DEVELOPMENT OF TRANSGENIC LINES OVERPRODUCING MANNITOL IN FINGER MILLET(*Eleusine coracana. Gaertn*) BY *Agrobacterium* MEDIATED GENE TRANSFER.

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This is to certify that the thesis entitled **"DEVELOPMENT OF TRANSGENIC LINES OVEREXPRESSING MANNITOL IN FINGER MILLET (*Eleusine coracana.* Gaertn.)" BY *Agrobacterium* MEDIATED GENE TRANSFER"** submitted in partial fulfillment of the requirement for the degree of **Master of Science (Agriculture),** in Crop Physiology to the University of Agricultural Sciences, Bangalore, is a record of research work carried out by, **Ms.Jayashree S.H.** under my guidance and supervision and that no part of the thesis has been submitted for the award of any other degree, diploma, associateship, fellowship or any other similar titles.

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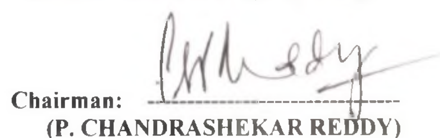

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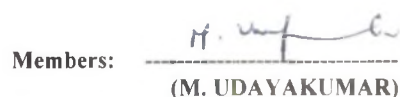

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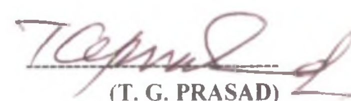

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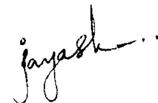

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INTRODUCTION

I. INTRODUCTION

Finger millet (*Eleusine coracana*_Gaertn.) is an important small millet food crop of south India. The annual planting area of our country is around 20 million hectares with an average productivity of around 1440 kg/ha. In India, this crop is mainly grown in southern states mainly in Karnataka, which contributes more than 50 percent of total production.

Finger millet is mainly grown in rainfed areas where intermittent moisture stress is a common problem, which affects growth and productivity of this crop. Low yields under rainfed conditions are primarily due to poor crop stand, because of poor germination and seedling establishment. Due to erratic rainfall and salinity condition of the soil, the plant growth and productivity levels are low and loss due to drought/salinity accounts nearly 50 percent of total production. Enhanced water uptake capacity by the germinating seeds and young seedlings and maintenance of cell metabolic activities assumes importance for maintaining high growth and developmental processes under stress. There is a need to increase moisture stress tolerance of this crop during germination and at early stages of development.

To counteract the effect of moisture stress, plants have evolved different adaptive mechanisms like drought escape, dehydration tolerance and dehydration postponement. Accumulation of compatible osmolytes is one of the most important dehydration tolerance mechanisms adopted by the plants under stress. The compatible osmolytes help in osmoregulation, considered to be one of the best mechanisms of drought resistance for crops exposed to dehydration stress specifically at the early stages of plant development. The important osmolytes produced by the plants are sugar alcohols like mannitol and pinitol ; amino acids like proline ; quarternary ammonium compounds like glycine betaine and sugars like trehalose and fructan. These osmolytes are produced in high concentrations under stress and help in maintenance of tissue hydration and cell turgor thereby maintaining cell metabolic activities.

Mannitol is the most widely distributed sugar alcohol in nature and has been reported in more than 100 species of vascular plants, including many important horticultural and agronomic crops. Mannitol is a six-carbon non-cyclic sugar alcohol. Plants producing mannitol exclusively are not known. In higher plants mannitol metabolism is not well understood. However pathway has been characterized in celery, where mannitol is produced by the reactions of mannose-6-phosphate isomerase, mannose-6-phosphate reductase and mannitol-1-phosphate phosphatase. Thus, mannitol biosynthesis, in celery proceeds via a different pathway which requires an additional enzyme-catalyzed step, than that in transgenic tobacco (Rumpho et al., 1983) where in, mtlD (mannitol-1-phosphate dehydrogenase) expression leads to mannitol biosynthesis by converting cytoplasmic fructose-6-phosphate to mannitol-1-phosphate. Evidences supporting the role of mannitol in stress tolerance have been obtained through genetic engineering of bacterial mtlD gene there by overproducing mannitol in the transgenic plants.

Plant genetic engineering has opened new avenues to modify crops and has provided new solution to solve specific needs. The combination of genetic engineering and conventional breeding programmes permits incorporation of useful traits into the commercial plants.

The ability to engineer transgenic plants is a powerful and informative means for studying gene function and regulation. Advances in tissue culture combined with improvement in transformation technology have resulted in increased transformation efficiency. Significant progress has been made in recent years in understanding stress tolerance using molecular biology approaches.

Several techniques have been developed to introduce foreign genes into the plants. The techniques like microprojectile, macroprojectile, biolistic, electroporation etc are in use. Among these methods *Agrobacterium* mediated transformation is the primary means of gene transformation in most plants. This system is advantageous because of the ease of

the protocol, higher efficiency, more predictable pattern of DNA integration and low copy number of integration. Transgenic plants obtained by this method often contain single copy insertion. These advantages were driving force to adapt this system to many different crops including monocots.

During the process of *Agrobacterium* transformation, a specific segment of the vector, T-DNA, which can be engineered to contain a selectable marker and genes of interest, is transferred from the bacterium to the host plant cells and inserted into the nuclear genome. These functions are mediated by a set of virulence genes with optimal expression occuring at acidic pH and in the presence of phenolic inducers, such as Acetosyringone, that are released by wounded plant cells.

Agrobacterium can efficiently transform dicots, but transformation of monocots is a problem since these have low wound response. But *Agrobacterium* can tranform monocots if the receptor substance Acetosyringone is supplemented in tissue culture media, even if they are generally not their natural hosts. Efficient *Agrobacterium* mediated transformation is developed in rice (Hiei et al., 1994) and in maize (Gould et al., 1991). Transformation of wheat immature embryo's and embryogenic calli is reported (Cheng et al., 1997). Barley transgenics were recovered after transformation of immature embryo's (Ishida et al., 1996).

The major focus of this study is to obtain the transformation and expression effectiveness of the gene construct mtlD. It was proposed to express mtlD in finger millet through *Agrobacterium* mediated transformation, to achieve transgenic plants synthesizing high levels of mannitol for drought tolerance.

Taking these into consideration the following are the objectives of the present investigation:

1. To develop efficient protocols for callus induction and regeneration of finger millet.
2. To develop efficient transformation protocol by using the *Agrobacterium binary* vector pCAMBIA 1380 having mtlD gene in finger millet.

3. Development of transgenic finger millet plants overproducing mannitol.

The finger millet variety used was GPU-28, one of the widely grown high yielding varieties recommended for southern India.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Plant productivity is greatly influenced by environmental stresses, such as freezing, drought, salinity and flooding. One of the ways in which tolerance to these factors can be achieved is by the transfer of genes encoding protective proteins or enzymes from other organisms. Key approaches currently being examined are, engineering alterations in the amounts of osmolytes or osmo-protectants, saturation levels of membrane fatty acids and rate of scavenging of reactive oxygen intermediates (ROS).

There are different techniques for transferring foreign genes into the genomes of plants, which include polyethylene glycol mediated gene transfer, microinjection, particle bombardment and *Agrobacterium* mediated gene transfer. *Agrobacterium tumefaciens* based gene transfer is most widely used system for introducing genes. Tissue culture stage is required in most current transformation protocols to ultimately recover plants. Indeed, it is the totipotency of plant cells that underlies most transformation systems. In the present study, attempts were made to develop transgenic plants overproducing mannitol, by transferring gene mannitol-1-phosphate dehydrogenase (mtlD), which is the rate-limiting enzyme in the mannitol biosynthesis. Therefore review was covered on the following topics.

I. Tissue culture studies in cereals

II. Transformation studies in cereals

III. Studies on Osmolyte- Mannitol

I. Tissue culture studies in cereals and millets

Various factors determining success of tissue culture optimizations in Graminae are discussed here separately both for callus formation and Morphogenesis.

(i) Callus induction

Factors affecting callus formation

(a) Explant

The choice of explant tissue is critical for successful induction of embryogenic callus. In the last few years, protocols have been developed for plant regeneration from *in*

vitro cultures for all major cereal species (Bright and Jones, 1985) and also for some of the millets like pearl millet (Lambe et al., 1999), foxtail millet (Osuna et al., 1995) and finger millet (George and Eapen, 1990). Practically the inter-play of explant, stage of differentiation, endogenous hormone concentrations and the genotype has significant effects on callus induction (Maddock, 1985). Different types of explants have been used successfully to establish totipotent cultures. The most widely used source of explant being embryo at specific stage of development, followed by young inflorescence and unexpanded leaves. Various explants for plant system were used to select the explant for callus induction and regeneration.

Green and Philips (1975) reported first about plant regeneration of maize from immature embryos, excised from 10-15 days after pollination but the frequency of embryonic callus initiation was higher in shoot apices in comparison with immature zygotic embryos (Lambe et al., 1999). In case of foxtail millet green and etiolated shoot apices produced embryonic callus (Osuna et al., 1995). Wang and Zapata, (1987) suggested that young inflorescence and scutellum of mature seed were the appropriate explants for establishing wild rice species via somatic embryogenesis.

(b) Genotypic Effects

Genotype plays an important role in the success or failure of the tissue culture experiments. Significant genotypic differences have been found in terms of efficiency of callus formation, somatic embryogenesis and plant regeneration.

In maize, regeneration from culture is genetically controlled by nuclear genes (Hodges *et al.,* 1986). William *et al.,* (1989) also suggested that at least one gene or a block of genes control the expression of somatic embryogenesis from maize tissue culture. Peng and Hodges, (1989) also presented evidence in rice cultures, that regeneration ability is under control of both nuclear and cytoplasmic genes. In sorghum Ma *et al.,* (1987) found that the ability to form regenerable callus varied among genotypes was heritable and acted as dominant trait. They felt at least two gene pairs were involved. Genetic analysis of *in vitro* characters of different lines of pearl millet showed that additive gene action was predominant for callus growth (Mythili et al., 1997). However, Close and Gallougher-Ludeman (1989) presented

evidence that the induction of regenerable callus in maize is largely physiological phenomenon that can be manipulated by the type and concentration of plant growth regulators, independent of the genetic back ground of the explant.

Genetic differences may be related to variations in endogenous hormone levels (Norstog 1970). Even explants from single genotypes do not respond identically in culture, most likely due to varying gradients of endogenous hormones (Werieke and Brettell, 1982). Immature embryos collected from the same inflorescence behave differently in culture, depending on size and location on the inflorescence. Response of explants from well-nourished plants is different from those of nutrient-deficient plants (Duncan *et al.*, 1985).

These examples indicated that something with an explant is as critical for given response as is its genotype. The variety of media and hormone concentrations used in cereal tissue culture adds testimony to the variations inherent in the explant and among genotypes.

(c) Plant Growth Regulators

Plant growth regulator requirement for callus formation and plantlet regeneration varied as explant and genotypes. Many cereal explants express embryogenesis competence in the presence of 2,4-D. A few other auxins have also been used with equal or greater success. Maggioni *et al.*, (1989) found that 2,4-D induced embryogenic rice callus. However, among other auxin sources tested, PCPA was not effective. But Dicamba and Kinetin resulted in enhanced wheat regeneration from callus cultures (Papenfuss and Carman, 1987). In finger millet somatic embryos from callus was induced by Picloram and low levels of kinetin (Eapen and George, 1989). Cultured caryopsis of finger millet produced callus from shoot apices or mesocotyl depending on picloram and cytokinin combinations (Eapen and George, 1990).

