PHYSIOLOGY AND SECONDARY METABOLITE PRODUCTION IN GENETICALLY TRANSFORMED BRAHMI (*Bacopa monnieri* L.WETTST.) WITH CYTOKININ SYNTHESIZING ISOPENTENYL TRANSFERASE (*ipt*) GENE.

VIGHNESHA

Thesis submitted in partial fulfillment of the requirement for the degree of

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DEPARTMENT OF PLANT PHYSIOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM 695522
KERALA, INDIA

DECLARATION

I hereby declare that this thesis entitled "Physiology and secondary metabolite production in genetically transformed brahmi (*Bacopa monnieri* L.Wettst.) with cytokinin synthesizing isopentenyl transferase (*ipt*) gene." is a bonafide record of research work done by me during the course of research and that it has not previously formed for the basis of award of any degree, diploma, associateship, fellowship or other similar title of any other university or society.

Vellayani
15/01/ -2008

VIGHNESHA
(2005-11-132)

CERTIFICATE

Certified that this thesis entitled "Physiology and secondary metabolite production in genetically transformed brahmi (*Bacopa monnieri* L.Wettst.) with cytokinin synthesizing isopentenyl transferase (*ipt*) gene." is a bonafide record of research work done independently by Mr. Vighnesha (2005-11-132) under my guidance and supervision and that it has not previously formed the basis for award of any degree, fellowship or associateship to him.

Vellayani
15-1-08

Dr. ROY STEPHEN
(Chairman, Advisory committee)
Assistant Professor & Head i/c,
Department of Plant Physiology,
College of Agriculture, Vellayani,
Thiruvananthapuram-695 522

APPROVED BY

CHAIRMAN

Dr. ROY STEPHEN
Assistant Professor &Head i/c
Department of Plant Physiology,
College of Agriculture, Vellayani,
Thiruvananthapuram-695 522

MEMBERS

Dr. R.V. MANJU
Assistant Professor,
Department of Plant Physiology,
College of Agriculture, Vellayani,
Thiruvananthapuram-695 522

Dr. K. RAJMOHAN
Professor and Head,
Department of Plant Biotechnology,
College of Agriculture, Vellayani,
Thiruvananthapuram-695 522

Dr. M.G. PURUSHOTHAMA
Scientist 'C'
Plant Molecular Biology Division,
Rajiv Gandhi Centre for Biotechnology
Jagathi, Thiruvananthapuram 695 014

EXTERNAL EXAMINER

Dr. N. NATARAJA KARABA
Assistant Professor (Sr. Scale),
Department of Crop Physiology
University of Agricultural Sciences,
GKVK, Bangalore -560 065

10/1/08

Dedicated

To

My Parents, Teachers

&

My Brothers

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LIST OF ABBREVIATIONS

2,4-D	D-2,4-dichlorophenoxyacetic acid
ADP	Adenosine diphosphate
AHK	*Arabidopsis thaliana* histidine kinase
AMP	Adenosine monophosphate
ARR	*Arabidopsis thaliana* Response Regulator
ATP	Adenosine triphosphate
BAP	Benzyl adenine purine
BHT	Butylated hydoxy toulene
BSA	Bovine serum albumin
CaMV 35S	Cauliflower mosaic virus 35 S promoter
CBB	Coomassie brilliant blue
cDNA	Complementary DNA
Chl	Chlorophyll
Co A	Coenzyme A
DEPC	Diethoxy pyrocarbonate
DMAPP	dimethyl allyl pyrophosphate
DMSO	Di methyl sulfoxide
DNA	Deoxy ribonucleic acid
DZ	Dihydrozeatin
ELISA	Enzyme Linked Immuno Sorbant Assay
et al.	And others
FW	fresh weight
GA	Gibberellin
gfp	Green fuorescent protein
gus	ß-glucuronidase
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
iP	N^6-(Δ^2-isopentenyl)adenin
iPDP	Isopentenyl 5-diphosphate
iPMP	isopentenyladenosine 5-monophosphate
IPT	isopentenyl transferase
ipt	Gene coding for isopentenyl transferase
iPTP	isopentenyl 5-triphosphate
MDA	Malondialdehyde
MS	Mass spectrophotometer
NAA	Napthyl acetic acid
nptII	Neomycin phosphotransferase
PCR	Polymerase Chain Reaction
POX	Peroxidase
RT – PCR	Reverse Transcriptase Polymerase Chain Reaction
RWC	Relative water content

Introduction

1. INTRODUCTION

Plant secondary metabolites represent an enormous value from economical point of view since most of them are used as specialty chemicals such as drugs, flavors, fragrances, insecticides and dyes. They play a major role in the adaptation of plants to their environment. These substances are also known as *secondary products*, or *natural products* (Taiz and Zeiger. 2006). The dictionary of natural products has about 85,000 entries for secondary metabolites (Verpoorte et al., 2000).

Medicinal plants are the most important source of life saving drugs for the majority of the world's population. Human beings have been utilizing plants for basic preventive and curative healthcare since time immemorial. The world health organization has estimated that more than 80 per cent of the world's population in developing countries depends primarily on herbal medicine for basic health care needs. Recent estimates suggest that over 50000 plants have known medicinal applications in various cultures and countries. Medicinal plants have played significant role in many ancient traditional systems of medicine in Asia such as the Ayurveda, Unani and Chinese medicine. Increasing demand for medicinal plants during the past 20 years has brought surging market and production opportunities for the plant species.

Past decade has witnessed a tremendous resurgence in the interest and use of medicinal plant products worldwide. International market for medicinal plants is over US$ 60 billion per year, which is growing at the rate of seven percent a year. India is exporting herbal material to the tune of Rs. 446.3 crores (Ghosh, 2000). The projected export marketing of herbal medicine is worth US$ 5 billion per year by 2010 in Kerala (Kumar, 2003).

Yield and quality of medicinal plants can be improved by biotechnological tools by gene alteration, introduction of a new gene, or hormonal regulation. Rapid progress in the area of crop biotechnology has enabled the development of efficient regeneration and suitable genetic transformation protocol which in turn could be used for enhancement of

their secondary metabolite content. Genetic transformation has been successful for various medicinal plants. Efficient transgene delivery system based on *Agrobacterium tumefaciens* are well established for several species like *Taxus spp, Echinacea purpurea, Scrophularia, Digitalis lanata, Thalictrum, Artemisia annua, Bacopa monnieri* and *Centella asiatica.*

Although the primary target for trait manipulation in medicinal plants is to improve the active compounds, the basic agronomic characters related to uniformity, stability, growth and development and resistance to biotic and abiotic stresses must also be improved. Metabolic engineering is emerging as one of the important approach to improve and modify secondary metabolite contents of medicinal plants. A major line of research has been to identify enzymes of a metabolic pathway and then manipulate them to provide better control of that pathway. Recently some examples of successful genetic manipulation of secondary metabolite pathway through metabolic engineering for increased metabolite content and exploitation of the plant as bioreactor for the production of natural or recombinant secondary metabolites of the commercial interest have been developed.

Bacopa monnieri, a member of the Scrophulariaceae family is also referred to as *Bacopa monniera, Herpestis monniera*, water hyssop etc. It is a small creeping herb with numerous branches. In India and tropics it grows naturally in wet soil, shallow water and marshes. It has been used in the Ayurvedic system of medicine for centuries. Traditionally it was used as a brain tonic to enhance memory development, learning, and concentration and to provide relief to patients with anxiety or epileptic disorders. Compounds responsible for the pharmacological effects of brahmi (bacopa) includes saponins, alkaloids and sterols. The constituents responsible for bacopa's cognitive effects are bacoside A and bacoside B. Recent research has focused primarily on bacopa's cognitive- enhancing effects, specifically memory, learning and concentration. Bacopa's antioxidant properties may offer protection from free radical damage in cardiac vascular disease and certain types of cancer.

Plant metabolism is generally affected by plant hormones, which act as messengers on developmental or environmental cues. Cytokinins are plant hormones promoting cell division and differentiation. They are involved in the regulation of a large number of physiological characters like chloroplast development, delaying senescence, shoot initiation, growth of lateral buds, leaf expansion, etc. Among the other functions, cytokinins can regulate some secondary metabolite pathways, including anthocyanin synthesis in maize (Piazza et al., 2002) and terpenoid indole alkaloid production in *Catharanthus roseus* suspension cells (Decendit et al., 1992; Yahia et al., 1998)

The major step in cytokinin biosynthesis involves the conversion of isopentenyl pyrophosphate to isopentenyl adenosine, which is mediated by the rate limiting enzyme, isopentenyl transferase (IPT). Much of the information about isopentenyl transferase has been obtained from the studies on *Agrobacterium tumefaciens* induced tumor tissues (Chilton et al., 1977). The *ipt* gene was isolated and characterized from plants only recently (Kakimoto, 2001: Takei et al., 2001a&b) hence genetic manipulation of cytokinin levels in plants were done using *ipt* gene from *Agrobacterium.*

The production of plant secondary metabolites is regulated depending on the expression of plant genome under developmental control or as a result of environmental factors such as biotic or abiotic stresses, pests and diseases etc. Exogenous application and tissue culture studies with different plant hormones for the metabolite formation and accumulation has been well established for many systems. Recently, a few reports were found about the relationship between the changes of endogenous plant hormones content and the production of secondary metabolites.

At this juncture, it was proposed to develop transgenic plants over expressing ipt gene in *Bacopa monnieri* to address the following objectives,

1. To over express *ipt* gene in *Bacopa monnieri* through *Agrobacterium* mediated transformation.
2. To regenerate the transformed plants through tissue culture
3. Analyzing the influence of overexpression of *ipt* gene on growth, physiology and secondary metabolite production.

Review of Literature

2. REVIEW OF LITERATURE

The plant secondary metabolites have high economical and pharmacological importance. Biosynthesis of metabolites although, controlled genetically, is also affected strongly by environmental factors. Modification of endogenous pathways to increase flux towards particular desirable molecules can be done by metabolic engineering. In some cases the aim is to enhance the production of a natural product, where as in others it is to synthesize a novel compound or macro molecule (Teresa and Paul. 2004). In this context a study was undertaken to see the overexpression of *ipt* gene on growth and secondary metabolite production in a medicinally important plant, *Bacopa monnieri*. Reviews related to cytokinins and its interaction with secondary metabolite production are discussed in this chapter.

2.1 *Bacopa monnieri*

Bacopa monnieri (also sometimes referred to as *Bacopa monniera* or *Herpestis monniera*) is a medicinal plant used for centuries in the Ayurvedic system of medicine. More recently it has gained popularity in western countries as a "Brain tonic" capable of improving mental ability and memory.

Bacopa is known as Brahmi in both Sanskrit and Hindi (Anonymous. 1998), Nirbrahmi in Kannada and Malayalam, Nirpirani, Piramiyapundu in Tamil and Sambranicettu in Telgu. The description of Brahmi appears in Hindu Vedas dating back to about 5000BC. Since about 500AD, Brahmi has been used in Ayurvedic preparations as a specific agent to develop and improve memory and concentration (Mukharjee and Day. 1966). It has been frequently mentioned in classical Indian texts since about 800 BC (Singh and Dhawan. 1997).

Botanically it is a glabrous somewhat succulent, creeping herb. It roots at the nodes, with numerous prostrate branches, each 10-30 cm long. Leaves are oblong to spathulate, sessile, decussate, rather fleshy, entire, punctuate, obtuse. Flowers are axillary, solitary, peduncles often much longer and deflexed in fruiting stage. Corolla are about one centimeter long, pale, lobes 5, oblong, obtuse, subequal, tips purple. Capsule is ovoid-acuminate or slightly beaked at the apex. Seeds are oblong, truncate, longitudinally

ribbed with transverse striations in between the ribs. It is propagated by seeds, stem cuttings and also *in-vitro* propagation technique.

Although bacopa is an important herbal drug in Ayurvedic medicine, some confusion exists regarding the botanical identity of bacopa. The problem arises because not only *Bacopa monniera*, but also *Centella asiatica*, is known as Brahmi.

According to Sivarajan (1994), the classical Ayurvedic texts provide scant botanical information about bacopa, stating that the leaves and flowers of the plant resemble the eyes of fish, a description insufficient for the determination of the drugs botanical identity. According to him, Charaka considered both drugs to promote general mental ability, but states that bacopa is also used for the treatment of insanity and epilepsy, while Mandukaparni helps to regain mental health. Further more, bacopa is said to promote fertility and sustain implantation and pregnancy, while Mandukaparni is said to have the opposite effect.

2.1.1 Medicinal role

Bacopa belongs to a group of medicinal plants classified as Medhyarasayana in Ayurveda; these are nervine tonics used to promote mental health and improve memory and intellect (Bhattacharya and Ghosal. 1998).

Bacopa has demonstrated anxiolytic, relaxing bronchodilatory, cognition-enhancing, antioxidant, anticancer, immuno modulating and anti-inflammatory effects in pharmacological studies (Dar and Channa. 1997, Singh et al., 1988, Tripathi et al., 1996, Elangovan et al., 1995, Dahankar and Thatte. 1997, Jain et al., 1994, Kidd., 1999)

2.1.2 Active constituents

Bacopa contains different types of saponins. Saponins are terpene glycosides with detergent properties. When agitated with water, saponins form stable soapy foam. The name saponin is derived from *Saponaria* (Soapwort). The herb contains saponins, monnierin, hersaponin, bacoside –A (Fig. 1) and bacoside –B. Monnierin, on hydrolysis, gave glucose, arabinose and aglycone whereas, bacoside A and B which are optical isomers (Rastogi., 1990) gave glucose, arabinose and bacogenines A, A2, A3 and A4. Bacogenines A1 and A2 are epimers, and A4 is an ebelin lactone. Smith-de Mayo degradation of bacoside A gave jujubogenin and pseudojujubogenin. Bacosides A and B

are regarded as the major active principles (Bone., 1996, Rastogi et al., 1994). The bacoside A content is about 2.5- 3.0 % on dry weight basis (Anonymous. 1998).

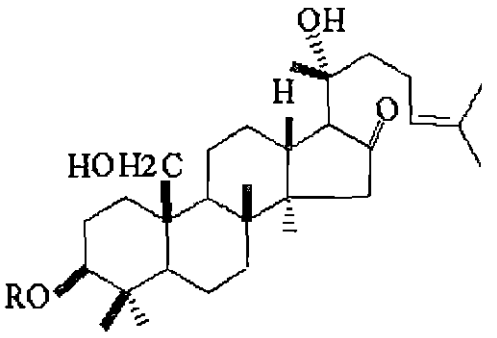


Fig.1. Chemical structure of bacoside-A

Other constituents present in this plant are D-mannitol, betulic acid, b –sitosterol, stigma-sterol and its esters, heptacosine, octacosane, nonacosane, triacontane, hentriacontane, dotriacontane, nicotine, 3-formyl-4-hydroxy-2H-pyran, luteolin and its 7-glucoside. The presence of amino acids like, alanine, aspartic acid, glutamic acid and serine is also reported (Anonymous. 1998, Kapoor. 1990).

Expression of growth and bacoside A in response to seasonal variation in *Bacopa monnieri* accessions was studied by Shalini et al. (2001). They reported a wide variation in bacoside A content in different season. The bacoside content was 1.8-2.0 % in summer (February-May) season, 2.1-2.9 % in the rainy (June-September) season and 0.7-1.5 % in the winter (September-December) season. Effect of method of drying on the bacoside A content was studied by Gupta et al. (1998). They reported that exposure to 80°C in an oven for 30 min followed by drying at 37°C in oven for 4 days has yielded 3.78 % bacoside A.

2.1.3 Bacoside estimation

Bacoside can be quantified mainly by two ways viz colorimetric and chromatographic techniques. Spectrophotometric methods were developed based on the hydrolysis of bacosides to an aglycone that has an absorption maximum at 278 nm (Pal et al., 1992. Singh et al., 1998). The major limitation of this method is that it lacks specificity and has interference from other hydrolyzed compounds.

High performance thin layer chromatography (HPTLC) method was developed for estimation of bacoside A in *Bacopa monnieri* plant extract and its formulations (Srikumar et al., 2004).

High performance liquid chromatographic (HPLC) methods were also developed for the quantification of bacosides in *Bacopa monnieri* extracts and formulations (Pal et al., 1998. Deepak et al., 2005). Ganzera et al. (2004) have reported a method for quantification of six saponins using gradient HPLC with PDA detector, but one of the major peaks obtained was not completely characterized. Deepak et al. (2005) determined bacoside A by HPLC which contained 4 compounds, but bacoside B was not considered. In another report bacosides were estimated by HPLC, coupled NMR, MS and bioassay methods (Renukappa et al., 1999). Murthy et al. (2006) developed a method for estimation of 12 Bacopa saponins in *Bacopa monnieri* extracts and formulation by HPLC method.

2.1.4 *In vitro* studies

Several *in vitro* studies for regeneration of *Bacopa monnieri* have been reported. Mohapatra and Rath. (2005) reported micro propagation for *Bacopa monnieri* on MS & B5 medium supplemented with BAP & NAA using leaf explants & nodal segments. Best result obtained with MS+BAP (2.0mg/l). Phytohormone self sufficiency for regeneration in the leaf & stem explants of *Bacopa monnieri* was reported by Shalini and Sushil. (1998). They obtained 2.6% of bacoside A in the MS media without growth regulators.

Srivastava and Rajani. (1999) reported multiple shoot regeneration and tissue culture studies on *Bacopa monnieri*. They found that the source of the explants as well as different gelling agent in the medium influence the shoot induction and eventual shoot growth. A mass *in vitro* propagation system for *Bacopa monnieri* has been developed by Vaibhav et al. (2001). A range of cytokinin has been investigated for multiple shoot induction with node, internode and leaf explants. Optimum adventitious shoot buds induction occurred at 6.8M thidiazuron. Tiwari et al. (1998) reported shoot regeneration and somatic embryogenesis from different explants of *Bacopa monnieri*. They cultured on MS basal medium supplemented with BA & Kinetin. Leaf explants gave the largest number of shoot buds.

2.2 Gene transfer methods in medicinal plants

The generally used gene transfer methods include *Agrobacterium* mediated transformation, particle bombardment, electroporation etc. Genes of interest, including marker or reporter genes, are vectored on short units of DNA called plasmids. The plasmids have various gene promoters and other sequences which affect the ability of the plant cell to transcribe the gene and to translate it into a protein product. Use of plasmid with inappropriate promoters and markers may mask the effectiveness of a specific gene transfer method (Kuehnle, 2000). The gene transfer method and the gene expression vector to be used must be compatible with the plant genotype and the tissue to be treated.

The *Agrobacterium* mediated gene transfer system requires physically injured plants cells that the bacteria can enter. The process also requires the regeneration of the explants into viable plant. Lievre et al. (2005) reported *Agrobacterium tumefaciens* mediated genetic transformation in *Ruta graveolens*. Transformation was obtained by co-cultivation of hypocotyls of 2-3 weeks old plants and *Agrobacterium tumefaciens* strain C58C1Rif^R containing a plasmid harboring neomycin phospotransferase and ß-glucuronidase encoding genes. Bae et al. (2005) reported an efficient transformation protocol for stable introduction of *hmgr* into *Taraxacum platycarpum* plants. The *Agrobacterium tumefaciens* strain EHA 105 containing binary vector, pCAMBIA 1301, with *gus* and *hmgr* genes, showed high transformation efficiency after 3-5 weeks of hygromycine selection.

