for enzymes in the biosynthetic pathway of ethylene (Hamilton *et al* ., 1990; Mattoo and White, 1991) is another revealing example. Sucrose Phosphate Synthase as the rate limiting enzyme in Sucrose biosynthesis has been well documented by over expressing the gene coding for SPS (Galthier *et al* ., 1993), further signifies the potential of this tool.

The significance of specific enzymes in the metabolic pathway is now being routinely analyzed by overexpression or down regulation of genes (Stitt and Sonnewald,1995). Apart from the enzymes involved in metabolic processes, more recently attempts are being made to alter the levels of signal molecules like growth hormones, by transgenic approach. Inspite of phenomenal progress in the area of hormonal research the primary sites of action of these growth hormones and how they initiate signal transduction path way is not clearly elucidated even on today. Except for ethylene the receptor sites for other growth hormones are yet to be identified and characterized (Binns, 1994).

The physiological response brought about by these growth hormones is very complex. However over the last three decades significant progress has been made in understanding the formative effects of different growth hormones.

The formative effects of Cytokinins, includes breaking of apical dominance (Sachs and Thimann., 1964), stimulation of Chlorophyll synthesis (Arnold and Fletcher., 1986), delaying senescence (Richmond and Lang ., 1957), counteraction of the effects of heat stress (Caers *et al* ., 1985) and regulation of stomatal movement (Fubeder *et al* ., 1992). These informations have been generated over the years by co-relative studies, which involves either spraying the hormone externally or relating the observed response to the endogenous concentrations. These methods suffer from lack of precision in uptake and entry of the hormone, which often varies depending on the experimental condition, and the quantification procedures used were not very efficient.

Even on today it is not clear how far the genetic variability observed in leaf senescence in field grown plants is mediated by cytokinins. Several relevant plant growth processes like protein turnover, stomatal conductance and nitrate reductase activity could be regulated by the signal molecules like cytokinins. However, there is no unequivocal evidence to prove these points under field grown conditions. From this context the transgenic approach to develop plants with varying cytokinin levels is very relevant. In view of this major approach in the present investigation has been to develop transgenic plants over expressing *ipt* coding for ISOPENTENYL TRANSFERASE, rate-limiting enzyme in cytokinin biosynthesis. Ultimately the objective was to develop transgenic plants with varying levels of cytokinins and assess the role of cytokinins on plant growth and few Physiological processes.

## ISOPENTENYL TRANSFERASE (ipt) the rate limiting enzyme in cytokinin biosynthesis

The initial step in cytokinin biosynthesis involves the addition of an iso-pentenyl side chain to 5$'$ -AMP from iso-pentenyl pyrophosphate (Chen and Melitz, 1979). The resultant compound isopentenyl adenine is converted to Zeatin and Zeatin riboside. This reaction is catalyzed by iso-pentenyl transferase that is encoded by the gene ,ipt (Barry *et al* ., 1984). This is the rate-limiting step in cytokinin synthesis (Akiyoshi *et al* ., 1984). However much of the work on isopentenyl transferase has been in Agrobacterium. Not much progress is made in cloning ipt from plant and the only progress, which has been made so far, is partial purification of isopentenyl transferase protein (Chen and Melitz, 1979).

## ***ipt* from *Agrobacterium tumefaciens* is the only source for genetic manipulation of cytokinins in higher plants.**

*Agrobacterium tumefaciens*, which causes crown gall, the neoplastic plant disease is known to transfer a well-defined DNA, segment from its large 200 kb Ti plasmid. This transferred DNA segment, called T-DNA encodes six genes, *iaaH* and *iaaM* for auxin biosynthesis, *ipt* for cytokinin bio synthesis, and nos for opine synthesis and a gene for a modulator, which controls tumor morphology. These genes are located between well-defined right and left border, which integrates in, to the plant genome on infection. In several studies, *ipt* from *Agrobacterium* has been extensively used to generate transgenic plants with enhanced levels of cytokinins (Smigocki and Owens,1988; Medford *et al* .,1989; Roeckel *et al* ., 1997). Significant information is available about Agrobacterium due to the effort of many researchers in this field (Sciaky *et al* .,1978; White and Nester.,1980; Thomashow *et al*., 1981 ; Chilton *et al* ., 1984).

Agrobacterium *ipt* being the only source, *ipt* from Ach5 strain (Hedikemp *et al* .,1983) was used for transformation in the present study. Stable transformants have been obtained in tobacco and other systems in several studies using ipt from Agrobacterium (Smigocki and Owens, 1988; Medford *et al* .,1989; Strabala *et al* ., 1996 ; Roeckel *et al* .,1997) . However in many of these studies *ipt* was cloned on constitutive or inducible promoters to obtain transgenic plants. *ipt* construct used in the present study has native promoter and terminator sequences and is about 1500 bp , we preferred native promoter as it easily gets expressed in plant system.