In addition to 2,4-D, cytokinins are also used for callus induction. Low levels of cytokinins were known to promote shoot growth in sorghum cultures (Wernicke *et al.*, 1982) and several investigators have used low levels of cytokinins in sorghum callus

induction medium (Bhaskaran and Smith, 1988; Bhaskaran *et al.*, 1983). Initiation of embryogenic callus from shoot meristem cultures of sorghum required 2,4-D and low levels of kinetin (Bhaskaran and Smith, 1988). In Sorghum, the shoot apical meristem with subtending embryogenic callus can be used to produce callus or shoots by varying the auxin or cytokinin ratios in the induction medium (Bhaskaran and smith, 1989).

A combination of auxins and cytokinins was found to be suitable for embryogenic callus initiations in several cultivars of rice (Ling *et al.*, 1983; Raghava Ram and Nabors, 1984). In maize, embryogenic callus formation and plant regeneration were accomplished in the absence of cytokinins (Vasil and Vasil, 1986). In Wheat, 2,4-D alone or in combination with cytokinins was used for callus initiation (Mathias *et al.*, 1986). Wheat cultivars produced regenerable callus on 2,4-D (Sears and Deckard, 1982). Shoot formation was improved when such callus was transferred to a medium containing cytokinin (Briman *et al.*, 1987). In many cases, a decrease in 2,4-D concentration or addition of cytokinin promoted but proliferation embryogenic callus formation of barley has been reported with the use of 2,4-D (Chu *et al.*, 1984; Thomas and Scott, 1985). Enhancement of embryonic callus formation with the addition of 6-benzyl adenine purine (BAP) in the medium was observed in barley by Rengel and Telaska, (1986). Norstog, (1970) also reported a positive correlation between kinetin concentrations and embryoid production. These reports signify that production of regenerable cultures could be explained by genotypes having different levels of endogenous cytokinin.

ii. Morphogenesis

Factors affecting organogenesis

a. Genotype

Grisham and Bourg, (1989) found drastic differences in shoot proliferation of two Sugarcane cultivars. Rajyalaxmi *et al.*, (1988) in Wheat, Rahaman *et al.*, (1991) in rice, Rao *et al.*, (1992) in sorghum documented genotypic variation for regeneration in different varieties. The genetic analysis of regeneration rate of different lines of

pearl millet suggested that additive gene action is predominant for frequency of regeneration (Mythili et al., 1997).

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b. Root formation

Root formation has been a easier task in most of the grasses with the exception of sugarcane. A medium with or without a lower concentration of any auxin was useful in most of the cereals. Bhaskaran and smith, (1990); Chin and Scott (1977) proposed that NAA (1.0 mg/l) was effective in inducing roots from wheat calli irrespective of their origin and age.

c. Age of Culture

A progressive decrease in morphogenetic potential usually results when plant tissues are maintained *in vitro* for prolonged periods through repeated sub-culturing (Chin and Scott, 1977; Gonzalez *et al.,* 1990). Henke *et al.,* (1978) observed that increased age of rice callus cultures almost completely inhibited shoot development.

d. Plant Growth Regulators

Bhaskaran and Smith, (1989) induced axillary buds in Sorghum callus on a medium with 2,4-D and high cytokinins, when the cytokinin concentrations was lowered, the same region predominantly produced roots. For regeneration, exogenous cytokinins are not required in all cases, probably because of adequate levels already present in some tissue (Norstog, 1970; Henke *et al.*, 1978). However those that do not contain adequate levels have to be supplied with exogenous cytokinins. Enhanced plant regeneration was obtained in pearl millet by using ethylene inhibitors like silver nitrate and cobalt chloride (Pinus et al.,1993). Papenfuss and Carman (1987) obtained enhanced regeneration from wheat callus cultures using kinetin. Cytokinin has been known to enhance tillering in Sorghum, which implicates a similar role in shoot bud initiation on tissue culture (Bhaskaran and Smith, 1990).

Hardening

It is recommended that removal of sugar from the support medium, pre conditioning to low relative humidity, high light intensity and high temperature can ensure high survival of root during transfer of plantlets to natural conditions. The gradual removal

of sugar is known to stimulate photosynthetic ability. Barbra *et al.*, (1981) indicated that potting with compost, plus rice husk, and white sand and keeping the tissue cultured plantlets under misted conditions were more favorable for plant survival.

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II. Tissue culture studies in Finger millet

Finger millet has not attracted much attention on the biotechnological front, except for the reports on callusing and plantlet regeneration in a few endemic varieties (Rangan, 1976; Thiru and Mohan ram, 1980; Mohanty *et al.*, 1985; Farook and Chander, 1989; George and Eapen, 1990; Selvi, 1990; Sivadas *et al.*, 1990; Kavi Kishore *et al.*, 1992). Rangan (1976) first showed that regeneration could be achieved using mesocotyl callus tissues. This was followed by successful attempts to induce calli form root, coleoptile and young leaves of 2 to 4 day old seedlings, but without plantlet regeneration (Thiru and Mohan Ram, 1980). Mohanty *et al.*, (1985) demonstrated formation of shoot buds from mesocotyl and leaf base tissue derived calli, on lowering the 2,4-D concentration. After shoots were regenerated, roots were induced by transferring them to media with half strength salts of MS medium. Hema, (2001) and Shivakumar, (2000) obtained successful callus formation from scutellar portion of the seed and regeneration by using different concentrations of 2,4-D and BAP. Genotypic differences with respect to callusing frequency and morphogenesis were noticed in leaf base and root explants of seedlings, also in glume explants form immature inflorescence from varieties Co 11 and 12 (Farook and Chander, 1989). The inflorescence explant was successfully induced to form calli capable of both organogenesis and somatic embryogenesis at the plant biotechnology section of BARC, Bombay (George and Eapen, 1990). Regeneration of plantlets occurred either at lower auxin concentrations (Thiru and Mohan Ram, 1980 and Mohanty *et al.*, 1985) or at specific auxin and cytokinin combinations (George and Eapen, 1990). Wakizuka and Yamaguchi (1987) reported kinetin to be the best cytokinin for regeneration. They obtained high frequency plantlet regeneration from 164 to 180 days old callus. Eapen and George, (1990) have studied the influence of phytohormones, carbohydrates, aminoacids and antibiotics on somatic embryogenesis and plant differentiation in finger millet. They observed that hormones enhanced somatic embryogenesis, carbohydrates produced the highest frequency of germinating

somatic embryos, and antibiotics like cefotaxime and carbenecillin enhanced plant differentiation, whereas, aminoacids had an adverse effect on germination of embryos.

Transformation studies

The plant transformation is a process where by DNA is introduced into plant cells. The proposal for plant transformation was initiated after the recognition of DNA is carrier of genetic information. Avery et al., (1994) showed that the DNA is the transforming principle in bacteria. Several procedures have been reported to accomplish gene transfer.

Direct gene transfer to protoplasts and genetic transformation

Transfer of a defined gene separated from the rest of the genome provides a unique method to achieve a well-defined genetic modification not achievable by normal breeding efforts. The development of direct gene transfer methods for cereals therefore was of great interest and importance, though it was achieved first with Tobacco, when isolated DNA was transferred to tobacco protoplasts and stable integration of the foreign gene was demonstrated into the genome of recipient cell (Paszkowski *et al.*, 1984). According to these experiments with model species, chimeric genes were transferred also into protoplasts of *Triticum monococcum*, *Zea mays* and *Oryza sativa* (Table).

Genetic Transformation of graminaceous protoplasts mediated by Direct DNA transfer

Species	**Method Of DNA Uptake**	**Reference**
Oryza sativa	PEG	Uchimiya *et al* .,1986
Triticum monococcum	PEG	Lorz *et al.* , 1985
Triticum monococcum	Electroporation	Ozias *et al* ., 1986
Zea mays	Electroporation	Rhodes *et al.,* 1988

The marker gene, Neomycin *Phosphotransferase in vitro* selection of transformed cells as well as the reporter gene GUS allowed biochemical assay of enzyme activity. DNA uptake into protoplasts can be achieved by treating the cells with PEG or by electroporation (Shillito e*t al.*, 1985).

Mechanical Transformation

Micro injection or Biolistics or Particle Bombardment

Serious limitations of direct gene transfer to protoplasts in cereals is the difficulty of regeneration. Protoplasts from most of cereals will not regenerate. Frequency of regeneration is low (Portrykus *et al.*, 1976). A major breakthrough and significant improvement in efficiency was achieved by delivering the DNA directly into the nucleus instead of cytoplasm (Reich *et al.*, 1986). In case of cereals only a limited number of cultures are available. Thus a chimeric tissue consisting of transformed as well as non-transformed cells may be the consequence.

Biolistics (Finner *et al.*, 1990) is the delivery of micro projectiles, usually of tungsten or gold coated with DNA and propelled into the target cells by acceleration. The acceleration can be provided by gunpowder, by gases such as Helium or CO_2 or by an electric discharge. This method can introduce DNA into virtually any tissue from any cultivar, success depends critically upon the ability of the target tissue to proliferate and give rise to a fertile plant.This technique was used in the cereals to transfer genes Gordan *et al.*, (1990), by which resulted in the successful transformation and regeneration in corn.

Agrobacterium mediated Transformation

The transformation of dicotyledons by *Agrobacterium* is well established. But in the case of monocots it is not general process. In the past monocots particularly graminaceous crop plants were considered to be recalcitrant to this technology and they were out side the *Agrobacterium* host range. However transformation methods based on the use of *Agrobacterium* are still preferred in many instances because of the

following advantages, (1) Easy to handle (2) High efficiency (3) more predictable pattern of foreign DNA integration.

Transformation of plants with *Agrobacterium* has advantages over microinjection or Biolistic method. This method generally results in higher rates of transformation (0.1-5% as compared with 0.01-1%) and more efficient and predictable for patterns of integration of the foreign DNA (Chan *et al.*, 1993; Wil mink and Dons, 1993; Binns, 1990).

Agrobacterium tumefaciens is a soil phytopathogen that genetically transforms host cells, causing crown gall tumors. The *Agrobacterium* plant cell interaction is the only known natural example of DNA transport between kingdoms. In this process DNA is transported from wild type *Agrobacterium* into the plant cell nucleus. Expression of this DNA (T-DNA) results in neoplastic growths on the host plant. The wild type T-DNA carries genes, involved in the synthesis of plant growth hormones and opines which are compounds formed by the condensation of an amino acid with a keto acid or a sugar. It is the induction of growth hormones in the transformed host cells which induces the formation of tumors. These tumors then synthesize opines, a major carbon and nitrogen source for *Agrobacterium. Agrobacterium* strains are usually classified based on the type of opines specified by the bacterial T-DNA, the most common strains being octopine or nopaline specific (Hooykaas and Beijersbergan, 1994). Opine import into and the subsequent catabolism within the bacterial cell requires specialized enzymes. Because these enzymes are encoded by the bacterium tumor inducing (Ti) plasmid, practically no other microorganism can mobilize opines, creating a favorable biological niche for *Agrobacterium*.