Giri et al. (2001) used different strains of *Agrobacterium rhizogenes, viz.*, A_4, 15834, K_{599}, LBA9402, 9365 and 9340 for induction of hairy roots in *Artemisia annua* using shoot tip meristem as explant. Hairy root lines induced by strain 9365 were found to contain highest amount of artemisinin (0.23%). Lee et al. (2004) produced transformation of hairy roots from the seedlings of *Taraxacum platycarpum* by infection with *Agrobacterium rhizogenes* strain 15834. Transgenic plantlets showed considerable differences in their morphology compared to the corresponding non transgenic plants. The differences reflect the modification of morphological root characters by introduction of *rol* genes.

Although the success of *Agrobacterium* vectors was paramount, there are other methods of gene transfer also. Microprojectile mediated transformation is another method by which gene is transferred to plant system. Genetically transformed *Catharanthus roseus* plantlets were obtained after bombardment of nodal explants, which were then micropropagated, with DNA coated particles with green fluorescent protein (*gfp*) or ß-glucuronidase(*gus*) reporter genes. Histological studies showed that the gene insertion method proved effective with many cells and different tissues displaying the reporter gene signals, showing that gene expression were rather stable (Zarate et al.,1999)

Leo et al. (2000) reported an efficient transformation system for *Hyoscyamus muticus,* an important medicinal plant of Solanaceous family. They developed a system using a plasmid carrying the *npt*II and *gus* A genes, which was delivered into leaf explants by particle bombardment. Ten percent of bombarded leaf explants formed kanamycin resistant callus, from which putative transgenic plants were recovered. The *npt*II gene conferring kanamycin resistance was found to be incorporated into the genome of all transgenic plant screened. Hosokawa et al. (2000) produced transgenic gentian plants by particle bombardment of suspension-culture cells.

2.3 Metabolic pathway engineering in medicinal plants

Genetic transformation is a powerful tool for enhancing the productivity of novel secondary metabolites of limited yield. Direct manipulation of DNA sequences to alter gene expression in medicinal plants, is an area that is expanding. One approach is to identify the key enzymes of a metabolic pathway and then manipulate these enzymes to provide better control of that pathway. Another approach is to introduce new genes for the increased yield of secondary metabolites. Metabolically engineered medicinal plants will complement conventional breeding efforts for the production of quantitatively and qualitatively improved pharmacological properties. Charlwood and Pletsch (2002) reported that pathway engineering will lead to improvement of potential value in the breeding of medicinal plants.

Different strategies are aimed at enabling transgenic plants to synthesize novel products,

- Completion of partial pathways
- Amplification of regular pathways
- Blockage of competing pathways
- Interdiction of regular pathways
- Revising metabolic regulation
- Minimizing response cascade

A number of plant species including many medicinal plants have been successfully transformed with *Agrobacterium rhizogenes*. Transformed hairy roots mimic the biochemical machinery present and active in the normal roots (Chilton et al., 1982). Yun et al. (1992) increased the production of scopolamine in *Atropa belladona* from the naturally occurring chemical precursor hyocyamine by transferring the enzyme hyoscyamine 6ß- hydroxalase from *Hyoscyamus niger*. Plant transformation and genetic modification using *Agrobacterium rhizogenes*, can be used to boost the production of those secondary metabolites, which are naturally synthesized in roots. Cai et al. (1995) reported the establishment of hairy root culture system of *Artemisia annua* by infection with *Agrobacterium rhizogenes*.

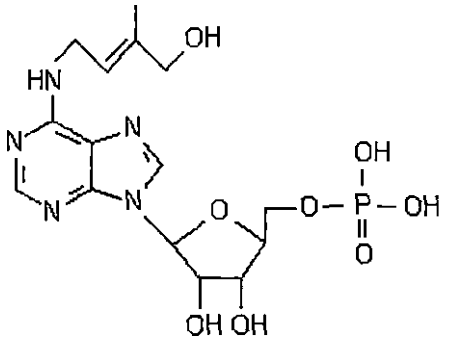
Pradel et al. (1997) developed a system for producing transformed plants from root explants of *Digitalis lanata*. They reported higher amounts of anthraquinones and flavanoids in the transformed hairy roots than in untransformed roots. Argolo et al. (2000) reported the regulation of solasodine production by *Agrobacterium rhizogenes* transformed roots of *Solanum aviculare*. Chen (2000) reported a three fold enhancement in production of the putative antimalarial, anticancer agent, artemisinin in transgenic *Artemisia annua* plants overexpressing farnesyl diphosphate synthase, the enzyme immediately preceding the first committed biosynthetic step. Souret et al. (2002) demonstrated that the transformed roots of *Artemisia annua* are superior to whole plants in terms of yield of the sesquiterpene artemisinin. Shi and Kintzios (2003) reported the genetic transformation of *Pueraria phaseoloides* with *Agrobacterium rhizogenes*. The content of puerarin in hairy roots reached a level of 1-2 mg g^{-1} dry weight

2.4 Cytokinin

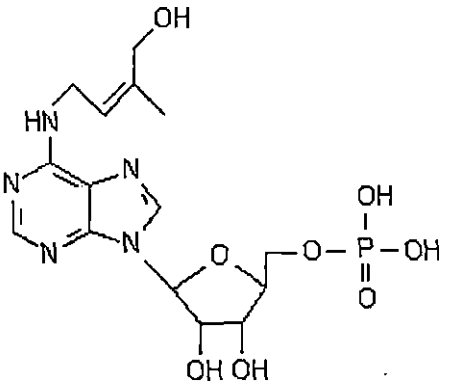
Cytokinins are adenin derivatives with an isoprenoid side chain and play an essentail role in plant development (Kakimoto, 2003). Their effects were first discovered through the use of coconut milk in tobacco callus pith culture in the 1940s by a scientist at the University of Wisconsin-Madison named Folke Skoog.

Zeatin is the most abundant naturally occuring cytokinin. In higher plants, zeatin occurs in both the *cis* and *trans* configurations and these forms can be interconverted by the enzyme zeatin isomerase. *Trans* form of zeatin is more active in biological assays. Apart from zeatin, N^6-(Δ^2-isopentenyl)adenin(iP) and Dihydrozeatin(DZ) were also found naturally in many plant and baterial species (Fig. 2). These amino purines differs from zeatin in nature of the side chain attached to the N^6 or in the attachment of a side chain to C^2 (Taiz and Zieger., 2006). Some plant tRNAs contain cis-zeatin as a hypermodified base. Cytokinins are not only confined to plant tRNAs but also they are part of certain tRNAs from all organisms, from bacteria to human.

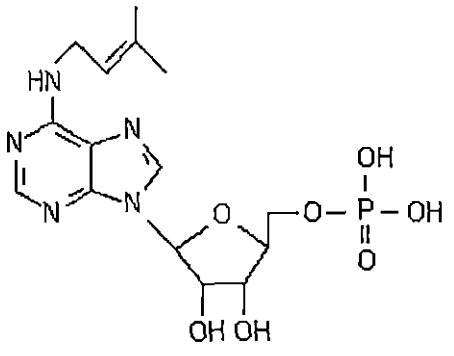
Cytokinins play a major role in different developmental and physiological processes in plants, such as cell division, regulation of root and shoot growth and branching, chloroplast development, leaf senescence, stress response and pathogen resistance (Mok et al., 2001) Additionally, it has been proposed that this class of plant hormones has a function in nutrient starvation and recovery response (Martin et al., 2000, Sakakibara et al., 1998)



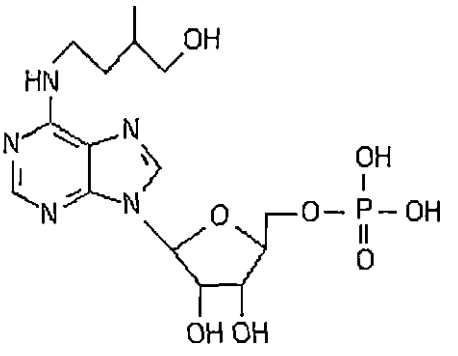
trans-zeatin riboside phosphate



cis-zeatin riboside phosphate



isopentenyladenosine phosphate



dihydrozeatin riboside phosphate

Fig. 2. Chemical structure of naturally occurring cytokinins

2.4.1 Biosynthesis

The biosynthetic pathway of cytokinin has been demonstrated in many of the species, *Dictyostelium discoideum, Agrobacterium tumefaciencs, Arabidopsis thaliana.* etc (Kakimoto., 2003). The first committed step in cytokinin biosynthesis is the transfer of the isopentenyl group of dimethyl allyl diphosphate (DMAPP) to an adenosine moiety. Enzyme that catalyzes this step is isopentenyl transferase (IPT). The plant and bacterial IPT enzymes differ in the adenosine substrate used. The plant enzyme utilizes both Adenosine Diphosphate (ADP) and Adenosine Triphosphate (ATP) and bacterial enzyme utilizes only Adenosine Monophosphate (AMP) (Kakimoto., 2001). The product of these

reactions, isopentenyladenosine 5-monophosphate (iPMP), isopentenyl 5-diphosphate (iPDP), isopentenyl 5-triphosphate (iPTP), is converted in to zeatin by an unidentified hydroxylase. The various phosphorylated forms can be interconverted and free *trans* zeatin can be formed from the riboside by enzymes of general purine metabolism. *Trans* zeatin can be metabolized in to *cis* and other forms of zeatin. The tRNA cytokinins are synthesized by the modification of specific adenine residues within the fully transcribed tRNA (Kakimoto., 2003)

The cytokinin biosynthetic enzyme first identified came from the gall-forming bacterium *Agrobacterium tumefaciens* (Akioshi et al., 1984; Barry et al., 1984). The *tmr* (ipt) gene was cloned and expressed in *Escherichia coli* and extract of *E.coli* shown to catalyze the production of iPMP from DMAPP and AMP (Akioshi et al., 1984; Barry et al., 1984). The purified *tmr* gene product isopentenylated AMP, but not ATP or ADP, and is DMAPP: AMP isopentenyl transferase (Morris et al., 1993)

DMAPP: AMP isopentenyl transferase activity was detected in partially purified enzyme samples from cytokinin- autotrophic cultured cells of tobacco (Chen and Metlitz, 1979) and from kernels of *Zea mays* (Blackwell and Horgan. 1994). The initial step in the *de novo* biosynthesis of cytokinin in higher plants is the formation of iPMP from AMP and dimethylallylpyrophosphate (DMAPP), which is catalyzed by adenylate isopentenyltransferase (IPT). Recently plant IPT genes were cloned after the *Arabidopsis* genome was analyzed for potential *ipt* like sequences (Kakimoto. 2001, Takei et al., 2001a & b). Nine different *ipt* genes were identified in *Arabidopsis thaliana*. Zubko et al. (2002) have identified a plant isopentenyltransferase gene SHO in a petunia line that showed CK-specific effects including enhanced shooting, reduced apical dominance and delayed senescence and flowering. Sho encodes a protein with homology to isopentenyl transferase (IPT), also causes CK-specific effects when expressed in other plant species. In contrast to the *ipt* gene from *Agrobacterium*, which primarily increases zeatin levels, Sho expression in petunia and tobacco especially enhanced the levels of certain N6-(DELTA2-isopentenyl) adenosine (2iP) derivatives.

Biosynthesis of cytokinin mainly occurs in the root tip regions, but cambium, the shoot apex, and immature seeds are also thought to synthesize cytokinin (Letham. 1994; Emery et al., 2000).

2.4.2 Metabolism

Hormone mediated cellular response needs to be regulated when 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 amino acids.

At present the only identified enzyme in plant that catalyzes the degradation of cytokinins to inactive product 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 cytokinin oxidase activity has been reported in several systems like maize (Whiny and Hall. 1974; Burch and Horgan. 1989), phaseolus (Chatfield and Armstrong. 1986) and in wheat (Laloue 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. 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 significant role in controlling cytokinin levels during plant development.

Conjugation of cytokinin to various sugars and amino acids 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, therefore, cytokinin O-glucosides are considered as intrinsically inactive forms of cytokinins which can be easily converted to free active cytokinins by the action of beta 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).

2.4.3 Transport

Based on the occurrence of cytokinins in the xylem sap and identification of the root tip as a major site of cytokinin biosynthesis, it is generally assumed that cytokinins are transported in the xylem (Haberer and Kieber. 2002). Biological role of cytokinin transport was provided by feeding of roots of nitrogen-depleted maize with nitrate (Takei et al., 2001b). In response to applied nitrate, cytokinin accumulated first in roots, subsequently in the xylem sap and finally in leaves.

2.4.4 Signal transduction pathway

According to the current consistent model for the higher plant *Arabidopsis thaliana*, the scheme for an immediate early response to the plant hormone cytokinin can be formulated as *Arabidopsis thaliana* histidine kinase (AHK) cytokinin receptor-mediated His -> Asp phosphorelay signal transduction (Yamada et al., 2004). Membrane-bound histidine kinases, including CRE1/AHK4, AHK2, and AHK3, perceive cytokinins. The signal is then transferred via histidine-containing phosphotransfer factors, AHPs, to transcription-factor-type response regulators, such as *Arabidopsis* Response Regulator (ARR1), which execute the signal-dependent transactivation of primary cytokinin-responsive genes, including those for other types of response regulator (Aoyama and Oka. 2003). Kiba et al. (2003) reported that in *Arabidopsis thaliana* AHK4 histidine kinase (also known as CRE1 or WOL) acts as a cytokinin signal transducer, presumably, in concert with downstream components, such as histidine-containing phosphotransfer factors (AHPs) and response regulators (ARRs), through the histidine-to-aspartate (His -> Asp) phosphorelay. Hwang et al. (2001) identified a eukaryotic two-component signaling circuit that initiates cytokinin signaling through distinct hybrid histidine protein kinase activities at the plasma membrane. Histidine phosphotransmitters act as signaling shuttles between the cytoplasm and nucleus in a cytokinin-dependent manner. Wainberg et al. (2005) reported that SPINDLY (SPY), a negative regulator of gibberellin (GA) responses involved in cross talk between the two hormone-response pathways. SPY enhances cytokinin responses and inhibits GA signaling through distinct mechanisms. GA3 and spy-4 inhibited induction of the cytokinin primary-response gene, type-A *Arabidopsis* response regulator 5, SPY may interact with and modify elements from the phosphorelay cascade of the cytokinin signal transduction pathway (Fig. 3).

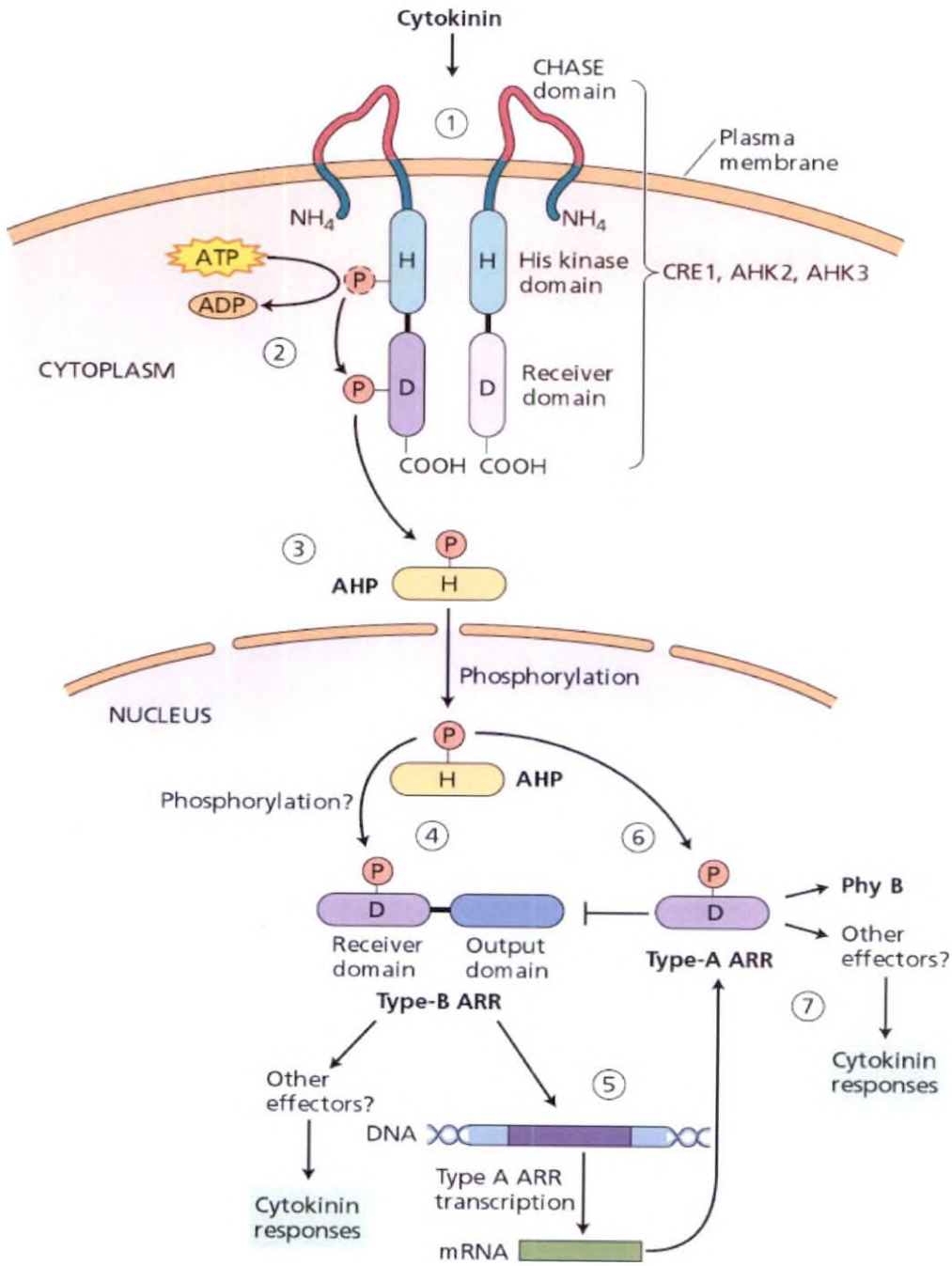


Fig. 3. Signal transduction pathway of cytokinins (Taiz and Zieger., 2006)

2.5 Isopentenyl transferase (*ipt* gene) overexpression

Agrobacterium Gene 4(T-cyt, ipt, roi) which codes for Isopentenyl Transferase that catalyzes the condensation of isopentenyl pyrophosphate and adenosine monophosphate giving iPMP was cloned by Barry et al. (1984). Since then *ipt* has been used extensively by number of researchers to express *ipt* either constitutively or 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.