Since our major emphasis was Agrobacterium mediated transformation, ipt was sub cloned to binary vector pBI101 (Clonetech, Fig- 3).

The insertion of the 1.5 kb ipt into the *EcoR*I-*Hind*III site of pBI101 was confirmed by analyzing the structure of the resultant plasmid pBI-*ipt* (Fig- 4) by restriction digestion of the plasmid with different restriction enzymes. *Pst*I digestion of the plasmid pBI-ipt released three fragments of size 8 kb, 5kb and 2 kb respectively as against the expected 13kb and 2kb (Plate- 5) fragments, but this is not according to the map of pBI101 (Fig-3). This could be due to the presence of another *Pst*I site in the vector back bone. The *Pst*I digestion of the resultant plasmid pBI-*ipt* released four fragments because of an internal site in ipt.

After transforming the ligated pBI-*ipt* , five colonies were selected randomly among several colonies appeared on kanamycin Plate-. Further, the cloning was confirmed by southern analysis (Plate- 6) of the DNA isolated from these five individual clones. Here the dig labeled *ipt* probe hybridized to the pBI-ipt DNA and not to the vector pBI101 DNA (Plate- 6).

## Transformation studies in tobacco

For any transformation study most crucial pre-requisite is regeneration. Tobacco is the widely used model system since the first report of successful transformation by Horsch *et al*.(1985). Since then number of transformants have been developed by several authors in different varieties of tobacco. We used a local cultivar Special Fcv and regeneration and transformation protocols were standardized (Plate- 1). Here the binary vector pKIWI 105 , which has nptII under nos promoter and *gus*A (uidA) driven by CaMV 35S promoter (Fig.1), was used.

The optimum hormonal concentration for obtaining maximum number of shootlets was found to be 0.2 mg/L NAA and 2.0 mg/L BA and the maximum number of shootlets obtained was 132 (Table- 1) which is more than the values reported earlier by Nauerby *et al* .(1997) where they obtained 92 shootlets per explant after an incubation period of 2 months with Petit Havana SRI leaf discs at 0.5 mg/L BA. Successful regeneration has been reported in Tobacco using different varieties and hormonal concentrations. The regeneration response varies depending on variety, cultural conditions and hormonal concentrations.

For standardization of transformation we used binary vector pKIWI105 with reporter genes *gusA* and *nptII*. However, for subcloning *ipt* the binary vector pBI101 (CLONETECH) was used instead of pKIWI105. The pKIWI105 is comparatively a larger vector (20kb) than pBI101 (15kb). This binary vector lacks unique sites for cloning *ipt* with native promoter The *ipt* used in the present study has native, promoter and terminator sequences, had to be subcloned to suiTable-Binary vector which lacked constitutive promoter (CaMV 35S or Nos) . The Binary vector pBI101 was found to be ideal as it has promoter less gus cassette with nos terminator sequences, adjacent to multiple cloning site. Here any functional promoter could be cloned in the Multiple Cloning Sites to express gusA to assess the promoter activity. By *EcoR*I-*Hind*III digestion the entire promoter-less gus cassette along with the terminator sequences could be removed from pBI101, whereas pKIWI105 lacks unique sites to release the entire gus expression cassette. Further, manipulation and handling of the larger sized plasmid would be difficult than the smaller pBI101. The *Agrobacterium* strain EHA105 mobilized with pBI-*ipt* by tri-parental mating, was used for transformation studies following the protocols standardized for EHA105(pKIWI105) and putative *ipt* transformants were obtained.

**Transformation efficiency**

The number of putative transformants obtained per explant in the present study was 10 in the EHA105 (pKIWI105) (Table-. 2) transformed leaf discs and varied from 0 to 5, incase of EHA105 (pBI-*ipt*) transformed leaf discs (Table-5). Transformation efficiency often depends on a number of factors, such as type of explant, *Agrobacterium* strain, binary vector and the structure of the construct. From several of our experiments on transformation it can be inferred that the transformation efficiency was very less with pBI-*ipt*. The higher transformation efficiency observed with EHA105 (pKIWI105) transformation studies as compared to pBI-ipt could be because of the differences in the size and structure of the two binary vectors used. Hiei *et al* . (1997) also observed variation in transformation frequency in case of rice where same *Agrobacterium* strain elicited differential transformation response when it harbored different binary vectors..

If the gene product has a direct role in regeneration or any specific metabolic process the transformation efficiency decreases. Since ipt gene product causes variations in cytokinin levels, which has a direct role in regeneration, the observed decrease in transformation efficiency may be due to altered hormonal balance at the regenerating points. In such situations low expression in vitro during regeneration is desirable.