Three genetic components of *Agrobacterium* are required for plant cell transformation. The first component is the T-DNA, which actually is transported from the bacteria to plant cell. T-DNA flanked by two 25-bp imperfect direct repeats known as the borders. The second component is the 35kb virulence region also located on the Ti Plasmid, which is composed of seven major loci (Vir A, Vir B, vir C, Vir D, Vir G, Vir E, and Vir H). The protein products of these genes, termed virulence (Vir) proteins, respond to specific compounds secreted by the wounded plant to generate a copy of the T-DNA and mediate its transfer into the host cell. The

third component is the set of chromosomal virulence (chv) genes, located on the *Agrobacterium* chromosome. chv genes are involved in the bacterial chemotaxis and attachment to the wounded plant cell (Citvosky *et al.*, 1920; Zambryski, 1992). Because its borders define the T-DNA element, the coding region of the wild type T-DNA can be replaced by any DNA sequences without any effect on its transfer from *Agrobacterium* to the plant. Thus, *Agrobacterium* is often used to produce transgenic plants expressing genes of interest.

Key factors involved in *Agrobacterium* - mediated gene transfer

Activation of vir genes

In prokaryotes, signaling often involves two families of signal transducing proteins. One family includes membrane sensor proteins and the other family comprises cytoplasmic proteins that transduce information from the membrane sensor. To regulate infection component, signal transduction system is composed of the virulence proteins Vir A and Vir G. Together these proteins sense signal molecules secreted by wounded plant cells and activate the expression of other vir genes, there by initiating the process of T-DNA transport (Winans *et al.*, 1994).

Wounded plants secrete sap with a characteristic acidic pH (5.0 to 5.8) and a high content of various phenolic compounds, such as lignin and flavanoid precursors. These conditions specifically stimulate *Agrobacterium* vir gene expression. The best-characterized and most effective vir gene inducers are monocyclic phenolics such as acetosyringone (Stachel *et al.*, 1985). Host specificity of *Agrobacterium* depends on the phenolic composition of plant exudates; few phenols are involved in wound signaling (Citvosky *et al.*, 1992). Monosaccharides such as glucose and galactose significantly increase vir gene expression when Acetosyringone is limiting or absent. Low opine levels enhance vir gene expression in the presence of acetosyringone (Veluthambi *et al.*, 1989).

The bacterium is attracted to wounded plants presumably of following signal molecules released by the plant cell (Hohn *et al.*, 1989; Stachel *et al.*, 1985).

Wounded tobacco (*Nicotiana sp.*) cells exude phenolic compounds such as acetosyringone and ∝-hydroxy acetosyringone that activate vir genes that are responsible for the transfer of T-DNA from *A. tumefaciens* to the wounded host cell (Stachel *et al.,* 1985). Bolton *et al.,* (1986) utilized seven phenolic compounds (catechol, gallic acid, progallic acid, p-hydroxybenzoic acid, protocatechuic acid and vanillin) to induce vir-gene activity. These phenols are important for *A. tumefaciens* to recognize suitable hosts and activate the vir loci on the Ti plasmid. The vir loci/gene mediate the T-DNA processing and delivery steps (Binns, 1990).

Grasses may not produce these compounds at sufficient level to serve as signal molecules. Usami *et al.,* (1987) worked with seven monocotyledons and Usami *et al.,* (1988) showed the presence of vir inducing compounds in tissue homogenates in *Triticium aestivum* L.

A. tumefaciens suspension to activate the vir genes prior to inoculation of the monocot tissue. The compounds include acetosyringone and nopaline for maize (Gould *et al.,* 1991) and potato wound exudate for yam (Schafer *et al.,* 1987) and rice (Chan *et al.,* 1993). Many investigators believe that inoculation of monocotyledons with *A.tumefaciens* treated with inducing compounds will significantly increase the number of transformation events in monocotyledons, resulting from *A. tumefaciens* treatment (Gould *et al.,* 1991; Chan *et al.,* 1993).

Explants

The development of transformation system for cereals using *Agrobacterium* mediated gene transfer is very crucial. Early experiments with *Agrobacterium* indicated that wounding of the host plants is required for development of tumors. Binns and Thomashow (1998) pointed out the distinct correlation in the literature between the wound-induced division of cells and competence of such cells to be transformed by *Agrobacterium tumefaciens*. They proposed that processes related to the synthesis of DNA and cell division are required for incorporation of foreign DNA into a host genome.

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Commonly used methods for the *Agrobacterium* mediated transformation of dicotyledons such as leaf-disk methods, depend strongly on wound responses of plants and are not very useful for monocotyledon plants. The main role of wounding in the transformation process are the induction of DNA synthesis and the rapid division of the cells, actively growing tissues from monocotyledons might be transformable in the presence of vir-inducing compounds.

The use of actively growing embryogenic calli is one of the most important factors in efficient transformation. Such calli can be obtained from mature or immature embryos, other tissues including shoot apex, immature inflorescence and young roots might also produce embryogenic calli but have not been tested extensively. Shoot apex are used as explant for developing transgenic rice, strong transient expression of GUS was observed in apical tissues of rice that had been co-cultivated with *Agrobacterium tumefaciens* (Li *et al.,* 1992). Putative transformants were generated by Godwin *et al.,* (1991) after inoculation of *Agrobacterium tumefaciens* into wounded coleoptiles of *In vitro* germinating seedlings. Expression of the GUS marker gene was detected histochemically in 23 plantlets (from 250 inoculated seedlings). Histochemical GUS expression was not observed in non transformed control sorghum seedlings.

Langridge *et al.,* (1992) generated transgenic plants of cereals by inoculating floret with *Agrobacterium*. This led to the production of embryos of wheat and barley with enhanced resistance to antibiotics. Plants of wheat, barley and maize were also recovered that gave positive hybridization signals with probes from within the T-DNA of the *Agrobacterium*.

Sung - Ho Lee *et al.,* (1999) used calli initiated from the scutellum of mature seeds for developing transgenic rice plants. Histochemical staining showed uniformly high levels of GUS expression.

Bacterial Strains and vectors

A large number of strains of *Agrobacterium tumefaciens* have been isolated and several of them have been modified for use in transformation. Strain A281 (Waston *et al.,* 1975) is a super virulent strain and its host range is wider and its transformation efficiency is higher than those of other strains (Hood *et al.,* 1987; komari, 1989). These characteristics are due to the Ti plasmid, PTiBo 542, that is harbored by this strain (Komari *et al.,* 1986). Two new types of system based on PTiBo 542 have been developed. The first involves strain EHA101 (Hood *et al.,* 1986) which carries a dis-armed version of PTiB0542. The second involves a super-binary Vir B, Vir C and Vir G from the virulence region of PTiBo542 has been introduced into a small T-DNA carrying plasmid (Komari *et al.,* 1990) that is used in a in a binary vector system. These systems have played important roles in studies of the transformation of monocotyledons.

Hiei *et al.,* (1994) listed the efficiency of various combinations of two strains and two vectors. The strains were the 'ordinary' strain LBA4404 (Hoekema *et al.,* 1983) and the super virulent strain EHA101. The vectors were pIG121 Hm, a derivative of the 'normal' binary vector pB1N19 (Bevan *et al.,* 1984) and pTOK 233 a derivative of a super binary vector pTOK 162 (Komari 1990). In transformation experiments, LBA 4404 (pTOK 233) was slightly more effective than both LBA 4404 (pIG12) and EHA 101(pIG121Hm) with the cultivar 'Tsukinohikari' and it was definitely the most effective with the cultivar 'koshikikari'. For unknown reasons, EHA 101(pTOK 233) was not very effective in transformation of rice. In recent results, LBA 4404 (pTOK 233) was found very efficient in transformation of Javanica Rice (Dong *et al.,* 1996).

Selectable Marker

Selection is an important part of the transformation process. In general, the gene of interest is co-delivered with a selectable marker to identify and encourage the growth of recipient cells. Selectable markers usually confer resistance to chemical agents, such as antibiotics or herbicides that inhibit various cellular functions (Wilmink and Dons 1993). Neomycin phosphotransferase (npt II) was used in many early attempts at the direct transformation of rice. This gene confers resistance to the amino

glycoside antibiotic kanamycin. While kanamycin can be used as selective agent during regeneration protoplasts. It is not effective for selection of transformed calli. In addition many calli recovered after kanamycin selection are unable to regenerate into green plants (Aryes and Park 1994). G418 is a related amino-glucoside antibiotic that is also inactivated by neomycin phosphotransferase. Not only with selection of transformed calli more efficient using G418 than Kanamycin (Aryes and Park 1994).

Another widely used, more effective selectable marker is hygromycin phosphotransferase (hpt) which confers resistance to the antibiotic hygromycin. The hpt gene has been used as an efficient marker gene for selection after *Agrobacterium*-mediated transformation (Dong *et al.*, 1996).Other potential selective agents include herbicides. Genes have been isolated for resistance to various commercially important herbicides. Among them the *bar* gene for phosphinotricin acetyl transferase might be the most valuable in rice (Aryes and Park 1994). The *bar* gene confers resistance to L-phosphinotricin (PPT), glyphosate and bialaphos. The *bar* gene has been successful used to select transgenic rice calli and plants (Cao *et al.*, 1992)

Scorable markers or reporter gene

Each of the transformation methods transient expression, generally performed with a reporter gene is a preliminary step used to identify conditions that will allow efficient DNA delivery. This simplest test provides the most efficient way of measuring the quantity of DNA introduced into the target cells. Several reporter genes are used in plants, including β-glucuronidase, Luciferase and genes involved in anthocyanin biosynthesis (Wilmink and Dons 1993).

Transgenic rice plants are developed and evidence for the regeneration of a large number of transgenic rice plants by the presence of introduced β-glucuronidase gene into rice plants gene was detected by DNA hybridization and then activity of β-glucoronidase detected in roots of transgenic plants (Zhang and Wu 1988). *Agrobacterium* mediated transformation of maize shoot tips from plants was identified by GUS assay (Gould *et al.*, 1991).

Transgenic rice plants are obtained through *Agrobacterium* mediated transformation and a. tumefaciens strains At 656 (PCNL56) and LBA 4404 (pTOK 233) to transform rice. Transgenic plants were produced with LBA 4404 (pTOK 233). Transformation was confirmed by southern blot analysis for the presence of GUS A gene and by transmission of GUS activity or progeny plants in a Mendelian manner (Aldemita and Hodges 1996).

Transgenic wheat plants are developed via *A. tumefaciens* from three kinds of explants of wheat. GUS assay showed that the plants are transformed GUS spots were present across all of the scutellum surface of freshly isolated immature embryos, GUS spots were localized on the areas starting to form callus in the precultured immature embryo's leaf sections showed GUS activity (Ming cheng *et al.*, 1997).
Transgenic rice plants are developed by *Agrobacterium* mediated transformation, where *Agrobacterium* strain used was LBA 4404 (pTOK 233) and AGLI (pCAMBI 1301). The Putative transformants are analyzed by GUS assay. Resistant calli grown in selection medium showed blue color. Plants regenerated from hygromycin resistant calli also showed GUS positive (Khanna and Raina 1999).

T-DNA Integration into host cell genomic DNA

The molecular mechanism by which T-DNA integration into host genomic DNA occurs is yet a lacking area. But techniques like PCR and Southern blot analysis etc. have given proof of T-DNA integration into host genomic DNA.

Bytebier *et al.,* (1987) demonstrated T-DNA integration into genomic DNA of *Asparagus officinalis* identical to T-DNA segments found in dicotyledonous plants transformed by *A. tumefaciens*. The *A. tumefaciens* strains were harboring wild-type nopaline and octopine tumor inducing plasmids. Selection of transgenic tissue on antibacterial, antibiotic-containing medium, followed by DNA blot hybridization was used to confirm transformation and integration of the T-DNA into the asparagus genome. They concluded that T-DNA was transferred and integrated into a monocot genome with *A. tumefaciens* as a vector, and T-DNA integration in asparagus was comparable to that in dicotyledons.