2.5.1 Effect of various promoters on the *ipt* gene overexpression

Smigocki and Owens (1988) placed *ipt* under the control of promoter of varying activities (the cauliflower mosaic virus 35 S promoter and nopaline synthase promoter) 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 a considerable variation in cytokinin content. In tobacco system cytokinin level varied from 36 picomolar/g fresh weight in control to 2416-4009 picomolar/g in transformed lines. In cucumber it varied from 31 picomolar/g to 5340 picomolar/g.

Gan and Amasino (1995) fused the *ipt* to senescence inducible auto regulatory promoter, SAG12. It was resulted in the suppression of leaf senescence. Transgenic tobacco plants expressing this chimeric 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.

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 in the medium supplemented with 2,4-D and kinetin. However further callus growth did not depend on the presence of the phytohormones. The regenerated transgenic plantlets had short stems with numerous leaves. Roots were initiated 3-5 days earlier than in wild

regenerated wild plantlets. They concluded that the morphological traits of the transformed regenerants were probably conditioned by the hormonal levels.

Cao (2001) analyzed the performance of the autoregulatory senescence-inhibition gene PSAG12-IPT in 422 transgenic plants from 134 independent resistant calluses obtained from rice cultivars (02428, Wuyujing 2, Taibei 309, Millin and Minhui) via *Agrobacterium tumefaciens* mediated transformation. Investigations on photosynthetic and agronomic characteristics of the R1 generation showed that the chlorophyll content and photosynthetic rates of flag leaves were higher than those of the wild-type rice by 41.23 and 60.24%, respectively. The number of grains per hill, seed-setting rate and 1000-grain weight of Millin, a cultivar known to exhibit leaf aging, increased by 40.44, 8.05 and 8.32%, respectively.

Garratt et al. (2001) reported the introduction of *ipt* gene under the control of the senescence specific promoter SAG12 from *Arabidopsis thaliana*, by *Agrobacterium*-mediated transformation into lettuce cv. Evola. This significantly delayed leaf senescence following harvesting of heads upto 60 days after sowing. Modification of carbohydrate partitioning also occurred in plants homozygous for the PSAG12-IPT gene. Glucose in the upper leaves of transgenic plants was higher by 5-fold at 96 DAS (bolting) and by 4-fold at flowering. Fructose in the upper leaves of transgenic plants was higher by 3-fold at 96 days and by 4-fold at 123 DAS than the non-transformed plants. Glucose was significantly higher in the upper middle, lower middle and lower quartiles of the stems of transgenic plants compared to non-transformed plants.

McCabe et al. (2001) reported over expression of *ipt* gene under control of the senescence specific SAG12 promoter from *Arabidopsis thaliana* (PSAG12-IPT) significantly delayed developmental and post harvest leaf senescence in mature heads of transgenic lettuce (*Lactuca sativa* cv. Evola) homozygous for the transgene. Apart from retardation of leaf senescence, mature, 60-days-old plants exhibited normal morphology with no significant differences in head diameter or fresh weight of leaves and roots. Induction of senescence by nitrogen starvation rapidly reduced total nitrogen, nitrate, and growth of transgenic and azygous (control) plants, but chlorophyll was retained in the lower (outer) leaves of transgenic plants. Harvested PSAG12-IPT heads also retained chlorophyll in their lower leaves. During later development (bolting and preflowering) of

transgenic plants, the decrease in chlorophyll, total protein, and Rubisco content in leaves was abolished, resulting in a uniform distribution of these components throughout the plants. Homozygous PSAG12-IPT lettuce plants showed a slight delay in bolting (4-6 days), a severe delay in flowering (4-8 weeks), and premature senescence of their upper leaves. These changes correlated with significantly elevated concentrations of cytokinin and hexoses in the upper leaves of transgenic plants during later stages of development.

Ma et al. (2002) reported seed-specific expression of a heterologous vicilin-isopentenyl transferase (ipt) gene in transgenic tobacco line. Seed cytokinin levels were 2-3-fold higher than control levels. Cytological analyses revealed a significant increase in the number of plerome cell layers and enlargement of diameter in transgenic embryos compared with controls. Dry weight of mature seeds and subsequent seedling growth was increased significantly. This correlated with the level of vicilin-ipt overexpression and increased cytokinin levels in transgenic tobacco seeds. The result suggests a crucial role for cytokinins in regulation of tobacco embryo development.

Sa et al. (2002) placed *ipt* gene from *Agrobacterium tumefaciens* T-DNA under the control of a TA29 promoter, which expresses specifically in anther. The chimeric TA29-ipt gene was transferred to tobacco plants. During flowering, mRNA of the *ipt* gene in the anthers of the transgenic plants accumulated and the level of iPA+iPs increased 3-4-fold in the leaves, petals, pistils, and stamens compared with those in the wild type plants.

Shen et al. (2004) driven *ipt* gene under the endosperm-specific rice prolamin promoter and they introduced it into tobacco by *Agrobacterium tumefaciens* mediated transformation. Cytokinin level in seeds were determined by ELISA kit. Results showed that the iPAs level in transgenic seeds was 4.17 fold higher compared with that in seeds of control plants, and the seed average weight increased by 12.14%.

Hu et al. (2005) have transformed the *Agrobacterium tumefaciens ipt* gene to embryogenic calli of *Festuca arundinacea,* driven by a maize ubiquitin promoter. Tillering ability, levels of chlorophyll a and chlorophyll b, and cold tolerance were greatly increased in the transgenic turfgrass, plants remained more vigorous and staying green longer under lower temperatures.

Zhang et al. (2005) reported developmental and physiological characteristics of the PSAG12-ipt transgenic rapes. The chimeric gene PSAG12-ipt was integrated into rape genome mediated by *Agrobacterium tumefaciens* EHA105. The transgenic plants grew normally with higher vigor than the controls with out any morphological variation. The senescence was delayed 15-20 days for leaves with petiole in the main stem and 20-30 days for those without petiole. In different leaf-positions of 2 transgenic plants, the contents of chlorophyll, cytokinins (zeatin ribosides and isopentenyladenine), malondialdehyde (MDA) and superoxide dismutase (SOD) activity were examined, which are the important physiological indexes of leaf senescence. The contents of chlorophyll, cytokinins and SOD activity were higher in the leaves below the newly full-extended leaf than in the controls, while those in the extending leaves were identical to those in the controls.

2.6 Cytokinin response to secondary metabolite production

Decendit et al. (1992) reported that cytokinins will stimulate the alkaloid synthesis by removing auxin from the medium of a cell line of *Catharanthus roseus*.

Deikman and Hammer (1995) reported in *Arabidopsis* that cytokinin treatment stimulates anthocyanin accumulation in tissue culture and plant organs. They concluded that, this increase was due to the coordinate increased accumulation of mRNAs encoded by four genes in the anthocyanin biosynthetic pathway.

Garnier et al. (1996) investigated the effect of cytokinins on accumulation of indole alkaloids in periwinkle callus cultures. They found that exogenously-applied cytokinin increased the ajmalicine and serpentine content of untransformed callus culture obtained from cotyledons. When they transformed periwinkle cotyledons with the isopentenyl transferase (*ipt*) gene under the control of a light-inducible promoter, they found that the *ipt*-transgenic tissues accumulated higher levels of isopentenyl transferase transcripts as well as zeatin riboside, even under non-inductive condition, but lower concentration of alkaloids compared to that of untransformed tissues. They inferred that

endogenously-produced cytokinin does not mimic the effect of exogenously-applied cytokinin on the alkaloid production in periwinkle calli.

Yahia et al. (1998) examined the interactions between cytokinins and ethylene on alkaloid accumulation in a periwinkle cell line. They found that either exogenously-applied cytokinins or ethylene greatly enhanced ajmalicine accumulation in cells subcultured in a 2,4-dichlorophenoxyacetic acid-free medium. The responses to exogenous cytokinin and ethylene, were additive and showed a different pattern of expression. They concluded that cytokinin and ethylene can up-regulate the alkaloid production in a periwinkle cells through independent pathways when added exogenously to the cultures.

Sa et al. (2001) have reported over expression of isopentenyl transferase gene (*ipt*) from T-DNA into *Artemisia annua* via *Agrobacterium tumefaciens*. The *ipt* gene was placed in a binary vector under the control of the CaMV 35S promoter. They observed an increase in cytokinins, chlorophyll and artemisinin contents to different degrees. Content of cytokinins (iPA and iP) was elevated 2- to 3-fold, chlorophyll increased 20-60% and artemisinin increased 30-70% compared with the control plants. A direct correlation was found between the contents of cytokinins, chlorophyll and artemisinin

Nicolas et al. (2002) isolated a full-length cDNA (CrCKR1) encoding a hybrid histidine kinase from a *Catharanthus roseus* cDNA library. Kinase belongs to the subfamily of cytokinin receptors in *Arabidopsis thaliana*. In cell suspensions the expression of CrCKR1 was not affected by various stress and hormonal treatments but was stimulated in the cells continually exposed to cytokinin. This gave evidence that CrCKR1 may take part in secondary metabolite production.

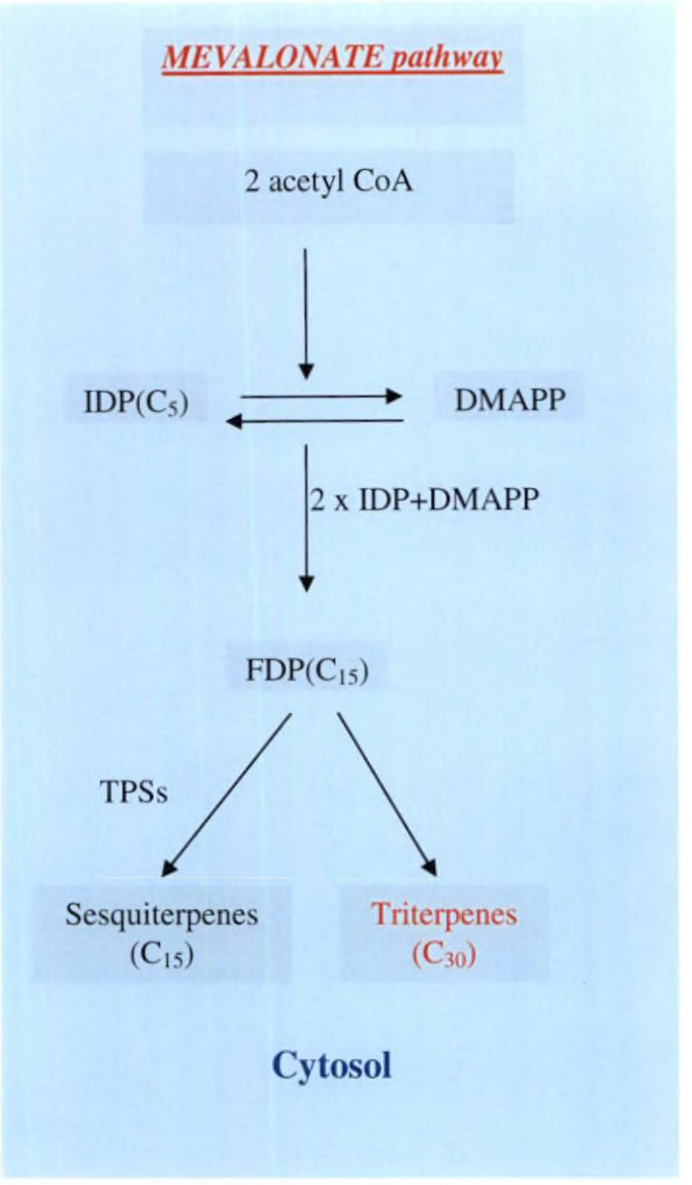


Fig.4 The mevalonate pathway producing different terpenoid classes in the Cytosol. TPSs- terpene synthases; IDP- isopentenyl diphosphate; DMAPP- dimethylallyl pyorophosphate ; FDP- farnesyl diphosphate;

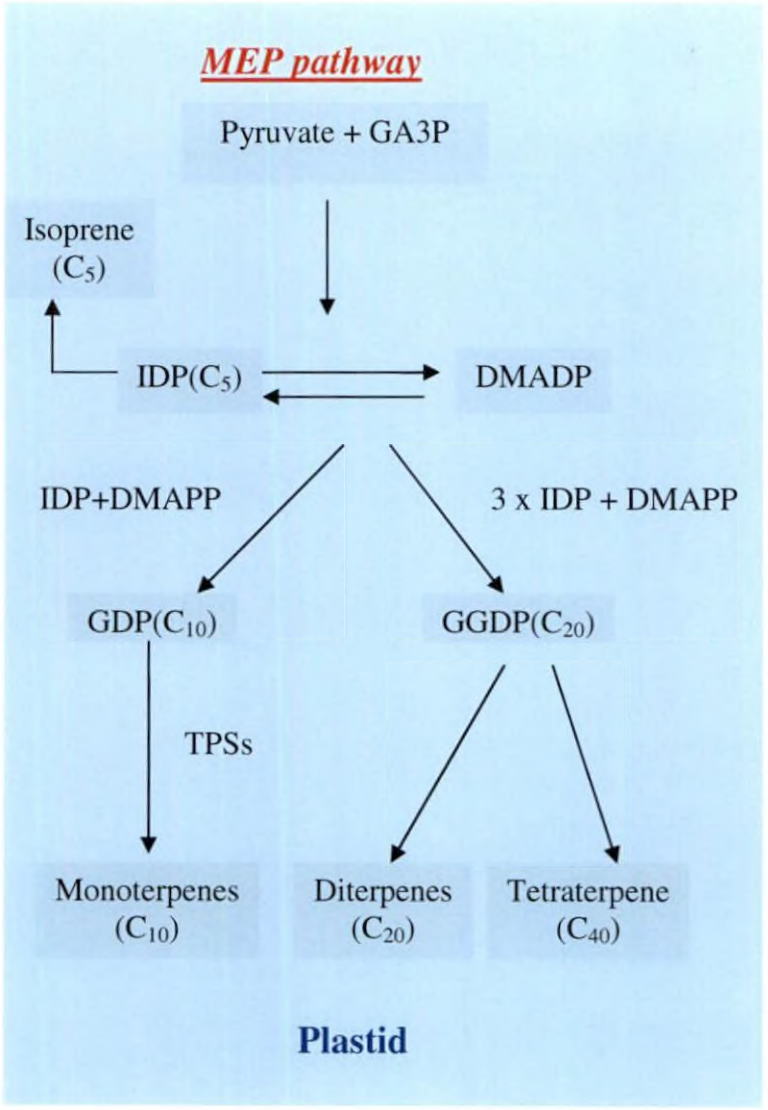


Fig.5 The MEP pathway producing different terpenoid classes in the Plastid. GA3P- D-glyceraldehyde-3-phosphate; TPSs- terpene synthases; IDP- isopentenyl diphosphate; DMAPP- dimethylallyl pyorophosphate ; FDP- farnesyl diphosphate; MEP- methylerythritol 4-phosphate; GDP- geranyl diphosphate; GGDP- geranylgeranyl diphosphate.

Materials and Methods

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Collection and maintenance of plant material

Bacopa monnieri cultures were obtained from Rajiv Gandhi Centre for Biotechnology, Jagathi, Kerala, India. The cultures were maintained in MS medium supplemented with 30g/l sucrose and 6g/l phytagar. The pH of the medium was adjusted to 5.8±0.1 and sterilized for 15min at 121°C with 15 psi. The cultures were incubated at 25°C under cool white fluorescent light (30μE m^{-2} s^{-1}, 16h photoperiod).

3.1.2 Chemicals

The chemicals and biochemicals used in the study were of either molecular biology grade or extra pure analytical grade.

Restriction endonuleases, including *Eco*RI, *Hind* III, *Sma*I, 100 bp ladder and 1 kb ladder were obtained from New England Biolabs Inc.,USA. The Biochemicals purchased from Promega Corp. (Madison, Wisconsin, USA) included MMLV- Reverse transcriptase, *Taq* DNA polymerase, Oligo dT_{18} primer , Nuclease free water, Diethyl-pyrocarbonate(DEPC), Bromophenol blue(BPB), Xylene cyanol FF, Glycerol, Tris, Isopropyl β-D-1-thigalactosidase(IPTG), 5-Bromo –4-chloro-3 -inolyl β-D-galactopyrenoside (X-Gal), Ampicillin and Tetracycline were purchased from US Biochemicals(USB), Kanamycin, Cetyl trimethyl ammonium bromide(CTAB), Ethidium bromide, Ethylene diamine tetra acetic acid (EDTA), Boric acid, Calcium chloride, Salmon sperm DNA, Sodium acetate, Thidiazuron(TDZ), Tween-20 , RNase A were purchased from Sigma, USA. agarose, Trizol™ reagent, alpha naphthalene acetic acid (NAA), Gibberellic acid (GA_3), Benzyl adenine (BA) and Phytagar were purchased from Gibco-BRL (Invitrogen) DNase I, dNTP set and Hybond N+ nylon membrane were purchased from Amersham Pharmacia , Orthophosphoric acid was obtained by from Merck and analytical grade absolute alcohol from Les Alcools De Commerce Inc., Ontario. All the primers used in this study were synthesized either by Gibco-BRL or from

Sigma- Genosys. The chemicals were purchased from Himedia or Qualigens. Cefotaxim(Alkem Laboratories Ltd.) used was obtained from chemists shop.

Radioactive α ^{32}P dCTP used in the study were obtained from Board of Radiation and Isotope Technology (BRIT), Anushakthi Nagar, Mumbai.

3.1.3 Glasswares and plastic wares

Glasswares were from either Borosil or Schot- Duran. Plastic wares were obtained from Nunc, Tarson or Axygen.

3.1.4 Bacterial strain and plasmid vectors

JM 109 Bacterial strain and vectors pBI B33 *ipt*, pBI SAG12 *ipt*, pRK 2013 and *Agrobacterium* strain EHA 105 were kindly spared by Rajiv Gandhi Center for Biotechnology (RGCB), Trivandrum, Kerala, India.

3.2 METHODS

3.2.1 Bacterial transformation

3.2.1.1 Preparation of competent of *E. coli* cells

A single colony of *E.coli* host cell (JM 109) was inoculated in 5ml of Luria Bertani (LB) broth. The cells were grown overnight with constant shaking at 200rpm at 37°C. To 50 ml of LB broth, 50 µl of overnight culture were added and cells were grown till they attained an OD_{600} = 0.5(in ~2.5h). The culture was transferred in to a pre- chilled sterile 50ml centrifuge tube. Care was taken that the tubes with the bacterial cells were always maintained on ice. The cells were harvested by spinning at 5000rpm for 5 min at 4°C. The bacterial pellet was resuspended in 10ml of ice cold 0.1M $CaCl_2$. The centrifugation was repeated and the pellet was resuspended in 5 ml of ice cold 0.1M $CaCl_2$ followed by incubation on ice for 30min. The cells were centrifuged again and the pellet was resuspended in 2ml of ice cold 0.1M $CaCl_2$ and kept on ice.