The observed reduction in the putative transformants obtained could be a dose response effect of externally applied cytokinin in combination with the endogenously produced cytokinins. Such dose response curves for cytokinins have been established (Nissen, 1988) and tobacco was shown to be ultrasensitive than species like *Phaseolus* or soybean.

To obtain better transformation efficiency and more number of putative transformants it is better *ipt* is expressed on some promoter whose expression is less in vitro. Promoters like rbcs, HSP70 and SAG are more desirable.

**Description of putative transformants**

The putative transformants, which were obtained on kanamycin medium, were phenotypically different from each other as well as with that of the wild type and pKIWI105 transformed FCV shootlets. The putative ipt plants were generally short in stature , had numerous tiny wrinkled leaves, the leaf shape was different from that of the wild type plants and they produced small shoots directly from the leaf lamina( Plate- 9) or the cut end of the stem. Some of the leaves were saucer shaped (Plate-. 8a and 8b) and never produced roots when transferred to medium with varying auxin concentration. The altered morphology of the putative transformants when ipt was over expressed by 35S promoter (Smigocki and Owens., 1988) further confirms that increased higher endogenous cytokinin levels are the causative factors for such abnormalities. Even in the *ipt* expressed by inducible promoter (maize hsp70) the transformed plants showed altered morphology (Medford *et al* .,., 1989). The observed abnormalities of the transformants are the unique feature in this system because the gene of interest is directly involved in morphogenesis. One of the striking features of the putative ipt transformants was their inability to root when transferred to optimum root induction medium.

Inhibition of rooting by the externally added cytokinins has been reported earlier (Svesson, 1972; Goodwin, 1978). Strabala *et al* .(1996) have shown that the cytokinin over expressing plants had diminished auxin transport. Even reduced synthesis and turn over of IAA in the transgenic *ipt* plants was reported by Ekloef *et al* ,(1997). Tissue culture rooting response depends on auxin to cytokinin ratio, if so transformed shootlets should have shown the rooting response with varying auxin concentrations in the medium. But our results shows that even with high concentrations (2.5 to 20 mg/L IBA) of auxin didnot elicit any rooting response (Plate-10). A high auxin to cytokinin ratio often induces rooting, however such a phenomenon is often seen at concentrations not more than 1-2 mg /L. In some plants even auxin free medium induces rooting. There fore for subsequent experiments IBA from 2-8mg/L was tried. But these treatments also did not elicit any root initiation response. In the next set of experiments lower concentrations of IBA (0.2 to 1.0 mg/L) on rooting was studied in cytokinin free medium. At lower concentrations leaf expansion was improved and occasional rooting was noticed only at 0.6 and 0.8mg/L IBA. The rooting response of the shootlets to the varying auxin concentrations was not consistent and by inoculating large number of shootlets we could retrieve few putative transformants with healthy roots. Even in the rooted plants the roots had less apical dominance and were highly branched (Plate- 14).

The plants developed in the present study also had higher cytokinin content (Table- 6) than the wild type plants grown *in vitro*. If the inhibition of rooting was direct effect of cytokinins, the addition of high concentration of auxins to the media should have elicited rooting response. But in our studies (Plate- 10) the addition of auxins at higher concentration resulted in excessive callus production and senescence of the shootlets. Yu *et al* .(1981) have reported about the evolution of ethylene in Mung bean hypocotyls with the external application of BA or kinetin and the level of ethylene evolved was increased with IAA or calcium in their study. Similarly Stenlid (1982) reported about stimulation of ethylene by auxins and cytokinins synergistically, in their study free cytokinins inhibited root growth than their ribosides. The inhibition of root growth was overcome by using ethylene synthesis or action inhibitors, and anticytokinins. From the information available about the interaction effect of auxin and cytokinin in stimulating ethylene synthesis it could be hypothesized that the lack of root initiation in ipt transformants is due to the indirect effect of cytokinin through stimulation of ethylene synthesis and that the higher levels of auxins in the media synergistically stimulated higher Ethylene biosynthesis than cytokinin alone and resulted in inhibition of root initiation. The additional support for such an inference could be drawn from the recent study by Aloni *et al* .(1998) where, in, the *Agrobacterium* induced tumor associated abnormalities were also seen in the wild tomato plants exposed to ethylene. Such type of ethylene induced abnormalities were not observed in ethylene insensitive Nr mutants of tomato. Stimulation of ethylene synthesis by cytokinins is well-documented phenomenon (Mattoo and White, 1991; Goren and Riov, 1989). Further, enhanced level of cytokinins has been shown in transgenic plants over expressing *ipt* and also in the tumor tissues caused by Agrobacterium (Weiler and Spanier,1981; Akiyoshi *et al.*,1983). In our experiments there was 100- 300 times increase in cytokinin levels in some of the putative transformed plants. The enhanced cytokinin levels there fore could have resulted in higher ethylene synthesis. The reduction in leaf size, decrease in internodal length (Plates 8a and 8b) and parageotrophic behavior of the shootlets could be the direct of effect ethylene mediated through enhanced endogenous cytokinin levels.