Small tumors were produced *in vitro* on bulbil tissue of yam (*Diascorea bulbifera*), after inoculation with *A. tumefaciens* (Schafer *et al.,* 1987), and the growth did not result if the bacterium was not pre-incubated with wound exudates from potato. The presence of nopaline was confirmed in the tumors; whereas, wounded bulbil tissue, bulbil tissue treated without the potato exudate, or the tissue treated with a virulent *A. tumefaciens* strain did not produce nopaline. A Southern blot demonstrated the integration of T-DNA into the monocotyledon nuclear DNA.

Raineri *et al.,* (1990) have demonstrated T-DNA expression and integration into the genomic DNA of rice following *A. tumefaciens* treatment. A super virulent strain A281 that contains pTiBo 542 was used to inoculate embryos of rice (*Oryza sativa*). Southern blots demonstrated the integration of the T-DNA. A second inoculation with an octopine strain produced rooty callus in which octopine was detected. Octopine was not detected in un- inoculated tissue.

Using *Agrobacterium* mediated transformation of maize shoot tips and expression of the *GUS* and *NPT II* genes was reported (Gould *et al.,* 1991). Foreign DNA was integrated into the host gnomic DNA and the expression were observed in progeny. Southern blot analysis of transformed rice seedling showed integration of T-DNA, and lack of hybridization with *vir* probes indicated *A. tumefaciens* was not present (Chan *et al.,* 1992).

Transformation and integration of T-DNA into genomic DNA was confirmed by Southern blot analysis of the progeny and sexual inheritance of the genes in a 3:1 ratio was confirmed. Transformation frequencies of 6.8% were obtained. Immature rice embryos were transformed using *Agrobacterium tumefaciens* containing *GUS* and *NPT II. Agrobacterium* strain LBA4404 (pTOK 233) Southern blots indicated two to three copies of the *GUS A* gene integrated in most transformants. *GUS* expression in progeny was due to the *GUS A* gene having integrated into the genome and was not due to contaminating *A. tumefaciens* (Aldemita and Hodges 1996). *Agrobacterium* mediated transformation of wheat developed by infecting immature embryos and embryogenic calli with *Agrobacterium* strain C58 harboring binary vector PMON 18365 which contains *GUS* gene with an intron and *NPT II* gene as a selectable

marker. *GUS* array of some explants showed that they are transformed. The genetic analysis of T_1 and T_2 progeny provided the evidence of the incorporation of T-DNA into the genome (Ming Cheng *et al.*, 1997). Scutellar-calli derived from mature seeds and root and shoot tips of rice were infected with *Agrobacterium tumefaciens* stain LBA 4404. The strain harboring super binary vector pTOK 233 which has *NPT II*, hpt and intron-*GUS* in the T-DNA region, is used to transform plants and the transformed plants are identified by *GUS* activity. Polymerase chain reaction using reverse transcriptase was used for confirmation of transgene integration into the plant genome Southern blots bands resulting from hybridization with respective gene probes confirmed the integration of hpt and *GUS A* genes into the rice genome (Khanna and Raina 1999).

Delbril *et al.*, (1993) re-examined *Asparagus officinalis* transformation by *A.* tumefaciens carrying the genes for *GUS* and *NPT II.* Assay for expression of the GUS gene confirmed β-glucoronidase activity in the cultures, and a Southern genomic blot of the cultures and regenerated plant supported T-DNA integration.

III. Mannitol

Improving crop tolerance to osmotic stress is a long-standing goal of agricultural biotechnology (Macue and Hanson 1990). Drought, salinity, freeze-induced dehydration constitute direct osmotic stress, chilling and hypoxia can indirectly cause osmotic stress via effects on nutrient uptake and loss. To with stand osmotic stresses, certain plants have evolved high capacity to synthesize and accumulate non toxic solutes (osmoprotectants or compatible solutes) predominantly in cytoplasm, As part of an over all mechanism to raise osmotic pressure and there by maintain both turgor and driving gradient for nutrient uptake (Rhodes and Samaras 1994). These osmoprotectants fall into four groups

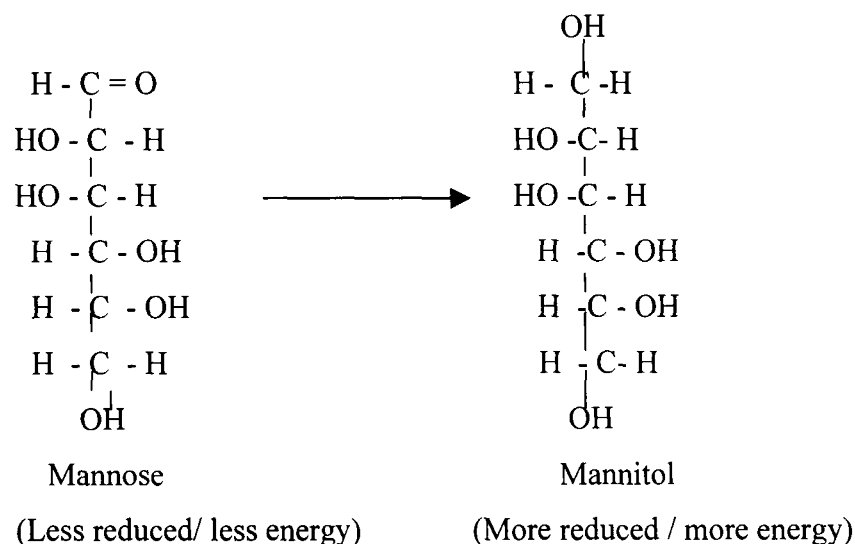
(1) Amino acids like Proline
(2) Polyols/Sugars alcohols like Mannitol and Pinitol
(3) Quaternary ammonium compounds such as glycine betaine.
(4) Sugars like Trehalose and Fructan

Osmoprotecants can accumulate to significant levels without disruption of metabolism; some of them can also protect enzymes and membranes against damage from high salt concentrations (Yancay, 1994) and other especially gives protection against reactive oxygen species (Smirnoff, 1988).

Mannitol is the most widely distributed non-cyclic sugar alcohol in nature and has been reported in more than 100 species of vascular plants, including many horticultural and agronomic crops. A sugar alcohol/ polyol means a chemically reduced form of an aldose sugar. Sugar alcohols, because of their water-like hydroxyl groups, may mimic the structure of water and maintain an artificial sphere of hydration around the macromolecules (Schobert, 1997). Sugar alcohols are chemically more reduced and high-energy storage compounds than their corresponding sugars.

The suggested physiological roles include osmoregulation (Lewis and Smith, 1967; Hellebust, 1976) and service as compatible solutes (Yancey et al., 1982; Brown and Simpson, 1972), storage of reduced carbon and energy (Lewis and Smith, 1967), regulation of co-enzymes (Lewis and Smith, 1967; Loescher, 1987) and neutralization of hydroxyl radiclas (Smirnoff and Cumbes, 1989).

Structure of Mannitol:



Accumulation of mannitol is correlated with tolerance to drought and/or salinity. Mannitol seems to function in two ways that are difficult to be separated: osmotic adjustment and osmoprotection. In osmotic adjustment polyols act as osmolytes, facilitating the retention of water in the cytoplasm and allowing sodium sequestration to the vacuole or apoplast. Alternatively, protection of cellular structure (by scavenging active oxygen species) might be accomplished through interaction of such osmolytes with membranes, protein complexes, or enzymes (Bohnert et al., 1995).

Mannitol is a major photosynthetic product and can accumulate to high levels in various higher plant species (Lewis and Smith, 1967; Bieleski, 1982; Loescher et al., 1985). Mannitol is translocated by some higher plants (Zimmermann and Ziegler, 1975), indicating the role as a storage compound.

Plants that produce mannitol exclusively are not known. Mannitol synthesis occurs simultaneously with either sucrose synthesis, as in Celery (Rumpho et el., 1983), or with raffinose saccharide synthesis, as in olive (Flora and Madroe, 1993). In celery, mannitol is the major photosynthetic product. Approximately half the fixed CO_2 in celery is converted to mannitol, while the other half is used to produce sucrose - both these translocated carbohydrates are used during the growth of non-photosynthetic sink tissues. Purification, characterisation and cDNA cloning of cytosolic non-reversible glyceraldehyde-3-P dehydrogenase suggests that this enzyme is the key contributor of the NADPH required for mannitol biosynthesis in celery (Zhifang Gao and Wayne, 2000).

Mannitol concentration progressively increased as the total salinity of the growth solution was increased in celery. Increased mannitol accumulation was observed in leaves, when celery plants were irrigated with 0.3 M NaCl. Celery grown in hydroponic nutrient solution with a salinity equivalent to 30 per cent sea water showed dry weight gains equal to control plants at normal nutrient levels (Stoop and Pharr, 1994)

A study was done on *Populus canadensis* ; where the cuttings were exposed for 12hrs to 0 mM, 50 mM and 150 mM mannitol, under stress. These closed their stomata at 50 and 150mM mannitol, whereas controls did not. At 50 mM mannitol, net

photosynthetic rate (PN) and chlorophyll and total solute contents remained stable; RuBiSCO activity, chlorophyll synthesis and turnover, ascarbate peroxidase and glutathione reductase activities were less affected. At 150 mM mannitol these showed higher PN and higher contents of antioxidants (Courtois et al., 1999).

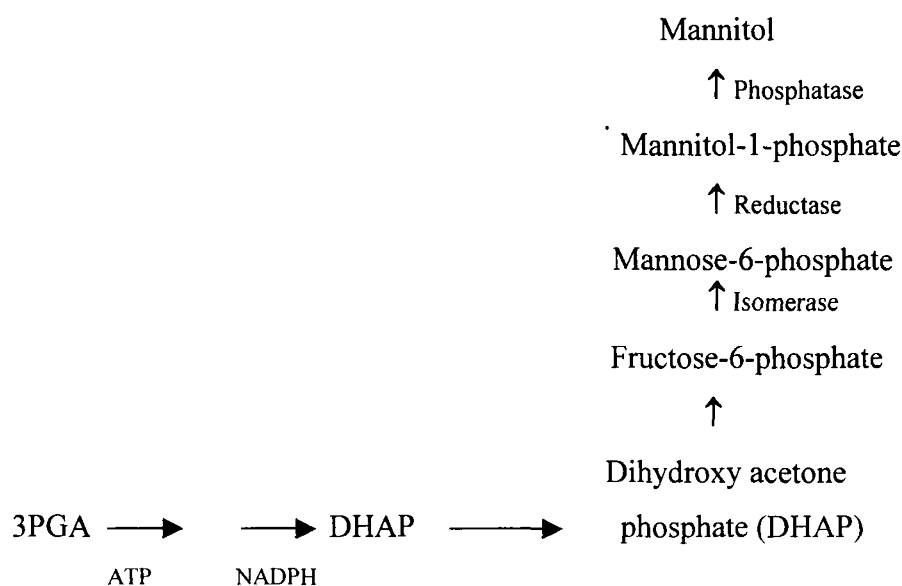
When ground nut embryos were cultured *in vitro* with and without seed coats and 8 per cent mannitol; mannitol inhibited precocious germination, maintained embryonic development, increased endogenous ABA content and promoted the synthesis and accumulation of storage proteins (Lin and Fu, 1994).

Mannitol metabolism:

In higher plants, mannitol metabolism is not well understood. However pathway has been characterized in celery, where mannitol is produced by the reactions of mannose-6-phosphate isomerase, mannose-6-phosphate reductase and mannitol-1-phophate phosphatase. Thus, mannitol biosynthesis, in celery, proceeds via a different pathway which requires an additional enzyme – catalysed step, than that in transgenic Tobacco (Rumpho et al., 1983).