3.2.1.2 Transformation

Hundred µl of the resuspended competent cells and 10µl of the ligation reaction were added in a prechilled 1.5ml microfuge tube and incubated on ice for 30min. A heat shock at 42°C was given to the cells for 90 s. The cells were quick chilled and incubated on ice for 5 min. and 400µl of LB broth was added to the cells and incubated with shaking at 37°C for 1h at 200rpm. Fifty µl of the culture was plated on the LB agar plates supplemented with 50µg/ml kanamycin. The plates were incubated at 37°C overnight.

3.2.1.2 Plasmid isolation

Plasmids were isolated from *Escherichia coli* (*E.Coli*) carrying independently the vectors, pBI B_{33} *ipt* and pBI SAG12 *ipt* by alkali lysis method (Sambrook et al., 1989). A single colony was inoculated in 5ml LB broth containing 50µg/ml kanamycin. The culture was incubated overnight at 37°C with vigorous shaking at 200rpm. The cells were harvested into a 1.5 ml microfuge tube by centrifugating at 10000rpm for 2min at room temperature. To pelleted cells, 100µl of ice-cold solution I and 10µl of Lysozyme, to break the bacterial cell wall were added and cells were resuspended by vortexing. To the dispersed cells, 200µl of solution II was added and mixed well by inversion and it was kept in ice for 10min. After 10min, 150µl of ice-cold solution III was added. Bacterial cell debris was precipitated by centrifugation of sample at 12000rpm for 5min at 4°C. The supernatant was transferred to a fresh microfuge tube and 10µl of 100ng/ml DNase free RNase A was added. The sample was incubated at 65°C for 15min to digest RNA present in the sample followed by extraction with equal volume of chloroform: isoamyl alcohol (24: 1) at 12000rpm for 5min in a microfuge. Chloroform: isoamyl alcohol step was repeated once. DNA was precipitated from supernatant by adding equal volume of isopropanol and centrifuged at 12000rpm for 10min. at 4°C. Pellet was collected and 1ml of 75% ethanol was added and spinned at 10000rpm for 6min at 4°C. Supernatant was removed by gentle aspiration. The pellet was air dried at room temperature and resuspended in 20µl sterile Milli-Q water. The DNA sample was checked by agarose gel electrophoresis (0.8%) and stored at -20°C.

3.2.1.3 Restriction digestion with *Hind* III and *Eco*RI

Plasmids were digested using *Hind*III and *Eco*RI in presence of Buffer 2. The digested DNA was checked on a 0.8% agarose gel.

3.2.2 Plant transformation using *ipt* gene

3.2.2.1 Triparental mating –*Agrobacterium* transformation

The binary vector pBI B33 *ipt* (Fig. 4) and pBI SAG12 *ipt* (Fig. 5) were mobilized from *E.coli* into the *Agrobacterium tumefaciens* strain EHA 105 by triparental mating. Triparental mating was carried out as per the method of Ditta et al. (1980). The procedure involved mixing two *E.coli* strains (one containing Binary vector and the other the helper strain) and the *Agrobacterium* strain. The bacteria was allowed to grow on a LB/AB plate at 30°C for 24h and the transconjugants were selected on antibiotic medium.

Bacterial strains used

Agrobacterium host	-EHA 105
E.coli donor	-JM 109: pBI B33 *ipt* and pBI SAG12 *ipt*
E.coli helper	-JM 109: pRK2013

Day 1- The *Agrobacterium* strain EHA 105 was streaked onto AB minimal agar plate supplemented with 20mg/l rifampicin and grown at 28 °C for 48h.

Day 2 – The two *E.coli* strains containing helper and donor plasmids were streaked on LB agar plates with 50mg/l kanamycin and grown overnight at 37°C.

Day 3 – Fresh LB agar plate was divided into four regions (one large and three equal small). In the large area, a loop full of three strains were placed and mixed thoroughly, while individual strains were streaked separately to the smaller areas. The plate was incubated at 30°C for 24h.

Day 4 – Fresh plate with AB minimal medium, supplemented with 50mg/l kanamycin and 20mg/l rifampicin was divided in to four sections as described above. A loop full of

bacterial mix from the large area of plate from day 3 was streaked onto the larger area, while the controls were streaked separately to the smaller regions and incubated at 30°C for 48h.

Day 6 – Single colonies of transformed *Agrobacterium* in the larger area were selected on AB minimal agar plated supplemented with 50mg/l kanamycin and 20mg/l rifampicin. The culture was maintained in AB minimal agar plates supplemented with the antibiotics.

3.2.2.2 Preparation of *Agrobacterium* cells for plant transformation

Day 1- Single *Agrobacterium* colony from the AB minimal medium was inoculated into 2ml of AB minimal liquid media with 50mg/l of kanamycin. The culture was incubated overnight at 30°C with shaking at 200rpm.

Day 2 –Five ml of the above culture was inoculated to 50 ml of AB minimal media supplemented with 50 mg/l of kanamycin and incubated overnight at 30°C with shaking at 200rpm

Day 3 – *Agrobacterium* cells from the overnight culture were harvested by centrifugation at 4000rpm for 5 min. The pelleted cells were re-suspended in MS salt solution (0.5 OD at 600nm) and used for plant transformation

3.2.2.3 Pre-Incubation

Leaf explants from *Bacopa monniera* cultured *in vitro* were used for transformation. The explants were pre-incubated for 48h in the regeneration medium (MS supplemented with 2mg/l BA)

3.2.2.4 Co-Culture

The pre incubated explants were infested with a suspension of *Agrobacterium* cells, harboring the binary vector, diluted to 0.5 OD (measured at 600nm) for 10 min.

The infected plants were blotted on sterile Whatman No.1 filter paper and co cultivated on the regeneration medium for 48h.

Control uninfected explants were maintained in the callusing medium.

3.2.3 Selection and regeneration of transformants

After co cultivation, the infected explants were washed with MS salt solution containing antibiotic cefotaxime, blotted on sterile Whatman No. 1 filter paper and transferred to the selection medium. The selection medium comprised of regeneration medium supplemented with 15mg/l of kanamycin and 300mg/l cefotaxime.

Control uninfected explants were also transferred to the selection medium without kanamycin, which served as infected control.

The explants were maintained in the regeneration medium for 4 weeks and the kanamycin resistant regenerants formed were sub cultured in to regeneration medium (MS basal medium) containing kanamycin 15 mg/l.

After two weeks, regenerants were transferred to another medium (1/2 MS supplemented with 1mg/l IAA and 1mg/l GA) for rooting and shooting.

The fully grown plants with roots were transformed to the green house for the hardening. The plants were grown in the potting mixture, with a composition of soil: cow dung and manure in the ratio of 1:1:1. The transformants and wild plant were kept in five replications for the analysis work. The weekly observation was carried out for the biometric characters and chlorophyll content.

3.2.4 Molecular analysis of putative *ipt* transformants

3.2.4.1 Genomic DNA isolation

Genomic DNA was isolated from the putative transformants by CTAB method (Rogers and Bendich., 1985).

Young leaves (100mg) were frozen in liquid nitrogen and grinded using a sterile mortar and pestle. One ml of hot (65°C) 2X CTAB buffer was added to the grinded tissue and incubated at 65°C for 10min in a water bath followed by incubation at room temperature (~25°C) for 10min. Five hundred µl of chloroform: isoamyl alcohol (24:1) was added to the microfuge tubes and mixed thoroughly to form an emulsion. The samples were centrifuged for 10min at 10000rpm. The supernatant was transferred to a new microfuge tube using cut tip. To the supernatant, 10µl RNase was added and kept in 65°C for 15-30min. The chloroform: isoamyl alcohol and RNase steps were repeated again. To this double the volume of ethanol was added and mixed properly and kept overnight. Next day the ethanol was aspired slowly and the microfuge was air dried and dissolved in 50µl sterile Milli-Q water.

3.2.4.2 PCR analysis

PCR amplification of the DNA was carried out using the primers nptF and nptR for *npt*II gene and iptF (5' TTG CAC AGG AAA GAC GAC GAC 3') and iptR (5' CGC GCA TGG ATG AAA TAC TC 3') for *ipt* gene independently. The reactions were performed in a thermal cycler (Eppendorf- Master cycler gradient) under the following conditions: Initial denaturation at 94°C for 6min; 34 cycles of 94°C for 1min, 60°C for 30sec and 72°C for 1 min and a final extension of 72°C for 6min. The PCR products were separated by 1.0% agarose gel electrophoresis and visualized on UV-transilluminator

3.2.4.3 RT-PCR analysis

The expression of the transferred gene was studied by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from a control plant as well as four positive plants using TRIZOL^{TM} reagent (Gibco BRL).

3.2.4.4 Total RNA isolation

Total RNA was isolated from 50 mg leaf tissue using Trizol^{TM} reagent. Young leaves were frozen in liquid nitrogen and grinded to a fine powder in RNase free mortar and pestle. Five hundred µl Trizol^{TM} reagent was added to the grinded tissue and was

kept at room temperature for 5min to permit the complete dissociation of nucleoprotein complexes. To this, 100µl of chloroform was added and it was shaken vigorously by hand for 15 seconds and incubated at room temperature for 2-3min. The sample was centrifuged at 12000rpm for 15 min at 4°C. The aqueous phase was collected in a new microcentrifuge tube and 250µl isopropanol was added and kept at room temperature for 10 min to precipitate the total RNA. The sample was centrifuged at 10000rpm for 10min at 4°C. The supernatant was discarded and the pellet was washed in 500µl of 75% ethanol by centrifugation at 7500rpm for 5min at 4°C. After centrifugation, ethanol was aspirated and the RNA pellet was air dried. The RNA pellet was dissolved in 30µl of nuclease free water (Promega). The quality of RNA was checked in a 2% agarose gel by loading 1µl sample along with loading dye.

3.4.4.4.1 Quantification of RNA

Total RNA was quantified spectrophotometrically at 260nm. The concentration of RNA in 1 µl of the RNA sample was calculated using following equation,

$$\text{Concentration } (\mu g/ml) = (40 \times A_{260} \times \text{Dilution factor})/1000$$

3.2.4.5 First strand cDNA synthesis (Reverse Transcription)

First strand cDNA was synthesized from mRNA by reverse transcription using M-MuLV Reverse Transcriptase (M-MuLV RT). The reaction mix was prepared for 40µl volume. Initially 8µl of total RNA, 14µl of DEPC water, 4µl of Oligo DT were added and kept at 70° C for 10min and snap cooled. To this 8µl of 5X buffer, 4µl of 10mM dNTP, 1µl of RNase and 1µl of MMLV RT were added and mixed, spinned and kept at 37°C for 1 hr, heat denatured at 80° C for 10 min finally stored at –20° C for further use.

3.2.4.6 Southern hybridization

3.2.4.6.1 PCR-Southern hybridization

The agarose gel with PCR amplified *ipt* gene transformants were transferred to nylon membrane using vacuum-blotting method.

3.2.4.6.2 Vacuum blotting

The DNA was transferred to a positively charged nylon membrane (Hybond N+) using alkaline transfer method. Initially, a Whatman filter paper #3 was placed over vacuum blotting apparatus and it was made wet with sterile water. Moist nitrocellulose membrane with 2X SSC was placed over the filter paper without air bubbles. A window of appropriate size (according to gel size) was placed on the nitrocellulose membrane upon that gel was placed (care should be taken to cover the sides of the gel with window sheet so that buffer will pass the membrane only through the gel and not through the sides). Apparatus was tightened and vacuum was checked and pressure reading was adjusted. The following solutions were poured over the gel in order.

Depurination solution ------ 20 minutes
Denaturation solution ------- 20 minutes
Neutralization solution ----- 20 minutes
2X SSC ------ 1 Hr

The gel should not be allowed to dry in this process. Well positions were marked with pencil, gel was removed and a cut was given at the upper right corner of nitrocellulose membrane. UV cross-link was done and stored at –20°C.

3.2.4.6.3 Hybridization

The wet nitrocellulose membrane with 2X SSC was inserted into the hybridization tube without any bubbles. 40 ml of pre hybridization buffer was poured and was pre hybridized for three hours at 65°C. To this, denatured radiolabelled probe was added and was kept for hybridization overnight at 65°C.

3.2.4.6.4 Washing

Buffer with probe was discarded in to a sealed tube and membrane was washed with following solutions,

2X SSC + 0.1% SDS for 10 min at 37°C (Twice)

1XSSC + 0.1% SDS for 10 min at 65°C

0.2X SSC + 0.1% SDS for 10 min at 65°C

The membrane was removed from the hybridization tube and was exposed for four hours and the signal was analyzed using phosphor imaging.

3.2.5 Physiological analysis

The transformants and wild plant were kept in five replications for the analysis work. The weekly observation was carried out for the biometric characters and chlorophyll content.

3.2.5.1 Growth parameters

3.2.5.1.1 Number of leaves

Weekly observations on number of leaves were taken.

3.2.5.1.2 Number of branches

Numbers of branches were observed at weekly interval.

3.2.5.1.3 Shoot length

Plant height was measured in weekly interval.

3.2.5.1.4 Root length

Equal length cuttings were kept in the 10 ml of tap water and the whole set up was maintained with such a condition where the rooting part will not get the light, thus the effect of light inhibition of rooting was avoided.

3.2.5.2 Physiological parameters

3.2.5.2.1 Chlorophyll estimation

Hundred mg of leaf sample was taken. It was chopped into pieces and 5 ml of DMSO (Dimethyl sulfoxide) was added and kept overnight. The supernatant was taken and absorbance was taken at 645 and 663 nm. The chlorophyll a, chlorophyll b and total chlorophyll were calculated using the formulae given below and expressed in mg of pigments / gram of fresh leaf weight.

Total Chlorophyll= {[20.2(OD at 645) +8.01(OD at 663)] x V}/ (W x 1000)

Chlorophyll a= {[12.7(OD at 663)-2.69(OD at 645)] x V}/ (W x 1000)

Chlorophyll b = {[22.9(OD at 645) - 4.68(OD at 663)] x V}/ (W x 1000)

3.2.5.2.2 Relative water content

Relative water content was calculated by measuring the fresh weight, dry weight and turgid weight of known number of leaf discs of the treatments taken. After measuring the fresh weight of the sample, it was submerged in distilled water for three hours and taken the turgid weight. The dry weight of the sample was measured after keeping the samples in oven at 70°C for three days. The relative water content of the treatments was calculated using the following formula.

RWC= [(Fresh weight - Dry weight) / (Turgid weight - Dry weight)] x 100

3.2.5.2.3 Total soluble protein

Total soluble protein of leaf was estimated using simple protein dye binding of Bradford (1976) using bovine serum albumin as the standard. This method is based on the principle that the coomassie brilliant blue (CBB) G-250 binds to protein and the protein dye complex has a higher extinction coefficient thus leading to great sensitivity in measurement of protein. This binding of dye to protein is a very rapid process (approx. 2 min). The method is devoid of interferences by other soluble compounds. One hundred

mg of CBB G 250 was dissolved in 50 ml of 95% ethanol. To this solution 100 ml of 85 % (w/v) orthophosphoric acid was added. The resulting solution was diluted to the final volume of 200 ml with distilled water.

One gram of leaf material was ground to a thin paste and soluble protein was extracted with 10 ml of phosphate buffer (pH 7.8) containing 1mM EDTA, 2% (w/w) PVP. The extract was centrifuged in cold (4°C) at 10,000 rpm for 10 minutes. To the 50µl of the supernatant 4 ml of Bradford reagent was added and mixed well. The absorbance of the solution was recorded after two minutes and within 30 minutes using spectrophotometer at 595nm.

The protein content was calculated using the BSA standard in the range of (10-100µg). The protein content was expressed as mg/g FW.

3.2.5.2.3 Stomatal frequency

Stomatal frequency refers to the number of stomata per unit area of leaf. A thick mixture of thermocoal and xylene was prepared and this was smeared on both surface of leaves and allowed to dry. It was peeled gently after drying, and the peels were observed under microscope and counted using a 45X objective and 10X eye piece. The field of the microscope was measured using a stage micrometer and stomatal frequency per unit area was calculated

Stomata frequency = Number of stomata /Area of the microscopic field (0.0086)

3.2.5.2.4 Stomatal resistance

Stomatal resistance gives an indication of the resistance offered by the stomata for gas diffusion and transpiration. Stomatal resistance was measured using the ΔT Porometer (ΔT Devices, UK) and expressed as s/cm.

3.2.5.3 Immunological quantification of cytokinins

The cytokinin estimation was done in the Department of Crop Physiology, GKVK Campus, Bangalore.

3.2.5.3.1 Extraction

Hundred milligram of leaf samples were weighed and frozen using liquid nitrogen. The samples were grinded in 5 ml of 80% methanol. Butylated Hydroxy Toluene (BHT) was added at a concentration of 10µg/ml. The extraction was continued at -70°C for 48 hrs.

3.2.5.3.2 Sep pack purification

Sep-Pack column was pre-wetted with 5 ml of 50% methanol followed by 5 ml of distilled water. The last traces of water were expelled by passing air. The clear methanol extract was passed through the column to remove lipids and pigments. The column was washed with 3 ml of 50 % methanol and collected. Pooled samples were evaporated under vacuum and residue was used for further purification.

3.2.5.3.3 Butanol partitioning

Water saturated n-Butanol was chosen because of high partitioning coefficient of the cytokinin into butanol at alkaline pH. The residue was dissolved in 1 ml of distilled water and partitioned three times against an equal volume of water-saturated n-Butanol (1:1 v/v). The organic phase was evaporated and the residue dissolved in 1ml of TBS buffer (pH 7.5). Aliquots from this were used in immunoassay for quantification of cytokinin.

3.2.5.4 Enzyme Linked Immunosorbant Assay (ELISA)

Cytokinin quantification was done using Phytodetek kit (Sigma) for isopentenyl adenosine (iPA). The quantity of iPA was calculated from the standard curve using graph pad software.

3.2.5.4.1 Phytodetek kit for Isopentenyl Adenosine

The iPA standards were prepared by dissolving 3.35 mg of iPA in absolute methanol. This makes stock solution with concentration of 0.1mole. The stock solution was serially diluted to 1000, 50, 20, 5, 2, 0.5, 0.2 picomoles using TBS buffer. 100µl of sample or standard (in duplicate) was added to the antibody coated well; 100µl of tracer

was added and mixed by gently tapping the plate. The test wells were covered with plate sealer and incubated at 4°C for three hours. After three hours of incubation the contents were discarded and the wells washed thrice with 200µl wash solution. 200µl substrate solution was added to the wells and incubated at 37°C for 60min after covering with plate sealer. After the incubation period, the reaction was stopped by adding 50µl of stop solution and absorbance was read at 405nm.

The mean of the optical densities (OD) of duplicate sample was calculated and % of binding was calculated by the formula,

Percent binding = [(Standard OD-NSB OD)/ (B_o OD- NSB OD)] x 100

where,

B_o = percent binding

NSB=Non specific binding (0 % binding)

OD=Optical density

Percent binding was converted to logit B/ B_o using the formula

Logit (B/B0) = (B/ B_o %)/(100-(B/ B_o %)

3.2.5.4 Bacoside estimation

Total bacoside was estimated using HPLC. The estimation was carried out in R&D section of Natural Remedies Private Limited, Bangalore. The bacoside content was expressed in percentage (w/w) basis.