## Endogenous cytokinin contents in transgenic and wild type plants

The cytokinin levels of the *in vitro* grown plantlets varied from 190-3756pM/g fresh weight (Table- 6) nearly 10 to 300 times more than the wild type plantlets. The corresponding values of the potted green house grown plants varied from 14 to 270pM/g (Table- 7) fresh weight nearly 3 to 50 times more than the wild type plants. The transzeatin riboside values of the present study are comparatively similar to the values reported earlier by Smigocki and Owens(1988) in transformed tobacco plants expressing *ipt* on 35S promoter. But values as high as 9000pM/g fresh weight as reported by Smigocki and Owens (1988) was not obtained in the present study. This could be due to the constitutive expression by the 35S promoter in their study. Weiler and Spanier (1981) and Akiyoshi *et al* .(1983) have reported about 1500 times increase in cytokinins in the tumor tissue. Though *ipt* was on a native constitutive promoter, the cytokinin levels in the transformed plants were not very high as in the case of tumor tissue. These low cytokinin levels could be because of the absence of regulatory effect of T-DNA encoded auxin and the modulator protein in the *Agrobacterium* strain used . The observed difference in the cytokinin values could be due to the absence of modulator gene which enhances the activity of the genes involved in auxin and cytokinin biosynthesis (Spanier *et al* .,1989). Further the effect of site of integration of the transformed T-DNA in the plant nucleus could be one of the reasons for the variability found in the cytokinin content of the different lines.

Yet another observation was that the pot grown plants had lower endogenous cytokinins than the corresponding plants grown in vitro (Table- 7). Ma *et al* ., (1995) observed similar reduction in the field grown plants expressing Glucuronidase, where *gus*A was expressed under native *ipt* promoter. These environmental factors which are quite different in pot grown plants may affect the level of expression. The other possibility is enhanced metabolism of cytokinins under field conditions.

## Characterisation of the pot grown *ipt* transformants

Few plants, which were successfully rooted *in vitro*, were hardened and were transferred to pots for further characterisation of these plants under green house conditions. The cytokinin over producing plants (Cyt1-5) were dark green with broad leaves, had thick stem with increased node number (Plate- 19). The p-CHS-*ipt* tobacco plants, developed by Wang *et al* .,(1997) where in the *ipt* was under the control of chalcone synthase promoter, also exhibited similar characters. The authors attribute this phenomenon to increased cell division and cell enlargement.

The transgenic plants developed in our study were classified as wilty, slightly wilty and never wilty, based on the relative extent of wilting symptoms exhibited by the plants. The wilting symptoms exhibited by these plants could be due to higher transpiration because of higher stomatal conductance (Table- 8). Wang *et al* .,.,(1997) also reported enhanced transpiration in p-CHS-*ipt* plants due to decreased diffusive resistance. In our study though *ipt* was expressed under native promoter the plants exhibited characters similar to the ipt transgenic plants developed using SAG promoter (Gan and Amasino., 1995) or under chalcone synthase promoter(Wang *et al* ., 1997). Though the *ipt* transformants obtained were with the native constitutive promoter, only the plants with low expression were selected since, they showed fewer abnormalities. The endogenous cytokinin levels of these *ipt* plants were also low. Our result indicate that the promoter with low constitutive activity under *ex vitro* conditions is desirable, as the cytokinin levels would be within permissible levels and bring about the formative effects at low concentrations, which is the main criteria for any hormone.

### Analysis of *ipt* plants for growth and physiological characters

Amongst the physiological traits the gas exchange parameters and leaf senescence were analyzed in the transformed plants differing in cytokinin levels.

### Photosynthetic efficiency

In general all *ipt* plants showed higher "A" compared to wild type plants (Table- 8 ). However, considerable variation amongst transformed plants was recorded. One of the transgenic plants, wilty, showed 27% increase in"A' in top leaves. Such an increase in "A" has also been reported earlier by Catsky, *et al* ., (1993 : 1996) in transgenic *ipt* tobacco and Sugar beet with external application of BA. In our study even the middle and bottom leaves also showed a similar increase in "A" More recently the changes in 'A' in tobacco was studied by Gan and Amasino., (1995), however in their experiments *ipt* was expressed on SAG promoter which results in enhanced amount of cytokinins only in aged leaves but not in young leaves. Therefore in their study enhanced "A" in ipt transformants was seen only in the lower leaves. In our experiments enhanced "A" was seen even in the young and old leaves. It is rightly so because *ipt* was constitutively expressed on its own promoter.