Mannitol production in higher plants:

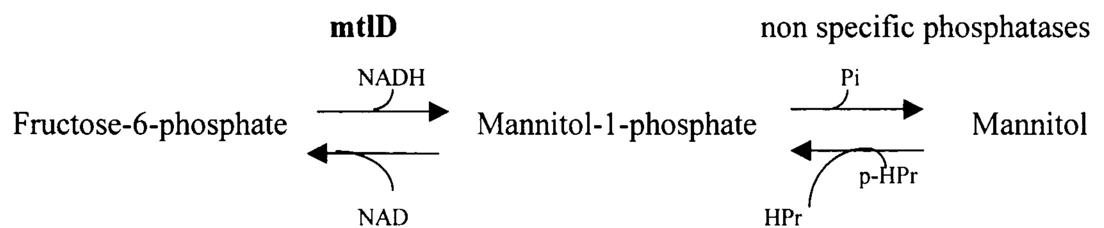
In celery



In celery mannitol is a photosynthetic product i.e., mannitol synthesis occurs simultaneously with sucrose synthesis. The photosynthetic product fructose-6-P is reduced to mannitol, by the action of the enzymes, mannose-6-P isomerase, mannose-6-P reductase and mannitol-1-P phosphatase respectively.

In *Escherichia coli,* mtlD (mannitol-1-phosphate dehydrogenase) expression leads to mannitol catabolism. The pathway involves phosphorylation of mannitol to mannitol-1-phosphate, which is oxidized to fructose-6-phosphate, which then enters glycolysis. In transgenic Tobacco, mtlD expression leads to mannitol biosynthesis. Cytoplasmic fructose-6-phosphate and NADH are utilized by mtlD to form mannitol-1-phosphate and NAD. Mannitol-1-phosphate is then dephosphorylated by a non-specific phosphatase enzyme

→ **In transgenic tobacco**



(pHPr – phosphorylated heat stable protein)

← **In *E.coli***

Additional evidences supporting the role of mannitol in salinity tolerance has been obtained through genetic engineering.

Transformation techniques offer the possibility of introducing new genes for specific traits, in a much shorter time-scale than any conventional breeding techniques. However, a corollary is the need to identify and isolate the genes that are needed.

The importance of osmolytes especially mannitol has been demonstrated by genetic engineering. Additional evidences supporting the role of mannitol in salinity tolerance has been obtained through developing transgenic plants over expressing mannitol.

Tobacco, a plant that does not normally contain mannitol was genetically engineered to synthesize mannitol through introduction of an *E. coli* gene, mtlD, encoding mannitol-1-phosphate dehydrogenase, results in the synthesis and accumulation of mannitol. Mannitol concentrations exceeded 6 μmol gm^{-1} fresh weight in the leaves and in the roots of the transformants (Tarczynski et al., 1992). Mannitol represented upto 25 per cent of the total soluble carbohydrate profile obtained for root material from the transgenic plants (Tarczynski et al., 1992). mtlD transformants containing relatively high amounts of mannitol were significantly salt tolerant than the untransformed tobacco (Tarczynski et al., 1993).

Transgenic tobacco showed decreased weight loss during salinity stress, in relative to that of control plants. Transformants increased in height to a mean of 80 per cent, while the control increased to a mean of only 22 per cent. New root and leaf growth occurred ina mean of 75 per cent of transformants, where as such growth occurred only in a mean of 33 per cent (Tarczynski et al., 1993).

Transformation of *Arabidopsis thaliana* using a similar strategy also resulted in mannitol accumulating plants. Seeds from third generation inbred mannitol synthesizing arabidopsis plants showed enhanced germination under high salinity of 400 mM NaCl, while the control seeds ceased germination at 100 mM NaCl (Thomas et al., 1995). Mannitol present in the seeds was probably acting as a compatible solute.

Transgenic expression of mtlD enhanced salt-stress tolerance of the transgenic rice seedlings. Bacterial mtlD was transformed into rice genome and three transgenic lines were obtained (M2, M5 and M8). In constast to the non-transgenic rice plants, the transgenics increased from 77 per cent to 141 per cent in shoot height, and 17 per cent to 50 per cent in the fresh weights of shoots and roots (Su jin et al., 1999).

To investigate the potential role of mannitol in oxidative stress protection, mtlD gene was targeted to chloroplast by the addition of an amino terminal transit peptide. The presence of mannitol in chloroplasts resulted in an increased resistance to methyl viologen (MV) induced oxidative stress, documented by the increased retention of chlorophyll in transgenic leaf tissue following MV treatment. MV treated mesophyll cells of transformants exhibited higher CO_2 fixation than the wild type (Bo Shen et al., 1997b).

Mannitol has protective effects on sulphydryl enzymes and other sulphydryl regulating chloroplast components (Ferredoxin, thioredoxin and glutathion) during oxidative stress in the cell, considering the sensitivity of sulphydryl enzymes to H_2O_2 and the hydroxyl radical scavenging ability of mannitol in chloroplasts. Cells from transgenic Tobacco plants that contained 100mM mannitol in the chloroplast maintained higher phospho-ribulo activity than the wild type cells, when treated with 1 mM H_2O_2 (Bo Shen et al., 1997b)

MATERIAL AND METHODS

III. MATERIAL AND METHODS

Plant transformation technology has become versatile platform for crop improvement as well as for studying gene function in plants. Plant transformation is an experimental tool for plant physiology. The most commonly applied and successful transformation procedure of higher plants is *Agrobacterium* mediated gene transfer.

Transformation without regeneration and regeneration without transformation are of limited value. Identification of target cell types for transformation is necessary. An effort was therefore made to study the target cell type for gene transfer without affecting normal developmental processes. The development of a transformation system is providing induced *Agrobacterium* with access to cells capable of dedifferentiation followed by regeneration.

Our objective was to develop transgenic plant over expressing mannitol by transferring mtlD gene into Finger millet. Keeping these in view studies were conducted on the following aspects

1. To develop efficient protocols for callus induction and regeneration of finger millet.
2. To develop efficient transformation protocol by using the *Agrobacterium binary* vector pCAMBIA 1380 having mtlD gene in finger millet.
3. Development of transgenic finger millet plants overproducing mannitol.

Preparation of glass wares and chemicals

Glassware like culture tubes, petriplates, conical flasks etc were procured from corning or Borosil. All chemicals and plant growth regulators of analytical grades were procured from standard chemical manufacturing companies.

Cleaning of glassware

Glasswares were cleaned by soaking in 0.15% chromic acid over night followed by rinsing with tap water and cleaning with detergent solution. The glasswares were thoroughly washed in tap water and rinsed with distilled water. The glasswares were then dried in hot air oven and stored in dust proof area till the use.

Sterilization of glasswares

Cleaned glasswares were put into autoclavable polypropylene covers and scalpel, forceps were wrapped in aluminum foil and then they are autoclaved at 121°C and 15 lbs. pressure for 20 minutes. Then the glasswares were transferred to sterile inoculation chamber for further use.

Preparation of stock solutions

The culture medium used for the study was MS medium (Murashige and Skoog, 1962). The composition of stock solution is given in table 1. The stock solutions were prepared by dissolving the chemicals in double distilled water and stored in reagent bottles. Stocks of auxins were prepared by dissolving in few drops of absolute alcohol. The required concentration was prepared using volumetric flask with double distilled water. Cytokinins were dissolved in a few drops of 0.1 N HCl and 0.1 N NaOH to adjust the pH and made up to a known volume in a standard volumetric flask with double distilled water.

Preparation of culture media

The required quantity of the stock solutions was transferred to a beaker with the help of a pipette along with the required quantity of sucrose and the required growth regulators. The pH was adjusted to 5.8 using 1 N NaOH and 1 N HCl. The volume was made up and the required quantity of agar was added and then media was autoclaved at 121°C and 15lb per square inch pressure for 20 minutes.

Sterilization of the inoculation chamber

All the aseptic requirements like sterilization, inoculation of the explant and subculturing are carried out in the laminar airflow cabinet. Before the laminar airflow cabinet is used, the working surface of the chamber is sterilized by spraying absolute alcohol. The chamber is then exposed to UV light for an hour along with the media to be inoculated, cotton, glassware and other accessories to ensure total sterility. In the course of inoculation, all the instruments were kept in a beaker containing absolute alcohol and were flamed before use.

I. Standardization of regeneration protocol in Fingermillet.

Optimization of callus induction

Mature Fingermillet seeds (variety GPU-28) were used as explant for callus induction. Seeds are soaked in water for 1 hour then seeds were washed with distilled water to remove the husk. After removing the husk further sterilization were done in laminar airflow cabinet. Seeds were then surface sterilized with Hg Cl_2 0.5% for 5 minutes. Then seeds were washed with the sterile water for 5 to 6 times to remove the sterilizing agent. After sterilization, seeds were inoculated on MS media supplemented with varying levels of auxin and cytokinin. Then the petriplates were incubated in 16hrs light and 8 hrs dark condition at the temperature of 26°C ±2°C.

Treatments

1. Control (MS media without hormones)
2. MS + 2.5 mg.L^{-1} 2,4-D + 0.2mg.L^{-1}Kinetin
3. MS + 2.5 mg.L^{-1} 2,4-D + 0.25 mg.L^{-1}Kinetin
4. MS + 2.5mg.L^{-1} 2,4-D + 0.3 mg.L^{-1}Kinetin
5. MS + 3.0 mg.L^{-1} 2,4-D + 0.2mg.L^{-1} Kinetin
6. MS + 3.0 mg.L^{-1} 2,4-D + 0.25 mg.L^{-1}Kinetin
7. MS + 3.0 mg.L^{-1} 2,4-D + 0.3 mg.L^{-1}kin

Observations

Callus weight and shoot weight were recorded after 16thday, 18thday and 20st day after inoculation

Standardization of morphogenesis

Callus (20 day old) was tested for their regenerability. Callus was inoculated on MS media supplemented with different composition of hormones.

Treatments

1. MS basal
2. MS + 0.5 mg.L^{-1} TDZ
3. MS + 0.75 mg.L^{-1} TDZ
4. MS +0.5 mg.L^{-1} Kinetin
5. MS + 0.75mg.L^{-1} Kinetin

After 30 days of inoculation in the regenerating media, observation was taken for number of shoot initials per calli. Later the regenerating calli were cut carefully into pieces having 4-5 shoot initials and transferred into the elongation media for 15-20 days.

Elongation media.

C. Control (½ MS without hormones)
1a. ½ MS +0.75 mg L^{-1} TDZ + 1 mg L^{-1}GA
1b. ½MS + 0.75 mg L^{-1} TDZ + 2 mg L^{-1}GA
2a. ½MS +0.5 mg L^{-1} TDZ + 1 mg L^{-1}GA
2b. ½MS +0.5 mg L^{-1} TDZ + 2 mg L^{-1}GA
3a. ½MS +0.75 mg L^{-1} Kinetin +1 mg L^{-1}GA
3b. ½MS + 0.75 mg L^{-1} Kinetin + 2 mg L^{-1}GA
4a. ½MS + 0.5 mg L^{-1} Kinetin + 1 mg L^{-1}GA
4b. ½MS +0.5 mg L^{-1} Kinetin + 2 mg L^{-1}GA

Observations

Shoot length were recorded 15-20 days after inoculation in the elongation media.