Table1. Primers and Tm used in molecular analysis of putative transformants

Serial no	Primer	Sequence	Tm (°C)
1	nptF	5' GAC GAG GCA GCG CGG CTA T 3'	71.9
2	nptR	5' AAG AAG GCG ATA GAA GGC GA 3'	64.9
3	iptF	5' TTG CAC AGG AAA GAC GAC GAC 3'	67.5
4	iptR	5' CGC GCA TGG ATG AAA TAC TC 3'	65.3

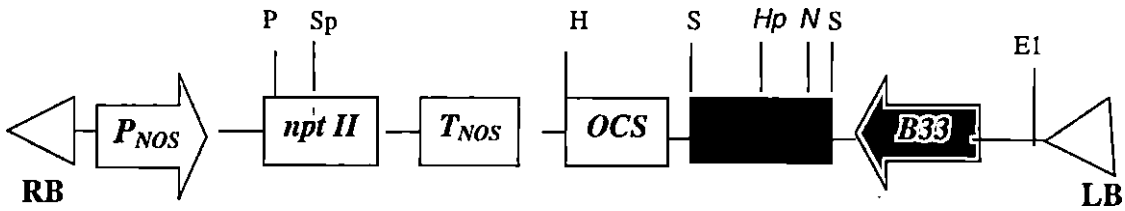


Fig. 6. Vector map of pBI B33ipt. RB-Right border; LB-Left border; P_{NOS} –Nopaline synthase promoter; *npt*II- Genes for neomycin phosphotransferase ; T_{NOS} –Nopaline synthase terminator ; OCS-Octopine synthase terminator; *ipt*- Gene for isopentenyl transferase ; B33- Promoter region of patatin B33 gene; P- *Pst*I; Sp-*Spe*I; H-*Hind*III; S-*Sma*I; Hp-*Hpa*I; N-*Neo*I; E1-*Eco*RI

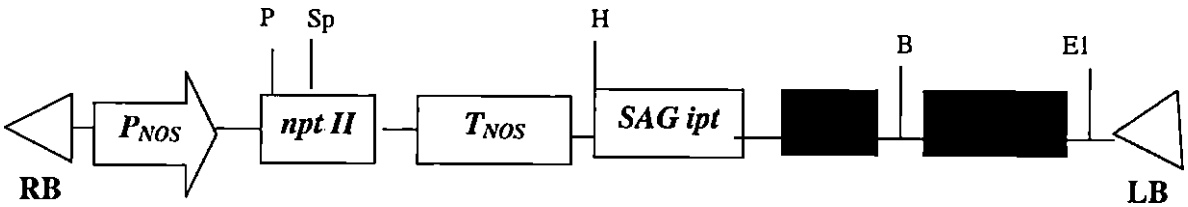


Fig. 7. Vector map of pBI SAG12 ipt. RB-Right border; LB-Left border; NOS_{pro} – Nopaline synthase promoter; *npt*II- Genes for neomycin phosphotransferase ; NOS_{ter} – Nopaline synthase terminator SAG *ipt*-Promoter region of senescence specific gene; *ipt*-Gene for isopentenyl transferase ; P- *Pst*I; Sp-*Spe*I; H-*Hind*III; E1-*Eco*RI

Results

4. RESULTS

An experiment was conducted to overexpress cytokinin biosynthesizing *ipt* in *Bacopa monnieri* through *Agrobacterium* mediated transformation and regenerate the transformed plants through tissue culture for analyzing the influence of overexpression of *ipt* on growth, physiology and secondary metabolite production in the Department of Plant Physiology, College of Agriculture, Vellayani. The experiment was carried out in collaboration with the Plant Molecular Biology Division, Rajiv Gandhi Centre for Biotechnology, Kerala-India, during 2006-2007. The results of different experiments done to address the above objectives are given below.

4.1 Agrobacterium mediated transformation of *Bacopa monnieri*

4.1.1Transformation of *Escherichia coli* cells with *ipt* construct

Escherichia coli strain JM 109 was transformed independently with pBI B33 *ipt* and pBI SAG12 *ipt*. The transformed *E.coli* cells were selected by growing on LB_{kan} media containing 50ppm kanamycin. A single colony was taken from each plate and transferred to LB_{kan} media for overnight growth.

4.1.2 Plasmid isolation and restriction digestion of *E.coli*

Plasmid was isolated from transformed *E.coli* cells using alkali lysis method. The isolated plasmid was double digested with restriction enzymes, *Eco*RI and *Hind*III. The release of insert from the samples confirmed the presence of the recombinant binary vector in the cells (Plate 1).

4.1.3 Triparental mating

Triparental mating was done using *Agrobacterium* strain EHA 105, pRK 2013 and *E.coli* cells with *ipt* gene construct (Plate 2a). The recombinant *Agrobacterium* cells were selected by growing it on LB media containing rifampicin 20ppm and kanamicin 50 ppm (Plate 2b).

4.1.4 Plasmid isolation and restriction digestion of *Agrobacterium* carrying pBI B33 *ipt* and pBI SAG12 *ipt*

Plasmids were isolated from triparental mated *Agrobacterium* cells, using the alkali lysis method. Double digestion was done with *Eco*RI and *Hind*III. The presence of binary vector construct in *Agrobacterium* was confirmed by insert release from the recombinant *Agrobacterium* (Plate 1).

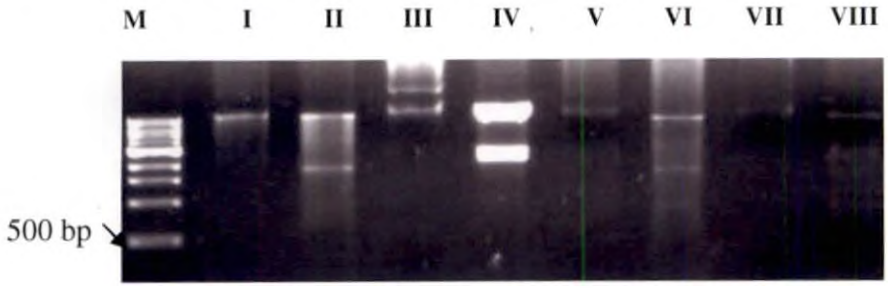


Plate1. Restriction digestion of binary vector showing insert release

M-Marker 1kb
Lane I- Uncut binary vector, pBI B33 *ipt* from *E.coli*
Lane II- pBI B33 *ipt* construct from *E.coli* digested with *Eco*RI and *Hind*III
Lane III- Uncut binary vector, pBI SAG12 *ipt* from *E.coli*
Lane IV- pBI SAG12 *ipt* construct from *E.coli* digested with *Eco*RI and *Hind*III
Lane V - Uncut binary vector, pBI B33 *ipt* from *Agrobacterium*
Lane VI- pBI B33 *ipt* construct from *Agrobacterium* digested with *Eco*RI and *Hind*III
Lane VII - Uncut binary vector, pBI SAG12 *ipt* from *Agrobacterium*
Lane VIII- pBI SAG12 *ipt* construct from *Agrobacterium* digested with *Eco*RI and *Hind*III

(2a) **(2b)**

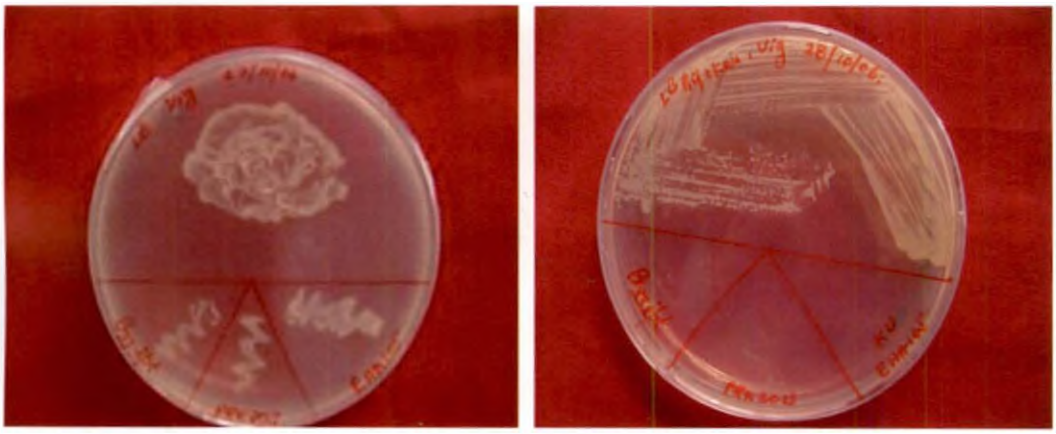


Plate 2a. Triparental mating of *Agrobacterium* strain EHA 105 with *E.coli* parents, JM 109 carrying binary vector and pRK 2013 carrying the helper plasmid

Plate 2b. Recombinant *Agrobacterium* growing on selection plate.

4.1.5 Transformation of *Bacopa monnieri* using recombinant *Agrobacterium* EHA105 carrying pBI B33 *ipt* and pBI SAG12 *ipt*

Leaf explants of *Bacopa monnieri* were co-cultivated with the recombinant *Agrobacterium* for two days and transferred to regeneration medium containing 15mg/l kanamycin and 300mg/l cefotaxime. Putative transformants were regenerated from co-cultivated explants when placed on the selection medium containing 15mg/l kanamycin and 300mg/l cefotaxime. Uninfected explants failed to regenerate in presence of kanamycin (Plate 3a, 3b and 3c). The regenerated shootlets were grown in MS medium devoid of growth hormones. The transformants showed no rooting in the plain MS medium. The transformants were then sub cultured in the medium containing1/2 MS supplemented with 1mg/l IAA and 1mg/l GA (Plate 9).

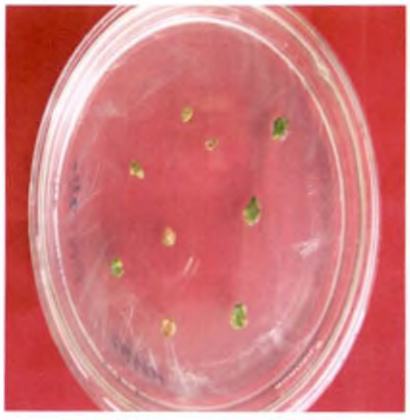


Plate.3a **Plate. 3b** **Plate. 3c**

Plate 3a. Negative control plates- uninfected explants growing on selection medium

Plate 3b. Infected explants regenerating on the selection medium

Plate 3c. Positive control plates- infected explants growing on non-selection medium

4.1.6 Molecular analysis of putative transformants

The successful integration and expression of transgenes in the putative transformants were confirmed by genomic DNA PCR, Southern blot hybridization and RT-PCR.

4.1.7 DNA isolation

Genomic DNA was isolated from the transformants and from the wild type brahmi plant using CTAB method. An agarose gel (0.8%) was run to confirm the DNA isolation. Required amount of genomic DNA was obtained by this method.

4.1.8 Polymerase chain reaction (PCR)

The integration of T-DNA region in the putative transformants was checked by PCR analysis using primers specific to *npt*II and *ipt* genes. PCR with *npt*II specific primers showed the amplification of 600bp fragment in all putative transformants except one (Plate 5), while the control wild plant did not show any amplification. The amplification of DNA using *ipt* specific primers also showed the presence of 600bp amplicons in all putative transformants (Plate 4). The control plant did not show any amplification with *ipt* primers.

4.1.9 RT-PCR analysis

4.1.10 RNA isolation

The total RNA was isolated from both transformant and wild using Trizol reagent. The amount of RNA was quantified spectometrically at 260 nm. The quantity of RNA was ranging from 400-600 ng/1µl of isolated RNA in transformant and wild respectively (Plate 6).

Four transformants along with wild type, which are confirmed with PCR analysis, were selected to study the expression of transgene by RT-PCR. First strand of c-DNA was synthesized from mRNA by reverse transcription using M-MuLV Reverse Transcriptase enzyme. RT-PCR analysis confirmed the expression of *ipt* (Plate 7a) and *npt*II (Plate 7b) gene in all the transformants, while there was no expression in the wild type. Expression of constitutively expressed plant gene –actin was used as loading control (Plate 7c).

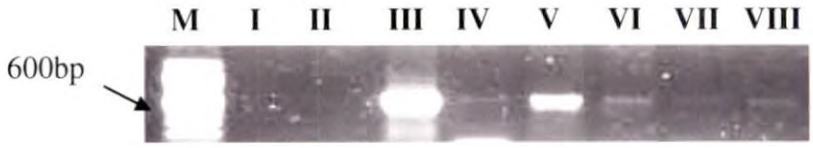


Plate 4. Agarose (1.0%) gel stained with Ethidium bromide showing PCR amplified product using *ipt* specific primer

M	Marker (1Kb)
Lane I	- Control (Water)
Lane II	+ Control (Wild type)
Lane III	+ (Plasmid DNA)
Lane IV-Lane VI	B33 *ipt* transformants
Lane VII &Lane VIII	SAG *ipt* transformants

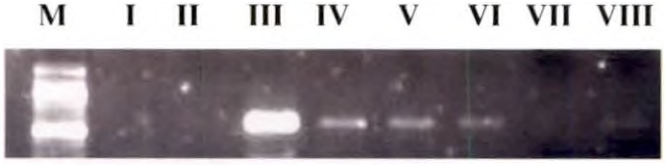


Plate 5. Agarose (1.0%) gel stained with Ethidium bromide showing PCR amplified product using *npt*II specific primer

M	Marker(1Kb)
Lane I	- Control (Water)
Lane II	+ Control (Wild type)
Lane III	+ (Plasmid DNA)
Lane IV-Lane VI	B33 *ipt* transformants
Lane VII &Lane VIII	SAG *ipt* transformants

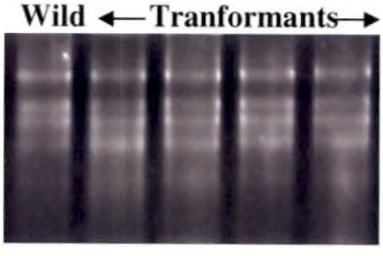


Plate 6. Agarose (1.0%) gel stained with Ethidium bromide showing total RNA isolated from wild and transformants of *Bacopa monnieri*

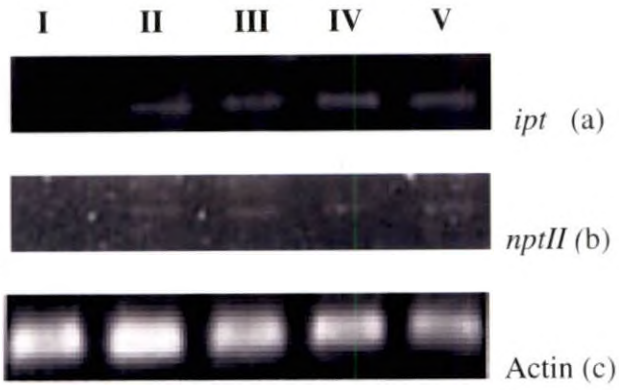


Plate 7. RT-PCR analysis

Lane I	Wild type
Lane II&III	B33 *ipt* transformants
Lane IV & V	SAG *ipt* transformants

4.1.11 Southern hybridization analysis

Southern hybridization analysis was done using the PCR amplified product for *ipt* gene. The DNA was transferred to nylon membrane by vacuum blotting method. The blot was hybridized with denatured radiolabelled probe specific for *ipt* gene. The phosphor imaging showed the presence of gene in all transformants and wild has not given the band specific for the *ipt* (Plate 8).

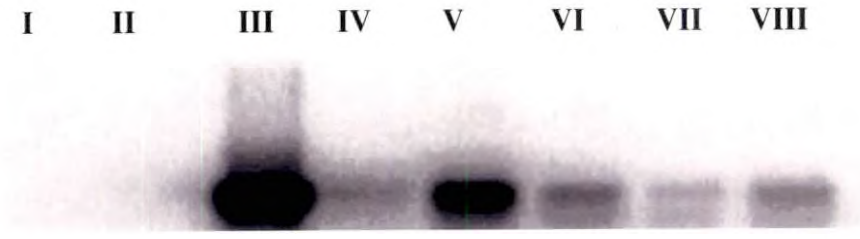


Plate 8. Southern blot showing the presence of transgene

Lane I	- Control (Water)
Lane II	+ Control (Wild type)
Lane III	+ (Plasmid DNA)
Lane IV-Lane VI	B33 *ipt* transformants
Lane VII &Lane VIII	SAG *ipt* transformants

4.1.12 Hardening and green house cultivation

The transformants and wild plants were hardened by growing them in the green house condition, which had transgenic containment facility (Plate 10). Transformants differed from wild type in morphological features. The weekly observation of biometric characters and physiological analysis were carried out.

Plate 9

Plate 10

Plate 9. Wild and transformants growing in the tissue culture medium

Plate 10. Transgenics and wild type plants growing in the green house with transgenic containment facility

4.2 Effect of *ipt* overexpression on growth, physiology and secondary metabolite content

4.2.1 Shoot length

Significant variation in shoot length was observed between wild type and transformants. Data on the effect of *ipt* overexpression on the shoot length of *Bacopa monnieri* is presented (Table 2). The shoot length was more in transformants than in the wild.

The mean length recorded for wild in the weekly interval ranged from 4.8 to 23.3 cm and in the transformants it was ranging from 5.1 to 27.1cm for B33 *ipt* and 5.9 to 31.7 cm for SAG *ipt*. SAG *ipt* recorded more height than B33 and wild type plants (Fig. 8).

4.2.2 Root length

Root length showed significant variation between the wild and transformants. The root length was recorded on the 6^{th} day of root emergence from the cutting. The root length was more in wild compared to the transformants (Table 3).

In the wild type the mean root length was 3.56 cm and the mean of transgenic lines, B33 and SAG were ranging from 2.16 cm and 3.36 cm respectively. Wild type had more root length than the transformants. Among the transformants, B33 *ipt* recorded lowest root length (Fig. 9).

4.2.3 Number of leaves

Significant variation was observed in number of leaves between the wild and transformants. Data on the effect of *ipt* gene overexpression on the number of leaves of *Bacopa monnieri* is presented in table 4. The number of leaves were more in transformants compared to the wild.

In the wild type, the average number of leaves in weekly interval was 7, 20.8, 46.6, and 104.2. Both B33 and SAG *ipt* overexpressed plants had 7.6, 33.6, 98.8, 209.4 and 11.2, 37.6, 115.8, 223.6 respectively (Fig 10).