Photosynthetic carbon gain is predominantly determined by $CO_2$ diffusive processes and intrinsic carbon fixation efficiency of chloroplasts (mesophyll efficiency). At the present ambient $CO_2$ levels, the substrate $CO_2$ is a constraint for most of the C3 plants . This signifies the importance of diffusive processes regulated by stomata. Under non stress and optimum light conditions assimilation rate is related to variations in the stomatal conductances. Any change in the **gs** therefore may have direct effect in enhancing photosynthetic rate. However, increase in the substrate by high '**gs**' can increase "A" only when the carboxylation efficiency and subsequent carbon reduction enzymes are efficient. Therefore, not only high gs but also high mesophyll efficiency , alone will increase net assimilation rate. In view of this to find out reasons for higher "A" in the transgenic plants in addition to "A", Ci and gs also Ci/gs, an indirect reflection of mesophyll efficiency (Sheshshayee *et al* .,1996; Krishna Prasad *et al* .,1996) was determined.

With concomitant increase in "A" there was increase in '**gs** 'of the *ipt* transgenic plants. It is well known that cytokinins have a direct effect on stomatal movement and enhances the gs. In addition to **gs**, the Ci was low in transformed plants and Ci/**gs** was also low. This is an indirect reflection of enhanced mesophyll efficiency of the transformed plants. In a similar study, Catsky *et al* ., ( 1993 and 1996) have shown increase in "A" with no change in **gs** either in transgenic system or in the plants with exogenously supplied cytokinins. These results indirectly confirm increase in mesophyll efficiency with cytokinins. From the fore going discussion, it could be inferred that, in the transformed plants increase "A" was because of increase in **gs** as well as mesophyll efficiency. Further the increase in mesophyll efficiency could be due to increased activity of RuBisCO. Kirichenko *et al* ,(1995) observed an increase in activity of RuBisCO in the field grown Wheat, sprayed with BAP and TDZ.

### Senescence pattern in *ipt* lines

The relative senescence of lower leaves was examined in cyt1 line , which intrinsically had high growth rate. In this transgenic plant significantly more chlorophyll content was seen only in the lower leaves and in the middle leaves it was marginally less (Table-10). These results clearly elucidate that over expression of cytokinins retards senescence,and by this approach desirable phenotypes can be obtained. Detailed analysis of these plants is in progress, where in the cytokinin levels in the lower leaves and other physiological traits like protein turnover rates, maintenance of nitrate reductase activity etc. are being quantified.

Yet another factor that is closely associated with higher reduced senescence was maintenance of higher photosynthetic carbon assimilation . The *ipt* transformants had 27% higher 'A' further signifying the role of cytokinins in maintenance of metabolic activity by retarding senescence.

The role of cytokinins in retarding senescence has been well documented in excised leaf discs. In recent elegant experiment by Gan and Amasino (1995) it was unequivocally demonstrated that senescence of lower leaves retarded by expressing ipt on SAG12 promoter which triggers expression in senescing leaves.

The most striking and interesting aspect of the present study is that even by transferring *ipt* with its native promoter and subsequently selecting desirable putative transformants phenotypically superior transformants can be identified. Since the *ex vitro* cytokinin levels in these plants was not high and expression was uniform in all plant parts including young and old tissues. These transformed plants will form unique experimental material in understanding the role of cytokinins in several growth processes.

## Growth studies in *ipt* lines

The transgenic ipt plants had more number of leaves with enhanced leaf area and this was more pronounced in the cyt1 plant which continued to grow 60cm taller than the corresponding wild type plant (Plate-20). Though in general the difference in plant height of the Wilty, Slightly Wilty and Never Wilty types was not significantly different from that of the wild type plant the *ipt* tramsformants had more number of leaves by virtue of increase in number of nodes. The basal stem circumference was also more in all the *ipt* transsformants when compared to wild type plants. Li *et al* . 1995), observed an increase in the plant height of the *Nicotiana plumbiginifolia* plants expressing *ipt* on wound inducible promoter and they also observed enhanced leaf number and leaf area only in the *Nicotiana tabaccum* plants. The general increase in leaf number could be due to the increase in the duration of vegetative growth of the plants. Flowering was delayed by one week in the transformed plants when compared to wild type plants. Wang *et al* . (1997) found similar flowering behavior of the transgenic *chs-ipt* plants. However SAG-*ipt* tobacco plants developed by Gan and Amasino (1995) did not exhibit any delay in flowering.