Hygromycin sensitivity test

Hygromycin phosphate transferase gene has been used for the transformation as a selectable marker. Hygromycin phosphate transferase provides resistance to hygromycin antibiotic. For successful selection, the target plant cells must be susceptible to relatively low concentration of antibiotic. This experiment is conducted to find out the concentration of hygromycin to be used for selection of transformed plants.

Sterile MS media was melted and cooled to which different concentration of hygromycin was added. Healthy calli (20 day old) were inoculated on the MS morphogenic media supplemented with different concentration of hygromycin.

Treatments

1. Control (MS morphogenic media without hygromycin)
2. 10 $\mu g.mL^{-1}$ hygromycin
3. 20 $\mu g.mL^{-1}$ hygromycin
4. 30 $\mu g.mL^{-1}$ hygromycin
5. 40 $\mu g.mL^{-1}$ hygromycin
6. 50 $\mu g.mL^{-1}$ hygromycin

Observation

Survivability of callus was recorded after 15 days of inoculation. Callus, which turned to black, was considered as dead.

II. Standardization of transformation protocol using binary vector pCAMBIA 1380.

Aim of the experiment was to develop suitable protocols for efficient transformation.

Details of Bacterial strain and plasmid

The standardization of transformation protocols in fingermillet system was carried out using Agrobacterium *tumefaciens* strain EHA 105 harboring a binary plasmid pCAMBIA 1380. The gene construct was obtained from Dr. Rajam's laboratory, South campus, New Delhi. The binary vector contains hygromycin resistant gene (hpt II) and 1.5kb mtlD gene, both driven by 35s CaMV promoter and the gene is targeted to chloroplast.

Map of the mtlD gene construct.

The mtlD gene was cloned in a binary vector having *hpt II* gene (Hygromycin phosphotansferase) as the plant selection marker. The mtlD gene of 1.5kb along with the CaMV 35s promoter is cloned into EcoR I – Hind III sites of pCAMBIA 1380 that has a Nos poly A site at its right border. The construct also has *hpt II* gene driven by 35s CaMV with a Poly A site at its left border (Fig 1).

Experimental detail

Preperation of glycerol stock

A single colony of Agrobacterium grown on AB minimal media,was inoculated into culture tube with AB glucose media containing Kanamycin (50 μg mL^{-1}),AB salt and AB buffer.The culture tube was kept on the shaker overnight.From this 500 μl of culture was inoculated into equal volume of 60 % glycerol and kept in -70°C for future use.

Preparation of culture for infection of plant material

The starter culture of *Agrobacterium tumefaciens* strain EHA 105 containing the binary vector pCAMBIA 1380 was grown in YEM (yeast extract mannitol) media supplemented with Kanamycin (50 $\mu g.mL^{-1}$), by inoculating 100 μl of the glycerol stock. The culture was grown at 28°C and 200 rpm shaking. Later a large culture was grown at 0.8 to 1.0

OD at 600 nm in YEM medium supplemented with 50μg.mL^{-1} Kanamycin. The grown bacterial culture was centrifuged at 10,000 rpm for 10 minutes and pellet was resuspended in the liquid callus induction media supplemented with acetosyringone (100μM) and Kanamycin (50μgmL^{-1})

Infection and co-cultivation

20 day old calli were soaked in bacterial suspension for different duration of infection. After infection calli were blotted on the sterile tissue paper and then transferred to co culture medium. Co-culture medium was regeneration media with 0.75mg.L^{-1} TDZ supplemented with acetosyringone (100μM). During co-cultivation *Agrobacterium* was found to grow on and around the callus explants.

Selection

After co cultivation, calli were washed with sterile water supplemented with cefotaxime to remove bacteria. Then calli were blotted on sterile tissue paper and then transferred to selection media. MS medium supplemented with 0.75 mgL^{-1} TDZ, hygromycin (40 μgmL^{-1}) and cefotaxime (400 μgmL^{-1}) served as selection media. Then they are incubated at 28°C.

Standardization of the OD_{600} of Agrobacterium culture and duration of infection

Transformation experiments were carried out by following above protocol with some alterations in the two factors, namely optical density of the *Agrobacterium* culture at 600 nm (OD_{600}) and the duration of infection of callus with bacterial culture. The different ranges of optical densities that were studied are as follows,

0.10 to 0.40

0.40 to 0.80

0.81 to 1.20

1.21 to 1.50

The infection period of 4,6,8 and 10 minutes were tested with bacterial cultures with different optical densities

Observations:

Relative growth of the bacteria around the callus over control, after 2 days of co-cultivation was recorded. Four categories of grading the bacterial growth was used namely, more growth, optimum growth, less growth and no growth.

Standardization of effective concentration of acetosyringone in the co-cultivation medium.

Calli infected with *Agrobacterium* culture in liquid callus induction medium were supplemented with different concentration of acetosyringone (0, 50, 100 and 150μM). Observation on the number of transformed (putative) calli to the total number of calli inoculated were taken and expressed as percentage. Observations were taken 15 days after inoculation.

Elimination of bacterium

To control the growth of *Agrobacterium*, cefotaxime antibiotic was used. After co-cultivation, calli were transferred to the selection media supplemented with different concentration of cefotaxime (100, 200, 300, 400, 500 and 600μg mL^{-1})

Observations regarding relative growth of bacteria was recorded 5, 10, 15 and 20 days after setting the experiment.

III. Transgenic plants overexpressing mannitol:

Development of putative transformants

The regenerating calli / green from the selection media containing Hygromycin (40μgm mL^{-1}) were obtained after 15 days of inoculation. The calli were carefully cut into small

pieces containing 4-5 developing shoots and were placed in fresh elongation media (half MS+1mgL^{-1}GA) containing cefotaxime (400μgm mL^{-1}). Hygromycin was not used in the elongation media. The plantlets were allowed to develop shoots and roots for 30 days. Later the plants were hardened.

Hardening and planting of the in vitro grown plants

The *in vitro* rooted plantlets of 50 days old were removed from the culture bottles without disturbing the roots. The plantlets were washed with sterile water, until the adhering media was washed off. The washed shootlets were transferred to perforated plastic cups with 1:1 mixture of soilrite and sand. The cups with the plantlets were covered with polythene bags and the mouth of the bag was tied with rubber band. Small holes were made on the polythene to avoid built up of humidity. The bags were kept in tissue culture incubation room for 1 week. After 1 week the polythene bags were kept open for 2 h daily, subsequently the bags were kept open for 4-6 h. The plants which did not show any wilting symptoms were transferred to 10 cm diameter pots with red soil and sand mixture (75:25). The pots were then transferred to mist chamber. The mist chamber is fitted with foggers with an electronic timer, which is set at 20 min off and 30 sec on time, so that the temperature is maintained around 30°C and RH 85%. After 2-3 weeks healthy looking plants were transferred to big battery containers and were kept in an enclosure, which was insect proof and the plants, were watered daily in the evenings.

Genetic analysis of the transgenic plants

Putative transformants were analyzed for the stable integration of pCAMBIA 1380. Plant DNA was extracted and PCR analysis was done.

Extraction of plant DNA by C-TAB method

(Extraction buffer – C-TAB-2%, 1.4M NaCl, 100mM Tris and 20mM EDTA; pH 8.0)

1. One gram fresh weight of young healthy leaf tissue from the hardened plants was ground with liquid nitrogen until it was powdered.

2. To the leaf powder, 5 ml of extraction buffer was added and the tissue was ground until it was thawed. The homogenate was added to 50 ml centrifuge tube.
3. The mortar was rinsed with additional 5 ml extraction buffer and the solution was added to the homogenate to make the volume to 10 ml.
4. To the above solution 60-65 μ lt of β- Mercaptoethanol was added and mixed thoroughly by swirling. And the centrifuge tubes were kept in the water bath for 1 h at 65°C. In between the solution must be gently shaked for 2-3 times.
5. After taking out from the water bath equal volume (10 ml) of chloroform: iso amyl alcohol mixture of 24:1 concentration was added.
6. After that the tubes were centrifuged at 5000 rpm for 20 minutes. The supernatant was collected.
7. To this supernatant 0.7 volume of propyl alcohol was added.
8. The resultant precipitated DNA was removed by spooling with Pasteure pipette.
9. The pellet was washed with 70% ethanol by spinning at 3000 rpm for 5-10 minutes. This procedure was repeated for 2-3 times.
10. This was air dried and dissolved in TE and was transferred to eppendorf tubes. The dissolved DNA was preserved in 4° C.
11. The DNA was quantified both by spectrophotometer and by running on an agarose gel.

Plasmid DNA isolation

Plasmid DNA was used as the positive control in the PCR analysis. The DNA was isolated from the *Agrobacterium* (pCAMBIA 1380) culture grown on YEM media in the following way.

Materials

1. Glycerol stocks of the *Agrobacterium* vector pCAMBIA 1380.
2. Sterile centrifuge tubes and tips.
3. Ice bucket with ice.

4. Solution I (25mM Tris HCl, pH 8.0, 50mM glucose, 10mM EDTA, 4mg/mL lysozyme).
5. Solution II (0.2N NaOH, 1.0% SDS, made fresh just prior to use).
6. TE (10mM Tris HCl, pH 8.0, 1mM EDTA)
7. 3M sodium acetate, pH 4.8
8. 0.3M sodium acetate pH 7.0
9. 70% ethyl alcohol.
10. Phenol chloroform
11. Chlro:isoamyl alcohol (24:1)
12. Vortex mixer
13. 37^0C water bath
14. Centrifuge
15. Vacuum drying apparatus
16. 100% ethyl alcohol.

Protocol

1. *Agrobacterium* cells were grown overnight in 50 mL YEM medium containing appropriate concentrations of antibiotics (Kanamycin 50 μ gm mL^{-1})
2. This was transferred to centrifuge tubes and centrifuged at 10,000 rpm for 10 minutes.
3. The supernatant was discarded and the cells were resuspended in 1.66 mL of ice-cold solution I and incubated for 10 minutes at room temperature.
4. Freshly prepared solution II 3.3mL was added and mixed by shaking. Incubated for 10 minutes at room temperature.
5. Solution III (3.3mL) was added and mixed by shaking. Incubated for 10 minutes at room temperature.
6. The tube was centrifuged at 11,000 rpm for 15 minutes and the supernatant was put into another fresh centrifuge tube.
7. To this tube equal volume of isopropanol was added and centrifuged at 11,000 rpm for 15 minutes.
8. Then supernatant was discarded and the pellet was dried in lyophilizer.

9. Pellet was dissolved in 500μL TE.

10. After dissolving the pellet, solution was transferred to 1mL eppendorf tubes.

11. DNAase free RNAase was added and incubated at 37^0C for 45 minutes.

12. To this phenol chloroform (500μL) was added vortexed for 1 minute and centrifuged at 12,000 rpm for 10 minutes.

13. The upper aqueous phase was transferred to a fresh tube to which chloroform: iso amyl alcohol (24:1) 500μL was added and vortexed and centrifuged at 12,000 rpm for 5 minutes.

14. The upper aqueous layer was transferred to a fresh tube and to that $1/10^{th}$ of the volume of 0.3M sodium acetate pH 7.0 and twice the volume of ethanol was added, mixed and allowed to precipitate for 1 hour at 70^0C.

15. The precipitate was centrifuged for 15 minutes at 12,000 rpm. The pellet was rinsed with 70% ethanol and the pellet dried in a vaccum desicator.