Table 2. Effect of *ipt* overexpression on shoot length of *Bacopa monnieri*

Treatment	I Week(cm)	II Week(cm)	III Week(cm)	IV Week(cm)	V Week(cm)
Wild	4.8	9.8	11.7	16.4	23.3
B33	5.1	10.3	14.5	21.0	27.1
SAG	5.9	11.3	17.4	24.7	31.7
Mean	**5.2**	**10.4**	**14.5**	**20.7**	**27.4**
CD (5%)	**0.99**	**2.06**	**3.56**	**3.94**	**4.16**

Table 3. Effect of *ipt* overexpression on the root length of *Bacopa monnieri*

Treatment	Root length (cm)
Wild	3.56
B33	2.16
SAG	3.36
Mean	**3.0267**
CD (5%)	**0.5331**

Table 4. Effect of *ipt* overexpression on the number of leaves in *Bacopa monnieri*

Treatment	I Week	II Week	III Week	IV Week
Wild	7	20.8	46.6	104.2
B33	7.6	33.6	98.8	209.4
SAG	11.2	37.6	115.8	223.6
Mean	**8.6**	**28.11**	**68.16**	**139.61**
CD(5%)	**1.779**	**10.098**	**14.446**	**30.012**

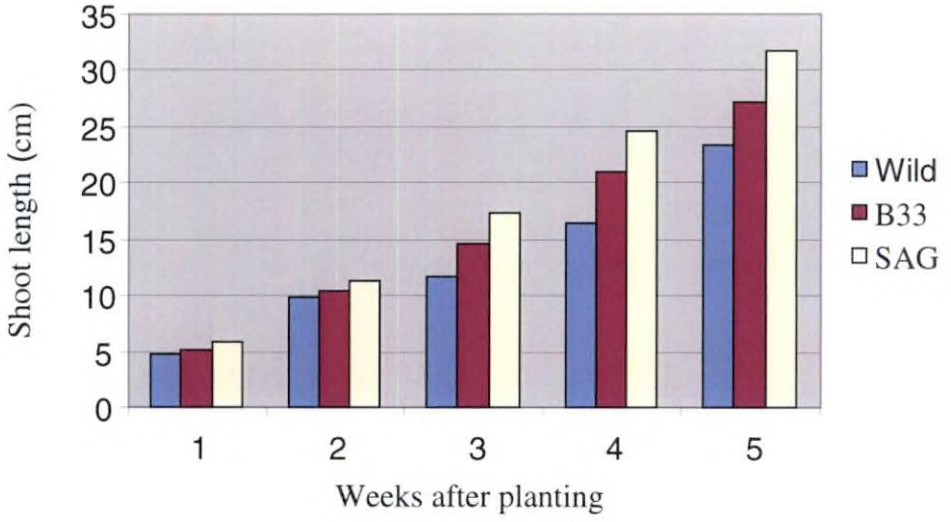


Fig. 8. Effect of *ipt* overexpression on shoot length in *Bacopa monnieri*

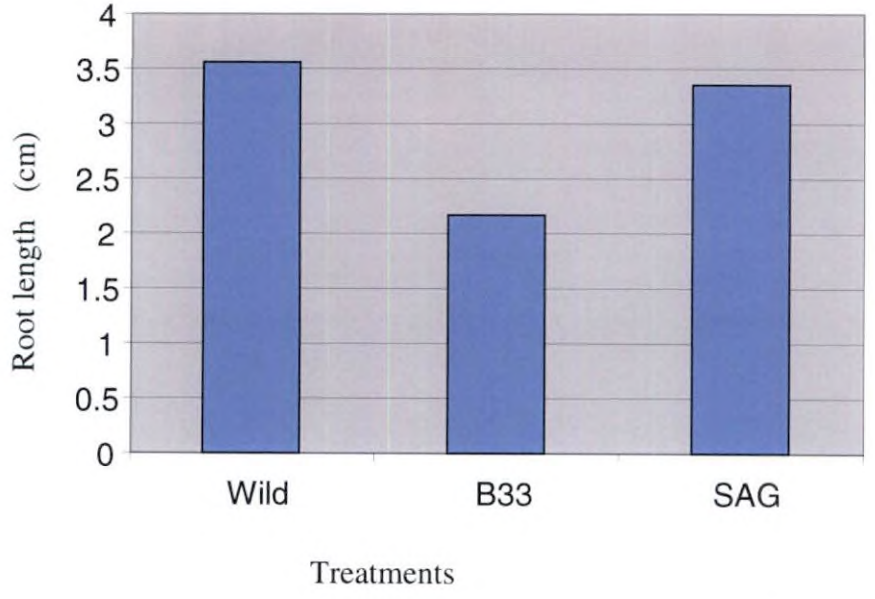


Fig. 9 Effect of *ipt* overexpression on root length in *Bacopa monnieri*

4.2.4 Number of branches

Data on number of branches showed a significant variation between the wild and transformants. The numbers of branches were more in the transformants compared to wild type (Table 5).

In the wild type the number of branches increased steadily from 1.2 in first week to 21.4 in fifth week and in both transformants viz, B33, it increased from 1 to 40 and SAG it was 2 to 46.6. SAG *ipt* transformant had given highest number of branches compared to wild type and B33 transformant (Fig. 11).

4.2.5 Relative water content

There was a significant variation in relative water content between the wild type and transformants. Data on the effect of *ipt* gene overexpression on the RWC of *Bacopa monnieri* is presented (Table 6). The relative water content was more in wild compared to the transformants at 7th week after planting.

The wild plant had an average RWC of 92.71% where as B33 *ipt* had an average of 88.85% and SAG had 88.74% (Fig.12).

4.2.6 Total chlorophyll content

Data on total chlorophyll content showed a significant variation between the treatments. Total chlorophyll content increased linearly till 5th week after planting for all the plants and after 5th week, only the transformants recorded an increasing trend for total chlorophyll content (Table 7).

SAG *ipt* recorded highest total chlorophyll content followed by B33 *ipt* and wild type. With increase in the senescence, decreasing trend for total chlorophyll was observed both for B33 *ipt* and wild type plants where as SAG *ipt* has recorded an increasing trend for total chlorophyll with a maximum of 0.64 mg/g of fresh weight (Fig. 13).

Table 5. Effect of *ipt* overexpression on the number of branches in *Bacopa monnieri*

Treatment	I Week	II Week	III Week	IV Week	V Week
Wild	2.0	3.8	7.4	14.0	21.4
B33	1.0	6.4	16.0	31.4	40.0
SAG	2.0	6.8	18.0	35.4	46.6
Mean	**1.4**	**5.6**	**13.8**	**26.9**	**36.0**
CD(5%)	**0.8715**	**1.180**	**2.846**	**5.006**	**6.116**

Table 6. Effect of *ipt* overexpression on the RWC of *Bacopa monnieri*

Treatment	RWC (%)
Wild	92.71
B33	88.85
SAG	88.74
Mean	**90.10**
CD(5%)	**1.68**

Table 7. Effect of *ipt* overexpression on the total chlorophyll content (mg/g FW) of *Bacopa monnieri*

Treatment	II Week	III Week	IV Week	V Week	VI Week	VII Week	VIII Week
Wild	0.44	0.41	0.47	0.48	0.45	0.43	0.38
B33	0.51	0.44	0.51	0.58	0.58	0.56	0.48
SAG	0.43	0.46	0.49	0.57	0.60	0.64	0.63
Mean	**0.45**	**0.43**	**0.48**	**0.54**	**0.54**	**0.54**	**0.49**
CD(5%)	**0.049**	**0.038**	**0.066**	**0.050**	**0.028**	**0.040**	**0.049**

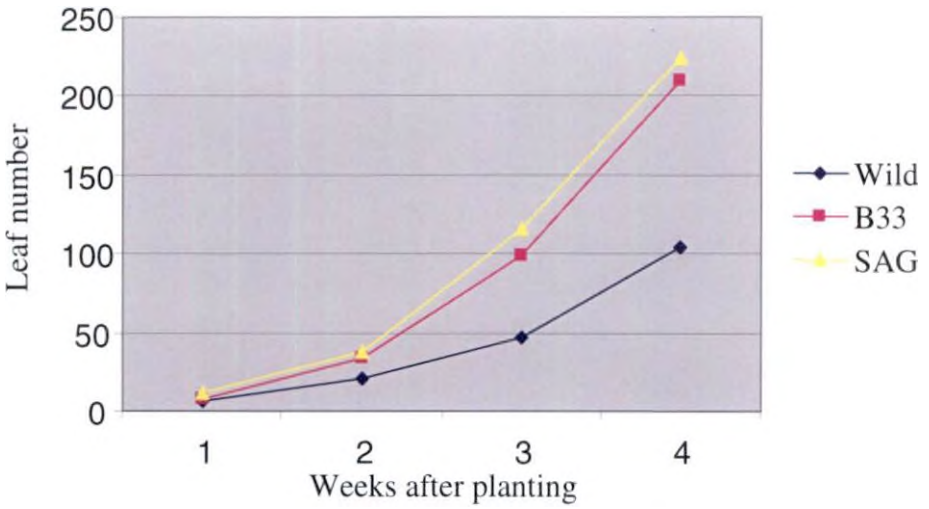


Fig. 10. Effect of *ipt* overexpression on number of leaves in *Bacopa monnieri*

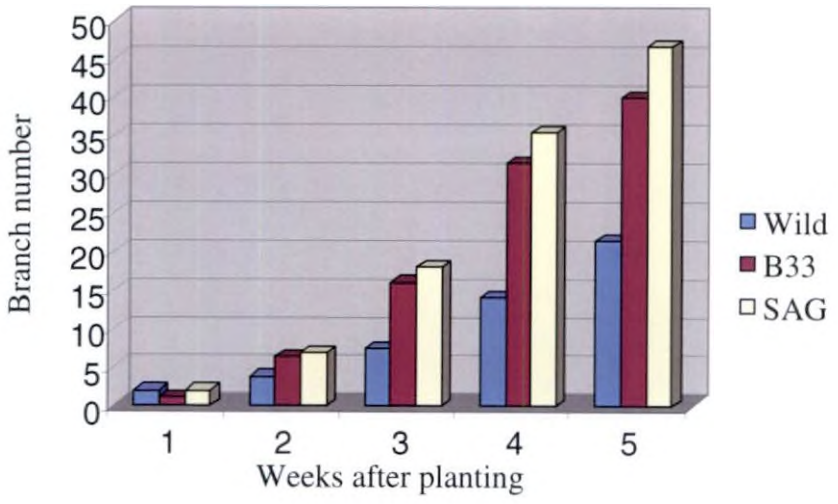


Fig.11. Effect of *ipt* overexpression on number of branches in *Bacopa monnieri*

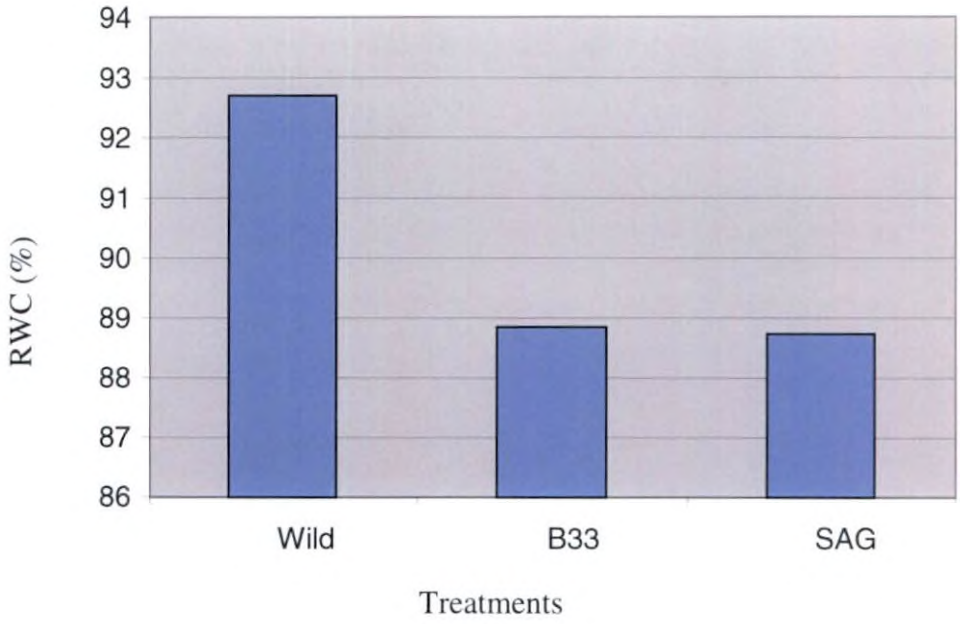


Fig. 12. Effect of *ipt* overexpression on the relative water content of *Bacopa monnieri*

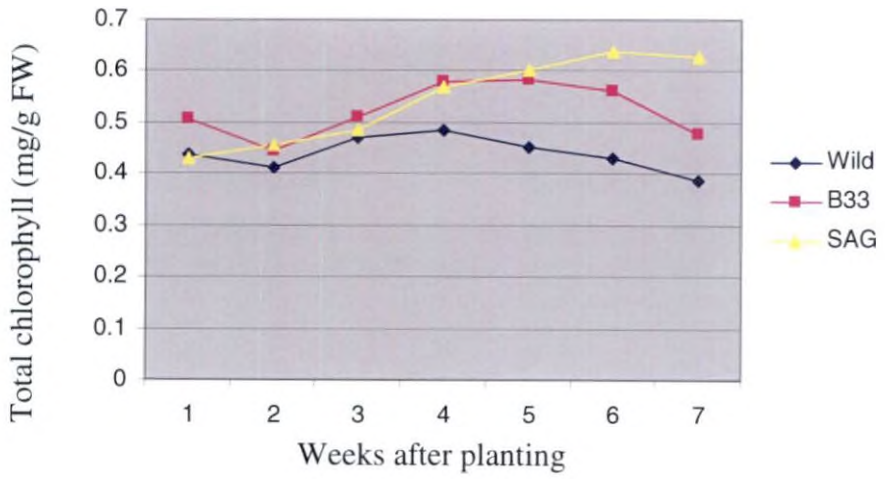


Fig. 13. Effect of *ipt* overexpression on the total chlorophyll content of *Bacopa monnieri*

4.2.7 Chlorophyll a content

There was a significant variation in chlorophyll a content between the wild and transformants. Data on the effect of *ipt* gene overexpression on the chlorophyll a of *Bacopa monnieri* is presented in table 8. The chlorophyll a was more in transformants compared to the wild. Transformant with SAG promoter recorded the maximum content of chlorophyll a during the period of 5-8th week (senescence stage).

The wild type of plant gave reduced yield of chlorophyll a (0.292 mg/g of fresh weight), with ageing transformants maintained a higher amount of chlorophyll a (0.374 mg/g and 0.49 mg/g of fresh weight in B33*ipt* and SAG *ipt* respectively) (Fig. 14).

4.2.8 Chlorophyll b content

The observation on chlorophyll b significantly varied between the treatments. Similar to chlorophyll a, the chlorophyll b also had the same trend for wild type of plant. There was no decrease in the SAG *ipt* transformants for chlorophyll b content (0.140mg/g of fresh weight). B33 *ipt* transformants gave decreasing trend after 7th week of planting (Fig. 15). Data on the effect of *ipt* gene overexpression on the chlorophyll b of *Bacopa monnieri* is presented (Table 9).

4.2.9 Total soluble protein content

A significant variation was found in the total soluble protein content between the wild type and transgenic plants with B33 and SAG promoters. The value of protein content estimated is shown in the table 10. Wild type recorded lowest amount of total protein content while both transgenics recorded similar protein content which was higher than the wild type (Fig. 16).

Table 8. Effect of *ipt* overexpression on the chlorophyll a (mg/g FW) content of *Bacopa monnieri*

Treatment	II Week	III Week	IV Week	V Week	VI Week	VII Week	VIII Week
Wild	0.34	0.31	0.36	0.37	0.35	0.32	0.29
B33	0.40	0.35	0.41	0.46	0.46	0.44	0.37
SAG	0.36	0.36	0.38	0.45	0.46	0.50	0.49
Mean	**0.36**	**0.34**	**0.38**	**0.43**	**0.42**	**0.42**	**0.37**
CD(5%)	**0.051**	**0.033**	**0.048**	**0.045**	**0.032**	**0.034**	**0.067**

Table 9. Effect of *ipt* overexpression on the chlorophyll b (mg/g FW) content of *Bacopa monnieri*

Treatment	II Week	III Week	IV Week	V Week	VI Week	VII Week	VIII Week
Wild	0.096	0.090	0.104	0.102	0.102	0.090	0.086
B33	0.108	0.10	0.114	0.122	0.126	0.116	0.108
SAG	0.104	0.104	0.106	0.126	0.134	0.138	0.140
Mean	**0.102**	**0.098**	**0.108**	**0.116**	**0.120**	**0.113**	**0.108**
CD(5%)	**0.020**	**0.009**	**0.015**	**0.016**	**0.010**	**0.015**	**0.016**

Table 10. Effect of *ipt* overexpression on total soluble protein content of *Bacopa monnieri*

Treatment	Total Protein (mg/g FW)
Wild	38.6
B33	58.4
SAG	56.0
Mean	**51.0**
CD(5%)	**5.162**

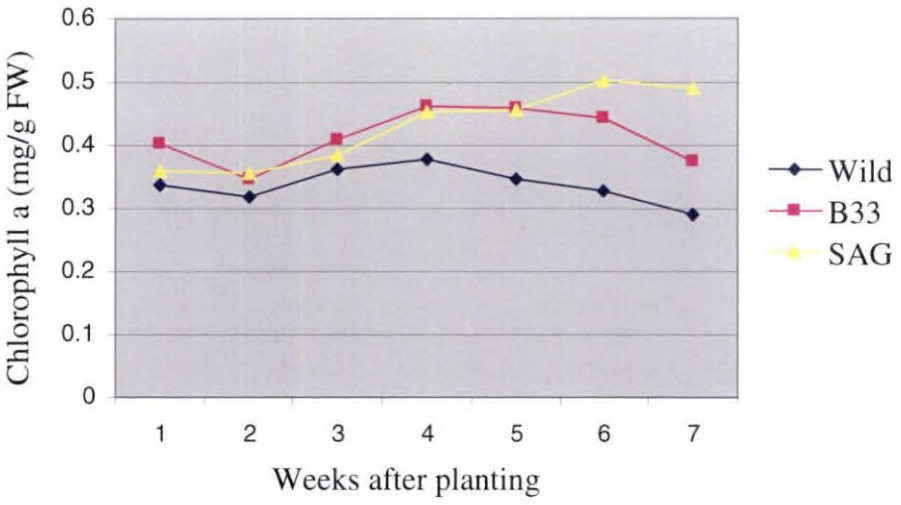


Fig. 14. Effect of *ipt* overexpression on the chlorophyll a content of *Bacopa monnieri*

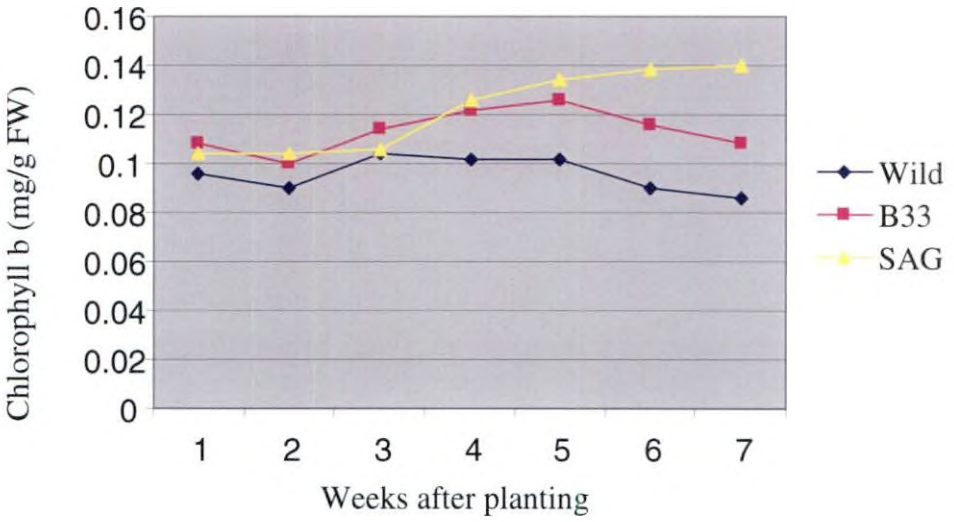


Fig. 15. Effect of *ipt* overexpression on the chlorophyll b content of *Bacopa monnieri*

4.2.10 Stomatal frequency

Stomatal frequency in the wild type and transgenic plants differed significantly. Wild type recorded lowest number of stomata in both upper and lower part of leaf surface. Both transgenic plants with B33 and SAG promoter recorded higher number of stomata in the lower side (2641 and 2528 for B33 and SAG respectively) than in the upper side (1890 and 1778 for B33 and SAG respectively) of the leaf. The stomata are present almost equal number on the both surface. Data on stomatal frequency in wild and transgenic plants are given in table 11.