The transgenic plants exhibited enhanced total pod weight per plant (Table-11) though there was decrease in pod number, some of the *ipt* transformed plants maintained higher pod weight compared to wild type and transgenic *npt gus* plants. This observed increase in pod weight could be due to enhanced assimilate mobilization to the developing sinks. Martineu *et al* .(1995) working with ipt transformed tomatoes have reported, an increase in total soluble solids in the fruits which is a reflection of enhanced assimilate mobilization to the sinks.

The data on growth in terms of biomass was not taken in these plants since these were continued till maturity to obtain seeds. However the observed increase in several parameters like leaf number, leaf area, stem circumference and assimilation rate which showed increase in these ipt transformed plants might have contributed to the enhanced biomass in these plants. These parameters are directly associated with Crop Growth Rate. Further analysis of these plants on several of the physiological parameters is needed.

From the fore going discussion the following inferences can be drawn. The *ipt* from Ach5 strain still is an useful construct for plant transformation. This construct on its own promoter can be successfully used to obtain transgenic plants. Though many putative transformants developed in vitro were abnormal due to enhanced expression of *ipt*, which could be due to constitutive nature of the promoter. This was probably one of the major reasons for shyrooting . Our initial interaction studies with auxin clearly demonstrates that the decrease in rooting efficiency could be possibly due to enhanced cytokinin induced ethylene production. If it is so this aspect could be over come by manipulating ethylene levels and its action by using suitable ethylene inhibitors *in vitro.*

Yet another characteristic feature of this study is larger variation in the endogenous cytokinin levels in the transformants. It was possible in the present study to identify putative transformants with normal phenotype (later confirmed by Southern and PCR analysis). These plants when established in green house conditions showed varying levels of cytokinins and this phenomenon was phenotypically expressed with respect to wilting symptoms in mid day, and hence we classified them as Wilty, Slightly wilty and Never wilty. Yet another characteristic feature of these plants has been higher photosynthetic efficiency and increased gs and ci/gs further suggests that the observed 'A' was both by increase in diffusive characters and mesophyll efficiency. The higher photosynthetic efficiency in lower leaves and chlorophyll content indicates that the senescence is delayed in lower leaves. The enhanced leaf size and inflorescence characters also suggest the direct and indirect role of cytokinins. These aspects needs in-depth analysis in the subsequent progenies to be obtained from these transforamnts.

It is one of the few studies where stable integration of *ipt* on its own promoter has been demonstrated and some of the transformants showed normal phenotype with enhanced growth rates. These putative transformants with different cytokinin levels could form excellent material for future studies to understand the role of cytokinins in several physiological processes under normal and stress full environments.

The $T_1$ seedlings developed from the seeds of $T_0$ plants showed cytokinin associated decrease in root growth. The cytokinin lines had higher shoot to root ratio (Table-15) than the wild type plants and this further proves the inhibitory action of cytokinins on root growth. This could be due to the enhanced ethylene, which decreases the root growth.

# SUMMARY

# VI SUMMARY

Recombinant DNA approaches have led to the development of transgenic plants with altered metabolism and has become powerful tools in understanding the relevance of the catalytic proteins or their products.

Growth hormones, are one group of such target molecules, that are attempted to be altered by the transgenic approach with the aim to elucidate their complex responses in plants. In spite of phenomenal progress, the complex physiological responses brought about by these growth hormones is still not completely understood. From this context transgenic plant with varying levels of endogenous growth hormones will form an excellent system to understand the formative effects of these hormones.

Cytokinin is an important growth hormone, that has been implicated in several growth processes. However, its role in regulating some of the plant processes like *insitu* senescence, leaf expansion and stomatal regulation is still to be unequivocally proven. Keeping this in view, the major emphasis in this study has been to develop transgenic plants overproducing cytokinins.

The rate-limiting step in cytokinin biosynthesis is conversion of isopentenyl pyrophosphate to isopentenyl adenine, and this reaction is catalyzed by ISOPENTENYL TRANSFERASE. This enzyme, though not very well characterized in plants, is fairly well characterized in *Agrobacterium*. It has been cloned and has been the only source for overexpressing *ipt* in plants. Often, it is overexpressed in plants either on constitutive or inducible promoter. We preferred to express *ipt* with native promoter since it gives more stable transformants without much molecular alterations.

The main approach therefore has been initially to standardize the regeneration and transformation protocols in a local tobacco cultivar FCV special. Subsequently, attempts were made to clone *ipt* to a suitable binary vector and to obtain transformed plants overexpressing *ipt* and therefore with different endogenous levels of cytokinins. These transformed lines were analysed for a few physiological processes.