16. The dried pellet dissolved in 50μL TE.

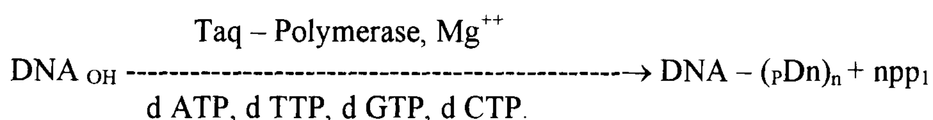
PCR analysis of putative mtlD transformants:

DNA from transformed and untransformed fingermillet plants was extracted as described above. PCR amplification was carried out in Gradient PCR machine using the specific primers for *hpt.*

Primer sequence for *hpt II* is:

`N` terminal primer 5` CGC ATG AAA AAG CCT GAA CTC ACC GCG 3`
`C` terminal primer 5` GCA GGC TCC CGT TTC CTT ATC GAT 3`
On amplification the expected product should be 350 bp.

PCR reaction:



$$DNA_{OH} \xrightarrow[\text{d ATP, d TTP, d GTP, d CTP}]{\text{Taq – Polymerase, } Mg^{++}} DNA - ({}_{P}Dn)_n + npp_I$$

Preparation of master mix

- 2.5µl PCR buffer with $(NH_4)_2$ SO_4 at 1X (supplied with 10X conc)
- 2.5µl $MgCl_2$ solution (supplied with 25m M conc)
- 1.0µl forward primer with the conc of 10 p moles
- 1.0µl reverse primer with the conc of 10 p moles
- 4.0µl d NTP's from 1.25 mM stock
- 1.0 unit reaction mixture of Taq – polymerase (recombinant)* (stock 3units/µl)
- 1.0µl template DNA with the conc of 200ng
- volume made up to 25 µl with sterile Milli-Q water

Protocol for *hpt II* amplification by PCR

- Step 1 95° C for 4 min (Denaturation)
- Step 2 95° C for 1 min
- Step 3 62° C for 1 min (Annealing)
- Step 4 72° C for 2 min (Extension)
- Step 5 GOTO step 2 for 30 more cycles
- Step 6 72° C for 5 min
- Step 7 hold at 4° C

Apart from the putative transformants and the control plants, the plasmid DNA was used as positive control in these PCR reactions. The PCR amplified DNA was observed through 1.5% agarose gel electrophoresis using 1Kb™ Gene ruler as the marker.

Southern blotting and hybridisation

Genomic DNA was isolated (as mentioned earlier) from wild and transgenic plants. PCR was performed using *hpt II* primers. PCR products were electrophoresed and transferred to the membrane as mentioned below for southern blotting.

Protocol

The PCR products were loaded on a 1.5 % agarose gel to resolve the expected fragments. Electrophoresis was carried out at 5V/cm for 3 hours.

The gel was visualized in a UV transilluminator. The gel was then processed for carrying out the southern analysis. The gel was transferred to a gel tray with 0.2 N HCl, and shaken in a rocker arm shaker for 30 min. During this step the bromophenol tracking dye turned yellow from blue.

The acid solution was decanted and the gel was transferred to denaturation solution (0.5 M NaOH) and rocked for 15 min during which time the dye turned blue from yellow.

Denaturation solution was decanted and the gel placed in neutralization solution (0.5 M NaOH + 1.5 M NaCl). The gel was rocked for 30 min.

The set up was made for blotting of the DNA onto the nitrocellulose membrane. In a glass tray the gel tray was placed in an inverted manner. About 300 mL of 20X SSC was poured into the tray. A wick was made by cutting one piece of Whatman 3 mm paper. The wick was 2 cm wider than the width of the gel and about 30 – 40 cm long. The wick was thoroughly wetted and put on the gel tray with both ends overhanging the plate and into the tray. Air bubbles were removed by rolling a glass rod over the wick.

The gel was removed from the neutralization solution and excess liquid drained off. The gel was placed on top of the wick. Air bubbles were removed by rolling a glass rod over the gel. The wetted nitrocellulose membrane was placed over the gel.

Four pieces of Whatman 3 mm paper, cut into pieces, 7 mm smaller in both dimensions of the gel were placed over the nitrocellulose membrane. Above this about 5 cm thick stack of filter paper, cut to the size of the Whatman papers were placed. A glass plate was placed over the filter paper and a weight was placed over the glass plate. The set up was left undisturbed overnight to facilitate transfer.

After transfer was complete, the stack of papers was removed and a mark was made at the right hand corner of the membrane to indicate the direction of the wells. The membrane was then carefully removed and washed with 2X SSC. The membrane was then subjected to UV cross-linking. The gel was viewed again in a transilluminator to confirm complete transfer of DNA. Absence of ethidium bromide fluorescence indicated complete transfer.

- Elution was done by using freeze – thaw method from agarose gel.

Nearly 50ng of purified *hpt II* insert DNA was denatured upon boiling water bath for 5 min and immediately kept on ice.

- Later performed following additions on ice in the following order.

  2.0μl d TTP (from stock of 0.5 m M)

  2.0μl d GTP (from stock of 0.5 m M)

  2.0μl d CTP (from stock of 0.5 m M)

  15μl random primer (hexamer 18 OD_{260} units/ml) buffer mixture

  5.0μl (Approximately 50 μ ci) [α - ^{32}P] ATP

  Made up to 50μl with double distilled water (supplied by Gibco BRL only). 1μl of Klenow fragment (3u/μl) was added mixed gently and centrifuged briefly.

- Incubated at room temperature for 3 hours.
- Purified using prepared Sephadex-G50 column.
- Proportion of incorporation and percent incorporation is calculated using the following formula.

$$\text{Proportion of incorporation} = \frac{\text{Activity in purified probe}}{\text{Total activity}}$$

☞ Percent incorporation = proportion of incorporation x 100

Prehybridization, hybridization and autoradiography

1. Pre-hybridization solution (~30 mL) was added to the big glass tube supplied by the manufacturer (Amersham) and pre-hybridization was carried out for 3 hours at 58° C.
2. The ^{32}P labelled probe was prepared by using random primer labelling kit supplied by Gibco BRL. The probe that would give around 10,00,000 cpm/mL was boiled for 5 minutes and chilled on ice. The labelled probe was added to the pre-hybridization solution and kept for overnight for hybridization.

2. The ^{32}P labelled probe was prepared by using random primer labelling kit supplied by Gibco BRL. The probe that would give around 10,00,000 cpm/mL was boiled for 5 minutes and chilled on ice. The labelled probe was added to the pre-hybridization solution and kept for overnight for hybridization.

3. The hybridized membrane was washed with different concentration of SSC in order to remove the non-specific binding of probe to the membrane.

   Washing: 6X SSC + 0.1 % SDS   37° C for 15 minutes
   4X SSC + 0.1 % SDS   37° C for 15 minutes
   2X SSC + 0.1 % SDS   65° C for 30 minutes
   Rinse with 2X SSC at room temperature

4. The autoradiography was done by exposing membrane to X-ray film and kept for 3 day at -80° C.
   The exposed film was developed by keeping it in developer for 30 seconds and transferred to distilled water for wash, again kept in fixer for 3 min and again washed in distilled water. After seeing signals on X-ray film it was photographed.

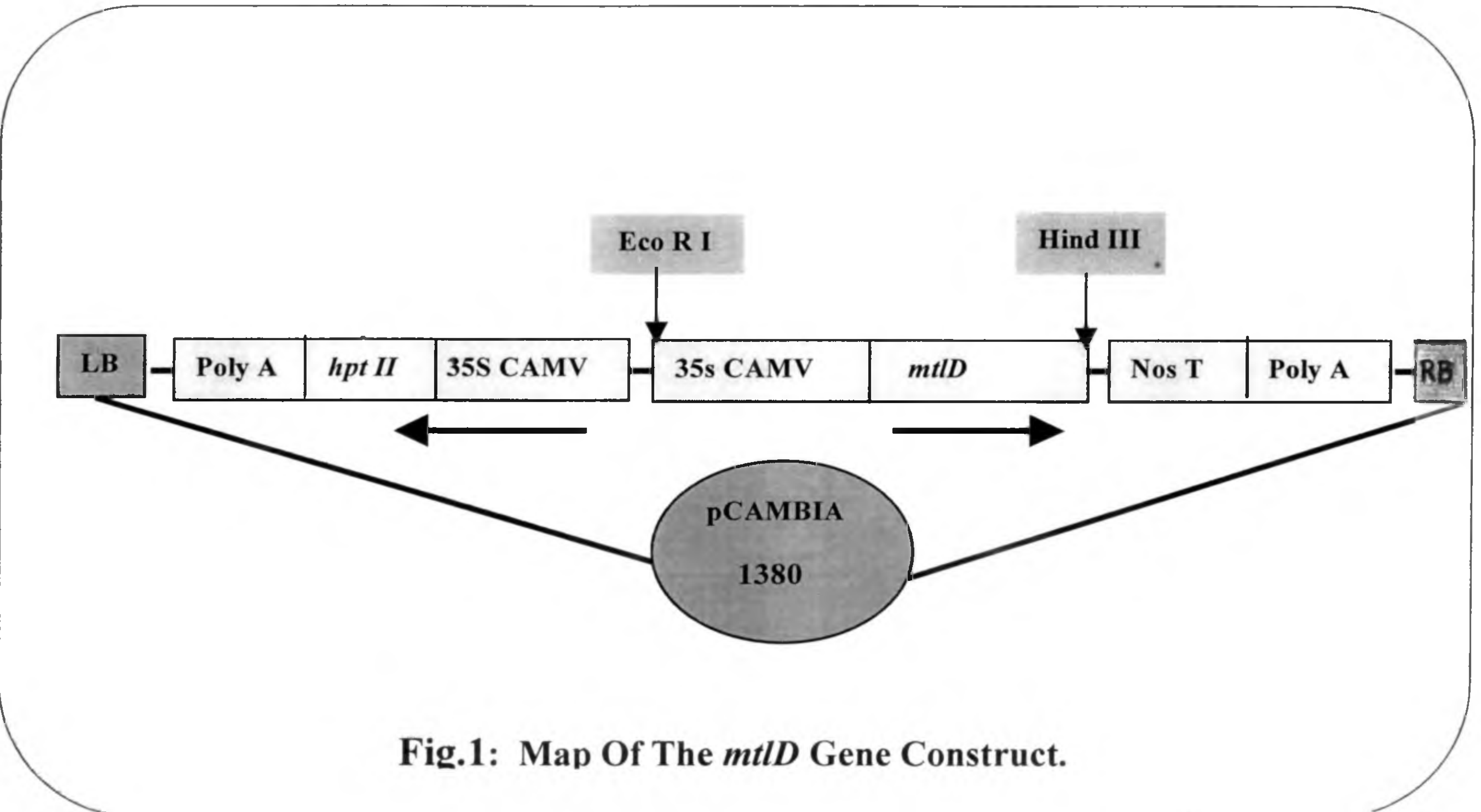


Fig.1: Map Of The *mtlD* Gene Construct.

RESULTS

IV. EXPERIMENTAL RESULTS

The major objectives of the investigation entitled "*Agrobacterium* mediated gene transfer for overproduction of Mannitol in Finger millet" were

1. To develop efficient protocols for callus induction and regeneration of finger millet.
2. To develop efficient transformation protocol by using the *Agrobacterium binary* vector pCAMBIA 1380 having mtlD gene in finger millet.
3. Development of transgenic finger millet plants overproducing mannitol.

The research findings are as presented below.