4.2.11 Stomatal resistance

There was no significant variation between the treatments in stomatal resistance.

4.2.12 Cytokinin content

Cytokinin content was estimated by ELISA using Phytodetek kit. The standard curve was plotted using 'graph pad' software programme and the concentration of iPA was calculated using the standard curve (Fig. 17). A significant variation in the cytokinin content was found between wild and transformants.

The average value of iPA concentration in wild was 142.6 picomoles/g of fresh weight and there was a considerable increase in iPA concentration in B33 *ipt* transformant (73.91%) and in SAG *ipt* transformant (64.51%) plants (Table 12).

4.2.13 Bacoside content

Bacoside was quantified using HPLC method. There was no significant variation in bacoside content between the wild type and transgenic plants. The data on effect of *ipt* overexpression on bacoside content of *Bacopa monnieri* is given below (Table 13).

Table 11. Effect of *ipt* overexpression on stomatal frequency of *Bacopa monnieri*

Treatments	Stomatal frequency	
	Upper	Lower
Wild	1198	1172
B33	1890	2641
SAG	1778	2528
Mean	**1622.1**	**2113.9**
CD (5%)	**78.51**	**30.48**

Table 12. Effect of *ipt* overexpression on cytokinin content of *Bacopa monnieri*

Treatment	iPA (pico moles/g FW)	% Increase
Wild	142.6	
B33	248	73.91%
SAG	234.6	64.51%
Mean	**208.4**	
CD(5%)	**25.72**	

Table 13. Effect of *ipt* overexpression on bacoside content of *Bacopa monnieri*

Treatment	Total bacoside % (w/w)*
Wild	2.90
B33	3.13
SAG	2.75
Mean	**2.93**

* mean of two samples

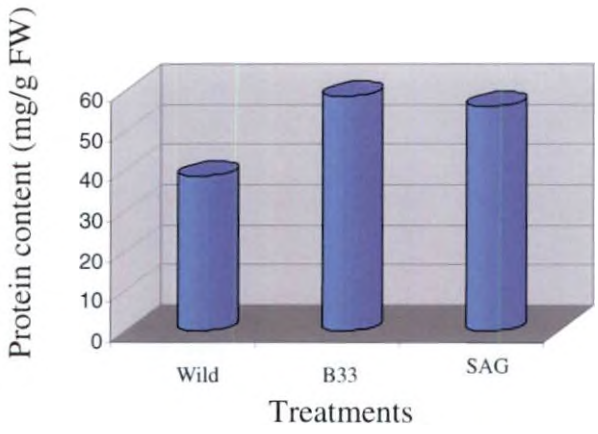


Fig. 16. Effect of *ipt* on total soluble protein content of *Bacopa monneri*

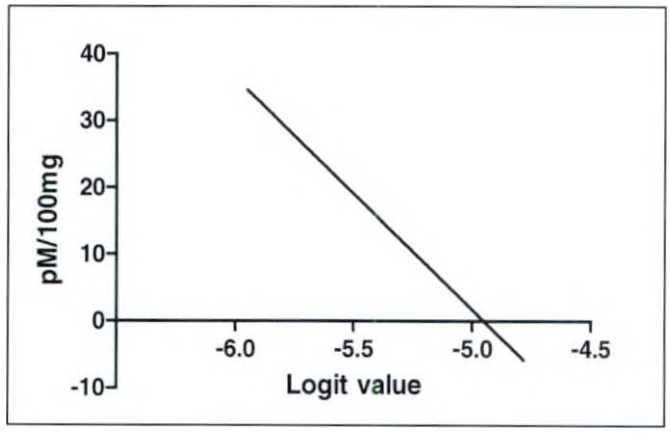


Fig. 17. Standard curve for iPA

Discussion

5. DISCUSSION

Growth and development is a result of genetic makeup of an organism and its interaction with environment. Most of the plant processes are triggered on receiving an appropriate signal which could be 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 processes 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. Cytokinins are one of the important groups of growth hormones, implicated in formative effects such as cell division, regulation of root and shoot growth and branching, chloroplast development, leaf senescence, stress response and pathogen resistance.

Because of memory enhancing ability of bacoside, a saponin found as secondary metabolite in the bacopa plant, bacopa is getting more popularity among the medicinal plants having both research and commercial value. Hence an experiment was proposed to overexpress *ipt* in *Bacopa monnieri* through *Agrobacterium* mediated transformation and to regenerate the transformed plants through tissue culture for analyzing the influence of overexpression of *ipt* gene on growth, physiology and secondary metabolite production.

The results of various experiments conducted to address the above objectives are discussed with sufficient supports from previous studies.

5.1 *Agrobacterium* Mediated Transformation of *Bacopa monnieri*

Among the different gene delivery systems available, *Agrobacterium tumefaciens* mediated gene transfer is most widely used method to introduce foreign genes into dicots (Weisings et al., 1988). Many plant species including several medicinal plants have been successfully transformed with *Agrobacterium tumefaciens* mediated gene transfer system.

Nisha et al. (2003) have standardized the genetic transformation of brahmi using *Agrobacterium tumefaciens* strain EHA 105. In the present study we have also used the *Agrobacterium tumefaciens* strain EHA 105 for transferring the *ipt* gene.

The *ipt* gene constructs were obtained from the Plant Molecular Biology Division, Rajiv Gandhi Centre for Biotechnology. The *ipt* constructs fused with SAG and B33 promoter were used for the present investigation to study the differential expression. Initially these constructs were transferred to *Escherichia coli* strain JM 109. Transformation was confirmed by restriction digestion of plasmid isolated from the recombinant cells with *Hind*III and *Eco*RI.

Triparental mating was done to transfer the gene construct from the *Escherichia coli* cells to *Agrobacterium tumefaciens* strain EHA 105. This was done by using pRK 2013 helper bacterial strain along with recombinant *E.coli* and *Agrobacterium* cells. Transformed cells were selected by growing it in the selection media. Transformation was confirmed by double digestion of plasmid isolated from the transferred cells with *Hind*III and *Eco*RI.

Pre incubated leaf explants of *Bacopa monnieri* were co cultured with recombinant *Agrobacterium tumefaciens* strain EHA for two days. This was grown in the selection media containing 15mg/l kanamycin and 300mg/l cefotaxime. The transformed plants showed reduced growth compared to non transformed plants in selection medium. Kuehnle and Sugii. (1992) and Belarmine and Mii (2000) also observed that transformed plants show slow growth compared to untransformed once.

5.2 Regeneration of transformed plants by tissue culture

The regenerated shootlets were grown in MS medium devoid of growth hormones. Nisha et al. (2003) have reported culturing of bacopa is possible in the MS medium without addition of growth hormones. Transformed shootlets in this study showed no rooting in the plain MS medium and hence it was sub cultured in the medium containing 1/2 MS supplemented with 1mg/l IAA and 1mg/l GA. The lack of rooting observed in this experiment is due to higher content of cytokinin in the transformed shootlets since cytokinin inhibits the root inhibition. Taiz and Ziegar (2006) reported inhibition of rooting in the cytokinin overexpressed plants. Rooting was observed in the media containing 1 mg/l IAA and 1 mg/l GA. This may be due to antagonistic effect of GA on cytokinin. Wainberg et al. (2005) also reported that GA3 and spy-4 inhibit the induction of the cytokinin primary-response gene, type-A Arabidopsis response regulator 5 which is important for the cytokinin response.

The DNA was isolated from selected plants using CTAB method. DNA samples were amplified by PCR with primers specific for the *npt*II and *ipt* gene. The sample showed a varied quantity of amplified product when the products were run on a 0.8% agarose gel. In the case of *npt*II gene, one of the transformant has not given amplification (Plate 5). The amplification product of *ipt* was appreciable in one of the transformant and other transformants were also shown a clear banding at 0.6 kb (Plate 4). As the initial concentration of the DNA sample used for the PCR was been same, this difference in amplification could be attributed to the increased number of integration of the transgene in the genomic DNA of the plant. Nan and Kuehnle (1995) also observed a dosage effect that may arise from gene duplication and multiple insertions in a varied quantity of amplified product for 0.7 kb *npt*II fragment. The same result was observed in the PCR analysis of Chrysanthemum (Seo et al., 2003).

RNA was isolated from the selected transformants using Trizol reagent. The amount of RNA was quantified spectometrically at 260 nm. This was further used for cDNA synthesis and RT-PCR.

The RT-PCR was done for further confirmation for transfer of *ipt* gene. Initially the cDNA was synthesized from mRNA by reverse transcription using M-MuLV Reverse Transcriptase enzyme. The analysis confirmed the presence of actin gene in all the transformants and also in wild plants. But the presence of *ipt* and *npt*II gene was shown only by transformants and not by wild type.

Southern hybridization analysis was done using the PCR amplified product for *ipt* gene. The vacuum blotting method was used for transforming the DNA from agarose gel to nylon membrane. The probe bounded only in transgenic plants and no band was found in wild type. This could be attributed to the presence of *ipt* only in the transformants.

5.3 Effect of *ipt* overexpression on growth, physiology and secondary metabolite content

The transgenic plants confirmed for the presence of gene was grown in a specific mixture of soil: cow dung: manure with 1:1:1 ratio. Wild type was also grown in the same condition along with the transformants in green house observing containment facility.

Knowledge on the overexpression of *ipt* on growth and physiology of medicinal plant will not only enhance the basic information on cytokinin action but also will be useful to regulating the growth and metabolism of the plant.

With this objective in mind, the leaf samples were taken in weekly interval for the different physiological and growth analysis.

5.3.1 Growth parameters

Cytokinins play an important role in regulating different developmental processes and growth parameters, such as shoot length, root length, number of leaves, number of branches etc.

In the present experiment a significant variation in plant height was found between the wild type and transgenic plants with B33 and SAG promoters. Zhang et al. (2005) reported developmental and physiological characteristics of the PSAG12-*ipt* transgenic rapes. They found that transgenic plants had higher vigor than the controls. Observed effect in these transformants is due to enhanced level of cytokinin in transgenic plants compared to wild type and it is well established that cytokinins regulate cell division in shoot cells (Taiz and Zieger., 2006).

Cytokinins at normal physiological concentration inhibit root growth (Werner et. al., 2001). In the present experiment the transgenics recorded low root length compared to wild type. Several reports were found on reduced root formation and stunted root growth in *ipt* transformed plants of different species (Taiz and Zieger., 2006).

The *ipt* overexpressed plants recorded a significantly increased number of branches. It is well documented that cytokinin modifies apical dominance and initiates growth of lateral buds. Cytokinin overexpressed plants tend to be bushy with more number of branches (Taiz and Zieger., 2006). Consequent to increased number of branches, the transgenic plants showed a higher number of leaves. The result of this experiment is supported by Werner et al. (2001) who indirectly showed by overexpression of cytokinin oxidase, a strong retardation of shoot development and leaf number owing to the reduction of cytokinin in transformants.

5.3.2 Physiological parameters

Cytokinins elicits a broad range of responses like retention of chlorophyll, incorporation of amino acids, protein retention in leaves, all of which indicates delayed senescence (Gardner et al., 1985).

Leaf samples were collected weekly up to 8th week of planting for estimating the total chlorophyll, chlorophyll a and b. Total chlorophyll content of wild type and transgenic plant with both promoters showed an increasing trend up to fifth week of

planting. After fifth week there was a decreasing trend in all the plants. This might be due to the lack of nutrient availability, since the plants were retained in the same pots up to 8th week. The transgenic plants showed higher chlorophyll content even after 5th week of observation. The transgenic plant with B33 promoter had 24% increase and transgenic plant with SAG promoter recorded 63% higher chlorophyll than the wild type on 8th week of observation (Table. 14). This could be attributed to the enhanced level of cytokinin in the transgenic plants particularly with senescence induced SAG12 promoter. It is well documented that cytokinin promotes chloroplast development and delay the senescence (Taiz and Zeiger., 2006). Sa et al. (2001) have reported over expression of isopentenyl transferase gene (*ipt*) from T-DNA into *Artemisia annua* under the control of the CaMV 35S promoter. They observed an increase in chlorophyll content up to 20-60%. Gan and Amasino (1995) reported that overexpression of *ipt* with SAG12 promoter in tobacco plant, delayed the senescence. Similar reports were also given by Garratt et al. (2001) and McCabe et al. (2001) in lettuce. Cao. (2001) also had the report of PSAG12-IPT overexpression in rice cultivars. They found that chlorophyll content and photosynthetic rates of flag leaves of transgenic rice lines were higher than those of the wild-type rice by 41.23 and 60.24%, respectively. A similar report was given by Zhang et al. (2005) in rapes.

Since there was a significant difference in the total chlorophyll content between the wild type and transformants and also between transformant plants developed with two different promoters, the analysis of chlorophyll a and chlorophyll b was done separately to know the effect of *ipt* overexpression on chloroplast development.

Both chlorophyll a and chlorophyll b were significantly differed in transgenic plants and wild type. At the onset of senescence, the senescence activated promoter SAG recorded 68% and 63% increase in the chlorophyll a and chlorophyll b compared to the wild, where as transgenic plant with B33 promoter recorded 28% and 26% increase in the chlorophyll a and chlorophyll b content over wild type. Hu et al. (2005) in *Festuca arundinacea* have reported a similar increase in chlorophyll a and chlorophyll b content

by transforming the embryogenic calli with *Agrobacterium tumefaciens ipt* gene using a maize ubiquitin promoter. A similar report was found by Smigocki et al. (1993) in tobacco system transformed with *ipt*. In the present study also transformants had higher cytokinin level compared to wild type which could be responsible for higher chlorophyll content in transgenic plants.

Table 14. Percentage increase in the chlorophyll content on 8th week of planting in B33 and SAG transgenic plants over wild type of *Bacopa monnieri.*

Treatments	Total chlorophyll	Chlorophyll a	Chlorophyll b
B33	23.83%	28.08%	25.58%
SAG	62.69%	67.80%	62.79%

The enhanced growth rate and higher chlorophyll content observed in the transgenic lines might help the plant to have a higher assimilation rate and protein synthesis.

Total soluble protein, which is the reflection of functional enzymes in the tissue, is an indication of the metabolic rate of the tissue. The protein content was higher in the transgenic plants than wild type. The transgenic plant with senescence specific promoter SAG had an increase of 45% and B33 promoter plant had 51% increase compared to wild type. Gardner et al. (1985) reported that cytokinins elicit incorporation of amino acids and protein retention in leaves.

Cytokinins which are transported from roots to the shoots via xylem, based on a signal from the shoot will regulate the water status of the shootlets (Taiz and Zeiger., 2006). In the present study the transformants recorded lower RWC (88.85% and 88.74% in B33 and SAG plants respectively) compared to wild type (92.71%). This result is in agreement with the observation on stomatal frequency in this experiment. The transformants had significantly higher stomatal frequency than in the wild type. Another interesting observation was on the distribution of stomata. Generally bacopa, a marshy

plant having almost equal number of stomata on adaxial and abaxial sides. But in this experiment the transformants produced using different promoters recorded higher number of stomata on the abaxial side. Stomatal frequency observed on the upper and lower sides of the wild plant was 1199 and 1172 respectively and that of B33 transformant was 1890 and 2641 and SAG transformant was 1779 and 2529 respectively (Fig. 18). Though the number of stomata was significantly differed, stomatal resistance was on par with transgenic plant and wild type owing to the reason that the plants were grown under uniform conditions. Wang et al. (1997) also have reported an increased number of stomata in the transgenic tobacco plant overexpressing *ipt*.

5.3.3 Effect of *ipt* overexpression on cytokinin content of *Bacopa monnieri*

Cytokinins are adenin derivatives with an isoprenoid side chain and play an essentail role in plant development (Kakimoto, 2003). Regulating the cytokinin level is one of the way by which we can control the plant growth and development including the secondary metabolism. Considerable reports are there for increasing the endogenous cytokinin level by overexpressing *ipt* (The gene coding for an enzyme regulating the first committed step in cytokinin biosynthesis) in tobacco (Makarova et al., 1997, Ma et al., 2002), in Lettuce (McCabe et al., 2001, Garratt et al., 2001), in Rice (Cao., 2001) etc. Controlled expression of *ipt* is possible by using specific promoters like seed specific (Ma et al., 2002), anther specific (Sa et al., 2002), endosperm-specific (Shen et al., 2004), senescence specific (McCabe et al., 2001) and constitutive expression by Ca35S promoter (Smigocki and Owens., 1988).