Efficient regeneration protocol for Fcv special cultivar of tobacco was found to be MS medium supplemented with 2.0mg/L BA and 0.2mg/L, NAA, which gave a maximum of 132 shootlets per explant. The transformation protocol was standardized using the construct pKIWI105, with *gus*A and *npt*II, mobilized to EHA105 *Agrobacterium* strain and on an average 10 shootlets per explant were obtained on the kanamycin selection media. The putative transformants were later confirmed for stable integration of the foreign gene by histochemical assay for glucuronidase activity both in $T_0$ and $T_1$ plants .

The source of *ipt* was Ach5 octopine strain. A 1.5kb *EcoR*I- *Hind*III *ipt* fragment from pRZ1 was cloned to *EcoR*I-*Hind*III site of pBI101, after releasing a 3kb *EcoR*I-*Hind*III fragment containing the promoterless gus fusion cassette. The binary vector pBI-ipt thus developed was mobilized to *Agrobacterium* strain EHA105. Transformation studies were conducted in FCV special cultivar of tobacco and putative *ipt* transformants were obtained on kanamycin media. The transformation efficiency of EHA105(pBI-*ipt*) was low when compared with EHA105(pKIWI105) though same protocol was used. Further, the integration of the ipt gene was confirmed by PCR and Southern analysis.

Low transformation efficiency which was observed with EHA105(pBI101-ipt) as compared to EHA105(pKIWI105) leaf disc transformation could be due to many factors influencing Agrobacterium mediated transformation. Results of our experiments and the results obtained in studies conducted by several authors suggests that variability in transformation efficiency could be because of many factors like Agrobacterium strain, the binary vector, its size and structure , explant, effect of transgene product etc. In addition to differences in the plasmid structure, the production of enhanced levels of cytokinins due to *ipt* seems to be the main reason for low transformation efficiency in our study.

Considerable variability was noticed among several transformants obtained in transformation experiments. The putative *ipt* transformants were generally short statured, had numerous tiny severe to moderately curled leaves or leaf like structures, had bulged stem, which did not have any visible roots. The wild type plants and the pKIWI105 transformed plants did not exhibit such abnormalities.

To achieve root initiation in *ipt* transformants, several experiments involving different concentrations of IBA were tried and few plants showed root initiation on MS medium supplemented with either 0.6 or 0.8 mg per liter IBA. Finally only few plants were obtained which had prominent roots, and the root morphology varied, in characters like, root length, thickness, branching etc.

The transzeatin riboside content in these putative transformants, which were randomly analyzed, varied from 196-3756 pM/g fresh weight, while, the wild type plants had only 11.8 pM/g fresh weight . Another revealing observation about the *ipt* plants, grown under green house condition was that the endogenous cytokinin content varied from 14.9-270 pM/g fresh weight. The wild type plants grown under similar conditions had 4.81 pM/g fresh weight and it was not significantly different from that of the values obtained under in vitro conditions.

The *ipt* transformants (Cyt 1-5) which were maintained under green house conditions exhibited visible wilting systems even with sufficient supply of water. Based on the relative extent of wilting characteristics the *ipt* transformants were classified as NEVER WILTY, SLIGHTLY WILTY AND WILTY. These plants were maintained under sufficiently watered condition and were grown till maturity to obtain T0 seeds.

The gas exchange studies of the different *ipt* transformants revealed that, the *ipt* transformants had intrinsically high photosynthetic efficiency and stomatal conductance. Further, high cytokinin levels in transformed plants resulted in maintenance of high ‘gs’ and 'A' even in leaves of lower canopy. The corresponding leaves in wild type plants failed to show this character. Further, Ci/gs (internal $CO_2$ concentration to **gs** ratio) was, less in *ipt* transformants when compared to wild type plants, suggests that the *ipt* plants had higher mesophyll efficiency. An additional interesting aspect of the *ipt* transformants developed in the present study was that, these plants had reduced lower canopy senescence. This was previously achieved only in the system where *ipt* was expressed under the control of the Senescence activated promoter. Our results suggest that it would be beneficial if the plants produce cytokinins constitutively at a slightly enhanced rate and the plants can perform better by retaining the greener leaves at lower canopy and would be photosynthetically active for a longer period during their growth. Though, there are few contradicting reports about the promotory role of cytokinins on gas exchange parameters in transgenic *ipt* tobacco system where *ipt* was expressed under the control of RubisCo small sub unit promoter, our studies show that cytokinins do help in this carbon assimilation process by indirectly affecting different processes, which needs thorough investigation in the progeny populations.

With regards to growth attributes, the *ipt* transformants had higher total leaf area per plant (due to enhanced leaf size and number) and there was increase in stem girth. The flowers were dark pink and showed delayed senescence. This would be one of the important characters, which has commercial importance, especially in floriculture industry and needs to be tested in crops of commercial importance.

Finally it can be concluded that, phenotypically normal transgenic plants were developed in this investigation, with desired physiological traits. The *ipt* when expressed on native promoter seems to be ideal, as phenotypically normal plants with enhanced cytokinin could be recovered. Though, *ipt* transformants showed reduced tendency to root on normal rooting medium, by subjecting large number of explants to various concentrations of auxins one could get rooted plants. The *ipt* plants showed wide variability in cytokinin content and this could be exploited to develop plants with required amount of endogenous cytokinins. The enhanced photosynthetic efficiency of the *ipt* transformants suggests the promotory role of cytokinins in this process. This character can be exploited for developing lines with enhanced crop growth rates and yields. Some of the cytokinin over producing transformants, showed better growth attributes, like, higher leaf number and total leaf area and these plants showed reduced lower leaf senescence. These plants produced more number of dark colored flowers with enhanced vase life and ultimately there was increase in capsule number and capsule weight though there was variability among the transgenics. Finally it could be concluded that phenotypically normal plants with interesting physiological and phenotypic characters could be obtained even when *ipt* is expressed on native promoter. Initial experiments suggest, the direct role of cytokinins in several physiological processes *in situ*.

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# APPENDIX

# Appendix

## COMPOSITION OF MS MEDIA (Murashige and Skoog, 1962)

| Ingredients | Salt concentration stock solution (g/L) | Aliquots taken for 1L of medium (mL) | Final concentration in 1L of medium (mg/L) | Group |
|---|---|---|---|---|
| **INORGANIC SALTS** | | | | |
| $NH_4NO_3$ | 66.00 | | 1650 | |
| $KNO_3$ | 76.00 | | 1900 | |
| $MgSO_4.7H_2O$ | 14.80 | | 370 | |
| $KH_2PO_4$ | 6.80 | 25 | 170 | I |
| $CaCl_2$ | 4.40 | 10 | 440 | II |
| $Na_2.EDTA$ | 7.45 | | 37.2 | |
| $FeSO_4.7H_2O$ | 5.57 | 5 | 27.2 | III |
| **MICRONUTRIENTS** | | | | |
| $H_3BO_3$ | 0.62 | | 6.2 | |
| $ZnSO_4.4H_2O$ | 0.86 | | 8.6 | |
| $MnSO_4.4H_2O$ | 2.23 | | 22.3 | |
| $Na_2MoO_4.2H_2O$ | 0.025 | | 0.25 | |
| KI | 0.083 | | 83.3 | |
| $CuSO_4.5H_2O$ | 0.0025 | | 0.025 | |
| $CoCl_2.6H_2O$ | 0.0025 | 10 | 0.025 | IV |
| **ORGANIC SALTS** | | | | |
| Glycine | 0.20 | | 2.00 | |
| Myoinositol | 10.00 | | 100.00 | |
| Thiamine HCl | 0.01 | | 0.10 | |
| Pyridoxine HCl | 0.05 | | 0.50 | |
| Nicotinic acid | 0.05 | | 0.50 | |
| Biotin | 0.005 | 10 | 0.05 | V |
| Sucrose | | | 30000.00 | |
| Agar | | | 8000.00 | |
| PH | | | 5.8 | |

CONSTITUENTS REQUIRED FOR **LB** AND **AB** MEDIUM PER LITRE

## A: LB – Medium

Bacto tryptone – 10 g
Bacto – yeast extract – 5 g
NaCl -- 10 g
PH to be maintained -- 7.5

(to prepare solid medium to the solution 15 g of Bacto agar should be added)

## B: AB – Medium

AB buffer - Solution I
$K_2HPO_4$ - 60 g
$NaH_2PO_4$ - 20 g
pH should be around 7.0

AB salts - Solution II
$NH_4Cl$ - 20 g
$MgSO_4,7H_2O$ - 6 g
KCl - 3 g
$CaCl_2$ - 3 g
$FeSO_4,7H_2O$ - 50 g

(100 ml of both these solutions are autoclaved separately and stored)

## Commonly used Electrophorosis and Transfer buffers:

***TAE (Tris Acetate Buffer)*** - 50X- 242g tris base; 57.1 ml glacial acetic acid; 100ml 0.5M EDTA (pH-8) – volume made up to 1L

***TE (Tris-EDTA)*** - pH 8.0 : 10mM Tris-Cl (pH 8.0)
1mM EDTA (pH 8.0)

**20 X SSC**:

175.3 g NaCl; Sodium citrate 88.2 mg , volume made up to 1L, pH adjusted to 7.0 with 10N NaOH.