In the present experiment two different promoters were used, B33 and SAG12 to analyze the effect of overexpression of *ipt* on cytokinin levels in brahmi. The isopentenyl adenosine (iPA), an important cytokinin was quantified in wild and transgenic plant at 5th week of planting by ELISA using iPA detection kit (Phytodetek kit from Sigma). The cytokinin content was more in the transformants than in the wild type (Fig. 19). Among the two different transformants, transformant with B33 promoter recorded 73.91% increase and transformant with SAG12 promoter recorded 64.51% increase over wild type. Since the senescence was initiated only in the 7th week of planting, the cytokinin

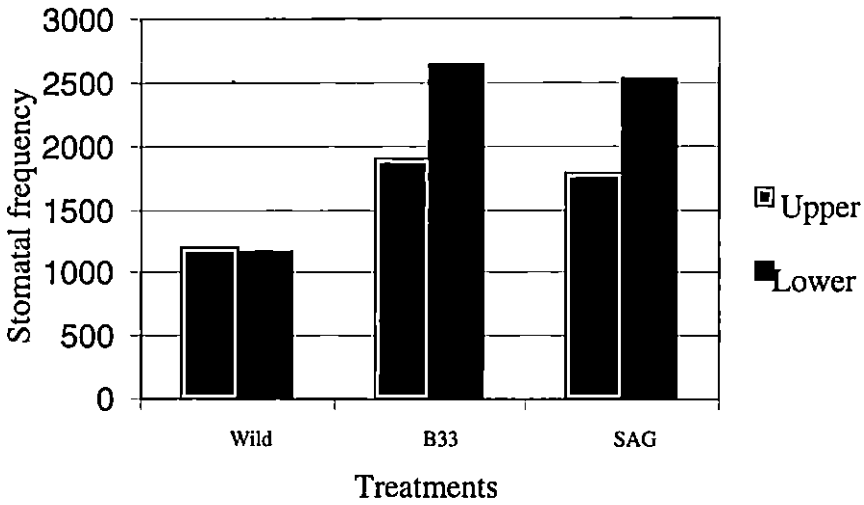


Fig. 18. Effect of *ipt* overexpression on stomatal frequency of *Bacopa monnieri*

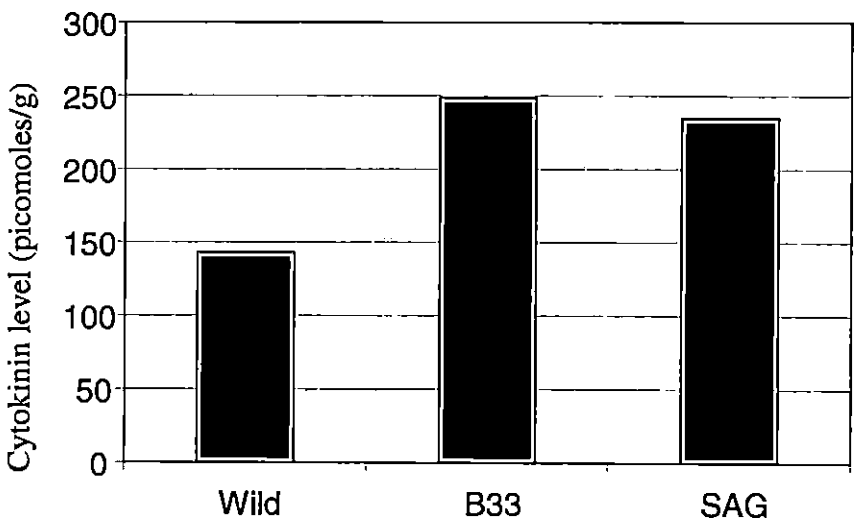


Fig. 19. Effect of *ipt* overexpression on the cytokinin content of *Bacopa monnieri*

level in the senescence induced SAG12 transformant was slightly less compared to the B33 trangenics. McCabe et al. (2001) have reported that an increased cytokinin content in the transgenic lettuce over expressed with *ipt* gene under the control of the senescence-specific SAG12 promoter from *Arabidopsis thaliana* (PSAG12-IPT). Sa et al. (2002) also have reported a 3-4 fold increase in the level of iPA+iPs in the leaves, petals, pistils, and stamens compared with those in the wild type plants overexpressed with ipt gene from *Agrobacterium tumefaciens* T-DNA under the control of a TA29 promoter. In another report, Shen et al. (2004) driven *ipt* gene under the endosperm-specific rice prolamin promoter (P) into tobacco and there was an increase in iPAs level in transgenic seeds compared with that in seeds of control plants.

5.3.4 Effect of *ipt* overexpression on total bacoside content of *Bacopa monnieri*

Bacoside is a triterpenoid saponin which comes under the terpenoid group of secondary metabolites. This is the active constituent found in the brahmi which is responsible for most of its medicinal value. Terpenoides are generally biosynthesized either in the plastid through glyceraldehyde phosphate/ pyruvate path way or in the cytosol through classical acetate/mevalonate pathway with sufficient cross talk between the two path ways. The triterpenoid bacoside biosynthesis can be enhanced by altering rate limiting enzymes in its metabolic path way or by enhancing plastid development. Lakshmi et al. (2006) have reported a two fold increase in the bacoside by overexpressing HMG-CoA reductase, the rate limiting enzyme in the mevalonate path way.

In this experiment we have tried an alternate approach by regulating the plastid regeneration and to study its effect on bacoside biosynthesis by overexpressing the *ipt* gene.

Cytokinin effect on secondary metabolism is species specific. Garnier et al. (1996) investigated the effect of cytokinins on accumulation of indole alkaloids in periwinkle callus cultures by overexpression of light inducible *ipt* gene and reported that

no correlation between endogenously produced cytokinin for the alkaloid production. Where as Sa et al. (2001) have reported over expression of isopentenyl transferase gene (*ipt*) from T-DNA into *Artemisia annua* via *Agrobacterium tumefaciens*. They observed an increase in artemisinin content up to 30-70% compared with the control plants. In this experiment the bacoside content was estimated by HPLC and variation was non significant between wild type and transgenic plants. A similar report was given by Garnier et al. (1996). The result from this experiment and previous reports shows that the bacoside biosynthesis may be mainly through the cytoplasmic mevalonate pathway.

Summary

6. SUMMARY

Plant metabolism is essential for growth and development of cells and tissues. Until recently, secondary metabolism was generally regarded as nonessential processes that produced as by- products. However, there is evidence that secondary metabolites have ecophysiological roles including plant defense against high light, drought, salinity and herbivores and microorganisms and their concentration was increased under such condition. In addition, the cytotoxic nature of some secondary metabolites has made them medicinally important.

Genetic transformation is a powerful tool for enhancing the productivity of novel secondary metabolite of limited yield. Direct manipulation of DNA sequences to alter gene expression in medicinal plants, is area that is ripe for expansion. One approach is to identify enzymes of a metabolic pathway and then manipulate these enzymes to provide better control of that pathway. Another approach is to introduce new genes for the increased yield of secondary metabolites. Plant growth hormones are mainly involved in the control of growth and metabolism of plant system. Only very few reports were found about the relationship between the changes of endogenous plant hormones content and the production of secondary metabolites. In view of this, the major emphasis of the present investigation was given to study the influence of overexpression of *ipt* gene on the growth and secondary metabolite production in *Bacopa monnieri*. To achieve these objectives, *ipt* gene was overexpressed in the *Bacopa monnieri* plant and the effect of this gene on the growth and metabolism was overlooked. The salient findings of the study are summarized below.

Agrobacterium* mediated transformation of *Bacopa monnieri

Bacopa monnieri cultures were obtained from Rajiv Gandhi Centre for Biotechnology, Trivandrum, Kerala, India. The cultures were maintained in MS medium supplemented with 30g l^{-1}sucrose and 6g l^{-1}phytagar.

Escherichia coli strain JM 109 were transformed with pBI B33 *ipt* and pBI SAG 12 *ipt* constructs independently and selected by growing on LB_{kan} media with kanamycin concentration of 50 ppm. Plasmids were isolated from recombinant *E.coli* cells using alkali lysis method. The isolated plasmids were double digested with restriction enzymes, *Eco*RI and *Hind*III. Triparental mating was done using *Agrobacterium* strain EHA 105, PRK 2013 and *E.coli* cells with *ipt* gene construct.

Pre incubated leaf explants of *Bacopa monnieri* were co-cultivated with the recombinant *Agrobacterium* for two days and then selected on regeneration medium containing 15mg/l kanamycin and 300mg/l cefotaxime. The transformants were sub cultured in the medium containing1/2 MS supplemented with 1mg/l IAA and 1mg/l GA.

Genomic DNA was isolated from the transformants and from the wild type brahmi plant using CTAB method. The integration of T-DNA region in the putative transformants was confirmed by PCR analysis using primers specific to *npt*II and *ipt* genes independently. In both PCR amplification, with specific primers showed the amplification of 600bp fragment in all putative transformants, while the control wild plant did not show any amplification.

Four transformants along with wild type, which are confirmed with PCR analysis, were selected for RT-PCR confirmation. The total RNA was isolated from both transformant and wild using Trizol reagent. The amount of RNA was quantified spectometrically at 260 nm. First strand of c-DNA was synthesized from mRNA by reverse transcription using M-MuLV Reverse Transcriptase enzyme. The presence of *ipt* and *npt*II gene was shown only by transformants and not by wild type plant. Southern hybridization analysis was done using the PCR amplified product for *ipt* gene. The phosphor imaged nylon membrane showed the presence of gene in all transformants but wild has not given the band specific for the *ipt*.

The Transformants and wild plants were hardened by growing them in the green house condition, which had transgenic containment facility.

Effect *ipt* overexpression on growth, physiology and secondary metabolite content of *Bacopa monnieri*

Significant variation in plant height was observed between wild type and transformants. Root length between the treatments also varied significantly. In the wild type the mean root length was 3.56 cm and the mean of transgenic lines, B33 and SAG were 2.16cm and 3.36cm respectively.

Number of leaves and number of branches were higher in transgenic plants compared to the wild type. Relative water content of transformants was lower compared to wild type and higher number of stomata was observed in the transgenics compared to the wild type. The distribution of stomata was also differed significantly, in wild type the distribution was equal in both upper and lower surface of leaf but in transformants a higher number of stomata was observed at the lower surface than the upper surface.

The levels of total chlorophyll, chlorophyll a and chlorophyll b were measured at weekly interval. There was an increasing trend was found in all the treatments till fifth week. From sixth week onwards, plants started to show a decreasing trend for the total chlorophyll and for chlorophyll a and b content, while transformants were able to retain the higher amount of total and also chlorophyll a and chlorophyll b content. Total soluble protein was also found to be higher in the transformants than in the wild type.

Quantification of cytokinin, iPA was done by ELISA by using iPA detection kit (Phytodetek kit from Sigma). The cytokinin content was more in the transformants than in the wild type. Among the two different transformants, transformant with B33 promoter recorded 73.91% increase and transformant with SAG12 promoter recorded 64.51% increase over wild type. Bacoside, the secondary metabolite of the plant was estimated by HPLC. There was no variation was found between the wild type and transformants.

Future line of work

Physiological and morphological evaluation of transgenic plants and their behavior under different environments. Quantification of other major cytokinin from transgenic plants. Relative expression of transgene under various inducers. Assessing *Hmg-CoA reductase* activity in transgenic plants. Knock out strategy to study the functionality of terpenoid biosynthetic path way genes.

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Appendices

APPENDICES

BUFFERS AND REAGENTS

AB minimal stocks

AB buffer, 100 ml

K_2HPO_4	6g
NaH_2PO_4	2g
pH	7.0
	Autoclaved

AB salts, 100ml

$CaCl_2$	0.3g
$FeSO_4.7H_2O$	5mg
KCl	0.3g
$MgSO_4.7H_2O$	0.6g
NH_4Cl	2g
	Autoclaved

AB minimal medium 100ml

AB buffer	5ml
AB salts	5ml
Glucose	0.5%
	Autoclved

AB minimal agar, 100ml

AB buffer	5ml
AB salts	5ml
Glucose	0.5%
Bacteriological agar	15g
	Autoclved

Antibiotics

Ampicillin	100mg/ml in sterile water
Cefotaxim	250mg/ml in sterile water
Kanamycin	50mg/ml in sterile water
Rifampicin	20mg/ml in 95% ethanol

LB agar, 1 liter

NaCl	10g
Tryptone	10g
Yeast extract	5g
pH	7.0 with NaOH
Bacteriological Agar	15g
	Autoclaved

LB broth

NaCl	10g
Tryptone	10g
Yeast extract	5g
pH	7.0 with NaOH
	Autoclaved

Molecular Biology

Chloroform: isoamyl alcohol	24:1
EDTA (pH 8)	0.5M, pH adjusted with NaOH pellets
	Autoclaved
Ethidium Bromide	10mg/ml in sterile water
Glucose	20% in water, autoclaved
Magnesium chloride	1M in water, autoclaved
RNase stock solution	10mg/ml RNase in sterile water
Tris -HCl	1M Tris, pH adjusted to 6.8, 7.5, 8 and 9.5 with concentrated HCl, Autoclaved

Gel Loading Dye (DNA/RNA tracking double dye), (10X)

Bromophenol blue	0.2%(w/v)
EDTA(pH 8)	10mM
Glycerol	50%(w/v)
Xylene cyanol FF	0.2%(w/v)
	Autoclaved

SSC (20X), 1 litre

Sodium chloride	175.3g
Sodium citrate	88.2 g
	Autoclaved

TAE (50X), 1 litre

0.5 M EDTA (pH 8)	100 ml
Glacial Acetic acid	57.1ml
Tris	242 g
	Autoclaved

TBE (5X), 1 litre

0.5 M EDTA (pH 8)	20ml
Boric acid	27.5g
Tris	54g

TE Buffer

EDTA(pH 8)	1mM
Tris-HCl(pH 8)	10mM
	Autoclaved

Genomic DNA isolation-CTAB method (all reagents autoclaved)

2X CTAB buffer

CTAB	2 %(w/v)
EDTA(pH 8)	20mM
NaCl	1.4M
PVP(M_r 40,000)	1%
Tris-HCl(pH 8)	100mM

10% CTAB solution

CTAB	10 %(w/v)
NaCl	0.7M

CTAB precipitation solution

CTAB	1%
EDTA (pH 8)	10mM
Tris-HCl(pH8)	50mM

High-salt TE

EDTA (pH8)	1mM
NaCl	1M
Tris-HCl(pH 8)	10mM

Plasmid Isolation

Solution I (Cell resuspension solution)

EDTA (pH 8)	10mM
Glucose	50mM
Tris-HCl	25mM

Solution II (Cell lysis solution)

NaOH	0.2N
SDS	1%

Solution III

Sodium acetate	3M in water, pH adjusted with glacial acetic acid to5.2, autoclaved.

Southern hybridization

1M Sodium phosphate (pH 7.2) 500ml

$Na_2HPO_4.7H_2O$	134g
85%H_3PO_4	4ml made to 500ml with Milli Q water. Filter sterilized and stored at 4°C

Alkaline transfer buffer

NaOH	0.4M
NaCl	0.6N

Sodium Phosphate buffer

Sodium Phosphate (pH 7.2)	0.5M
SDS	7 %(w/v)
EDTA (pH 7.0)	1mM

Denaturation solution	1.5M NaCl 0.5M NaOH
Depurination solution	0.25 N HCl
Neutralization solution	1.5 M NaCl 0.5M Tris HCl(pH8)
Salmon sperm DNA	10mg/ml in sterile water, sheared by sonication, stored as aliquots in –20°C
Stripping solution	0.1% SDS with boiling water

Plant Tissue Culture

Growth regulator

1 mg/ml of all growth regulators were made in sterile water

BA	1mg/ml in 1N NaOH
IAA	
GA_3	1mg/ml in 1N NaOH
MS (Murashige and Skoog) powder	Sigma

Cytokinin estimation

TBS buffer

Tris base	0.303g
NaCl	0.584g
$MgCl_2.6H_2O$	0.02g
$Na.N_3$	0.1g
pH	7.5
H_2O	100ml

Washing buffer

NaCl	8g
KCl	0.2g
KH_2PO_4	0.2g
$Na_2HPO_4.2H_2O$	2.3g
Tween-20	0.5ml
H_2O	1000ml

PHYSIOLOGY AND SECONDARY METABOLITE PRODUCTION IN GENETICALLY TRANSFORMED BRAHMI (*Bacopa monnieri* L.WETTST.) WITH CYTOKININ SYNTHESIZING ISOPENTENYL TRANSFERASE (*ipt*) GENE.

VIGHNESHA

Abstract of the
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DEPARTMENT OF PLANT PHYSIOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM 695522
KERALA, INDIA

ABSTRACT

ABSTRACT

An experiment was conducted in the Department of Plant Physiology, College of Agriculture, Vellayani, to overexpress cytokinin synthesizing *ipt* gene in *Bacopa monnieri* through *Agrobacterium tumefaciens* mediated transformation and to regenerate the transformed plants through tissue culture for analyzing the influence of overexpression of *ipt* gene on growth, physiology and secondary metabolite production. The transformation and molecular works were done in Rajiv Gandhi Center for Biotechnology, Trivandrum. *Escherichia coli* strain JM 109 was transformed independently with pBI B33 *ipt* and pBI SAG12 *ipt*. Triparental mating was done using *Agrobacterium tumefaciens* strain EHA 105, pRK 2013 and recombinant *E.coli*. Plasmids were isolated from recombinant *E.coli* and recombinant *Agrobacterium* cells to confirm the successful transformation of constructs. Both have showed the insertion release when double digested with restriction enzymes *Eco*RI and *Hind*III. Pre incubated leaf explants of *Bacopa monnieri* were co-cultivated with the recombinant *Agrobacterium* for two days and transferred to regeneration medium containing MS supplemented with $2mgl^{-1}$ BA, $15mgl^{-1}$ kanamycin and $300mgl^{-1}$ cefotaxime. Putative transformants were regenerated from co-cultivated explants when placed on the selection medium containing 15mg/l kanamycin and 300mg/l cefotaxime. Uninfected explants failed to regenerate in presence of kanamycin. Rooting was not found in the MS medium devoid of growth regulators. Sub culturing of shootlets was done in MS medium supplemented with 1ppm GA and 1ppm IAA. Hardening was done to the fully rooted plants and were kept in five replications for further analysis. DNA was isolated from both wild type and transformants. PCR amplification for *npt*II and *ipt* gene specific primers showed presence of gene in transformants but not in the wild type. From the selected transformants, RNA was isolated and RT-PCR was done. RT-PCR analysis confirmed the expression of *ipt* and *npt*II gene in all the transformants, while there was no expression in the wild type. Expression of constitutively expressed plant gene –actin was used as loading control. Southern hybridization of PCR amplified products gave the evidence for the presence of *ipt* gene only in transformants but not in wild type. Physiological and biometric

observations were performed on both transformants and wild type which served as control over the transformants. Plant height was more in transformants compared to the wild. Both root length and relative water content was more in wild compared to the transformants. Other parameters like number of branches and number of leaves were higher in the transformants than in the wild. Total chlorophyll, chlorophyll a and chlorophyll b were found to increase for first five weeks in all treatments, after that there was a decrease in the total chlorophyll, chlorophyll a and b in wild type but transformants were able to retain higher contents throughout the period of study. Total soluble protein content was higher in the transformants than the wild type. Stomatal frequency showed a significant difference between the treatments. Higher number of stomata was observed in the transgenics compared to the wild type. The distribution of stomata also differed significantly. In wild type the distribution was equal in both upper and lower surface of leaf but in transformants a higher number of stomata were observed at the lower surface than the upper surface. Cytokinin content was estimated using ELISA. There was a significant variation in cytokinin, iPA concentration between wild type and transformants. Transformants had higher cytokinin content than the wild type. The transformant with B33 promoter had more cytokinin content than transformant with SAG promoter. Bacoside, the major secondary metabolite of the plant was estimated by HPLC and its content between the wild type and transformants were found to be on par. In this experiment, the overexpression of *ipt* gene in bacopa resulted a higher amount of cytokinin in transgenics and hence had higher growth rate, protein and pigment content. Overexpression of *ipt* may not increase the bacoside content in the bacopa.