I. Standardization of efficient callus induction and regeneration protocol in Finger millet

Optimization of callus induction

Callus induction response of seed explant was tested on media supplemented with varying levels of auxin and cytokinin. Callus growth started one week after inoculation. In control (MS media without hormones) there was no growth of callus, only there was growth of the shoot. Across all the treatments, maximum growth of the callus was observed at 2.5 mg L^{-1} 2,4-D and 0.25 mg L^{-1} Kinetin. At all the stage of callus growth presence of growth regulator in MS media in general reduced the growth of shoots (Table1) (Plate 1).

Optimization of morphogenesis:

An experiment was conducted to induce organogenesis from the calli from seed explant. Callus was transferred to MS morphogenesis media supplemented with different concentrations of Kinetin (0.5, and 0.75mg L^{-1}) and TDZ (0.5, and 0.75 mg L^{-1}), 30 days after inoculation; observation was taken for number of shoot initials (Table 2, Plate2). Later each callus was cut into pieces containing 4 -5 shoot initials and was transferred to elongation media (1/2 MS with 1mg L^{-1} and 2mg L^{-1}GA) for 15-20 days. Shoots and roots were developed. After 15-20 days of inoculation in the

Table - 1: Effect of different concentration of growth hormones on callus induction of finger millet seeds. (Fresh weight of 10 calli in grams)

Treatments		Days after inoculation		
		16	**18**	**20**
1.	Control (MS media without hormones)	-	-	-
2.	MS + 2.5 $mg.L^{-1}$ 2,4-D+ 0.2$mg.L^{-1}$Kinetin	0.40	0.68	0.80
3.	**MS + 2.5 $mg.L^{-1}$ 2,4-D+ 0.25 $mg.L^{-1}$Kinetin**	**0.42**	**0.78**	**0.82**
4.	MS + 2.5$mg.L^{-1}$ 2,4-D+ 0.3 $mg.L^{-1}$Kinetin	0.24	0.56	0.64
5.	MS + 3.0 $mg.L^{-1}$ 2,4-D + 0.2$mg.L^{-1}$ Kinetin	0.15	0.27	0.38
6.	MS + 3.0 $mg.L^{-1}$ 2,4-D+ 0.25 $mg.L^{-1}$Kinetin	0.22	0.34	0.40
7.	MS + 3.0 $mg.L^{-1}$ 2,4-D+ 0.3 $mg.L^{-1}$Kinetin	0.36	0.60	0.75
	lsd values for treatments	0.036	0.036	0.042

Seeds were inoculated in different media and callus growth (fresh weight) was determined at regular intervals on16th, 18th and 20th day after inoculation.

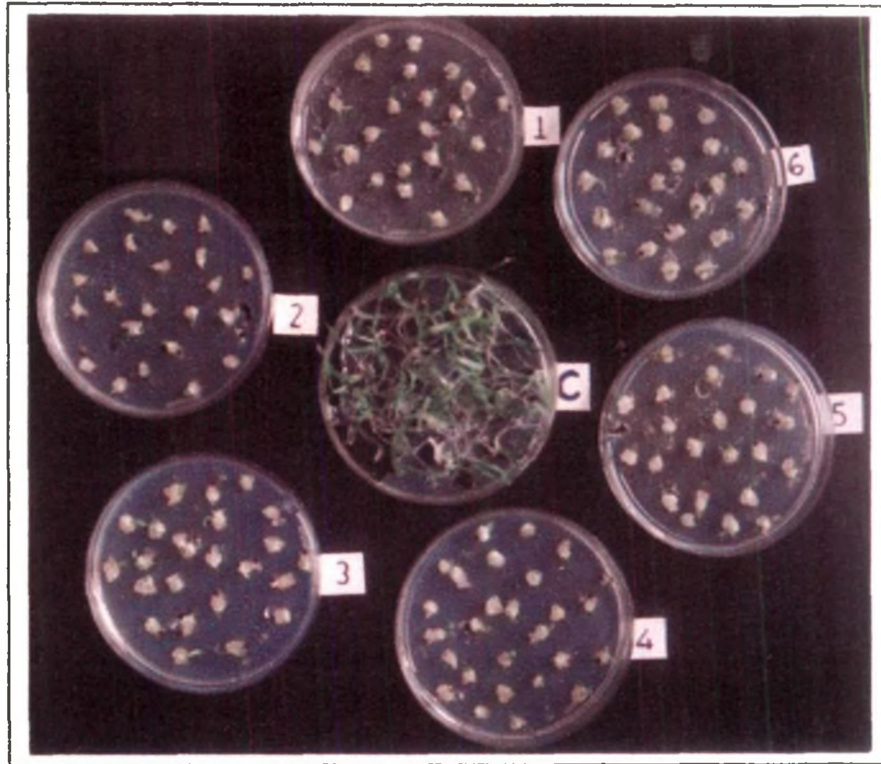


Plate 1: Effect of different concentrations of 2,4-D and Kinetin on callus growth from germinating finger millet seeds.

C. Control (MS media without hormones)
1. MS + 0.2mg.L^{-1}Kinetin + 2.5 mg. L^{-1} 2,4-D
2. MS + 0.3 mg. L^{-1}Kinetin + 2.5mg.L^{-1} 2,4-D
3. **MS + 0.25 mg. L^{-1}Kinetin + 2.5 mg. L^{-1} 2,4-D**
4. MS + 0.2mg.L^{-1} Kinetin + 3.0 mg. L^{-1} 2,4-D
5. MS + 0.25 mg. L^{-1}Kinetin + 3.0 mg. L^{-1} 2,4-D
6. MS + 0.3 mg. L^{-1}kinetin+ 3.0 mg. L^{-1} 2,4-D

Table- 2: Number of shoot initials regenerated from the callus on the regeneration MS media.

Treatments	Average number of shoot Initials / calli
1. Control (MS media without hormones)	14
2. MS + 0.5 mg L^{-1} TDZ	15
3. MS + 0.75 mg L^{-1} TDZ	**18**
4. MS + 0.5 mg L^{-1} Kinetin	14
5. Ms + 0.75 mg L^{-1} Kinetin	16
lsd value for treatments	2.547

Twenty day old callus was inoculated into different regeneration media. Thirty days after inoculation observation was taken for the number of shoot initials per regenerating calli.

elongation media observation was taken for shoot length (Table 3, Plate 3). Maximum shoot initials per calli was observed at 0.75 mg L^{-1} TDZ (18 shoots/callus). Shoot length was maximum at 0.75 mg L^{-1} TDZ with 1mg L^{-1} GA.

Hygromycin sensitivity test

Hygromycin is used as a selectable marker to select the transformed cells since in presence of hygromycin, untransformed cells cannot grow. To identify the concentration of hygromycin, which kills the untransformed callus, twenty days old calli were inoculated on the media supplemented with different concentrations of hygromycin. Observations were recorded 15 days after inoculation. At 40μg mL^{-1} of hygromycin, there was complete inhibition of the callus growth (Table 4) (Plate 4).

II. Development of efficient transformation protocol by using the *Agrobacterium* binary vector pCAMBIA 1380 having mtlD gene in finger millet.

A. *Standardization of the transformation protocol in finger millet with pCAMBIA 1380.*

Agrobacterium tumefaciens strain EHA105 (pCAMBIA1380) was used to standardize the protocols for transformation in finger millet variety GPU-28. Transformation studies were done by using calli as explant (twenty days old). Callus was co-cultivated on regeneration media for 2 days with *Agrobacterium tumefaciens*. After two days of co-cultivation, calli were thoroughly washed with sterile water and then were transferred to the selection medium containing 40μg mL^{-1} of hygromycin and 400μg mL^{-1} cefotaxime. After 15 days, observation was recorded on the number of hygromycin resistant calli, the calli which remains green and regenerates (Table 8).

Table 3: shoot length of the rooted plantlet obtained on the elongation media

Treatments	Shoot length (in cm)
C	8.6
1a	**9.9**
1b	9
2a	4.6
2b	8.6
3a	4.8
3b	8.3
4a	10.9
4b	7.8
lsd value for treatments	0.516

Thirty days after inoculation in the regeneration media, the regenerating calli were cut into pieces containing 4 - 5 shoot initials and inoculated into elongation media. Observation was taken for shoot length. 20 days after inoculation in the elongation medium.

C. Control (½MS without hormones)
1a. ½MS +0.75 mg L^{-1} TDZ + 1 mg L^{-1}GA
1b. ½MS + 0.75 mg L^{-1} TDZ + 2 mg L^{-1}GA
2a. ½MS +0.5 mg L^{-1} TDZ + 1 mg L^{-1}GA
2b. ½MS +0.5 mg L^{-1} TDZ + 2 mg L^{-1}GA
3a. ½MS +0.75 mg L^{-1} Kinetin +1 mg L^{-1}GA
3b. ½MS + 0.75 mg L^{-1} Kinetin + 2 mg L^{-1}GA
4a. ½MS + 0.5 mg L^{-1} Kinetin + 1 mg L^{-1}GA
4b. ½MS +0.5 mg L^{-1} Kinetin + 2 mg L^{-1}GA

Plate 2: Regeneration of the shoot initials from the callus.

1. Control (MS media without hormones)
2. MS + 0.5 mg L^{-1} T
3. **MS + 0.75 mg L^{-1} TDZ**
4. MS + 0.5 mg L^{-1} Kinetin
5. Ms + 0.75 mg L^{-1} Kinetin

Plate 3: Development of shoots and roots on elongation media.

C. Control (½ MS without hormones)
1a. ½ MS +0.75 mg L^{-1} TDZ + 1 mg L^{-1}GA
1b. ½ MS + 0.75 mg L^{-1} TDZ + 2 mg L^{-1}GA
2a. ½ MS +0.5 mg L^{-1} TDZ + 1 mg L^{-1}GA
2b. ½ MS +0.5 mg L^{-1} TDZ + 2 mg L^{-1}GA
3a. ½ MS +0.75 mg L^{-1} Kinetin +1 mg L^{-1}GA
3b. ½ MS + 0.75 mg L^{-1} Kinetin + 2 mg L^{-1}GA
4a. ½ MS + 0.5 mg L^{-1} Kinetin + 1 mg L^{-1}GA
4b. ½ MS +0.5 mg L^{-1} Kinetin + 2 mg L^{-1}GA

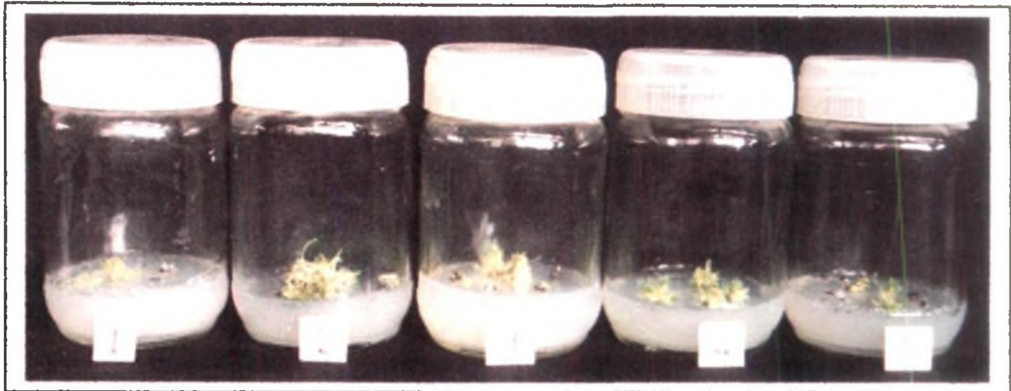


Plate 2.

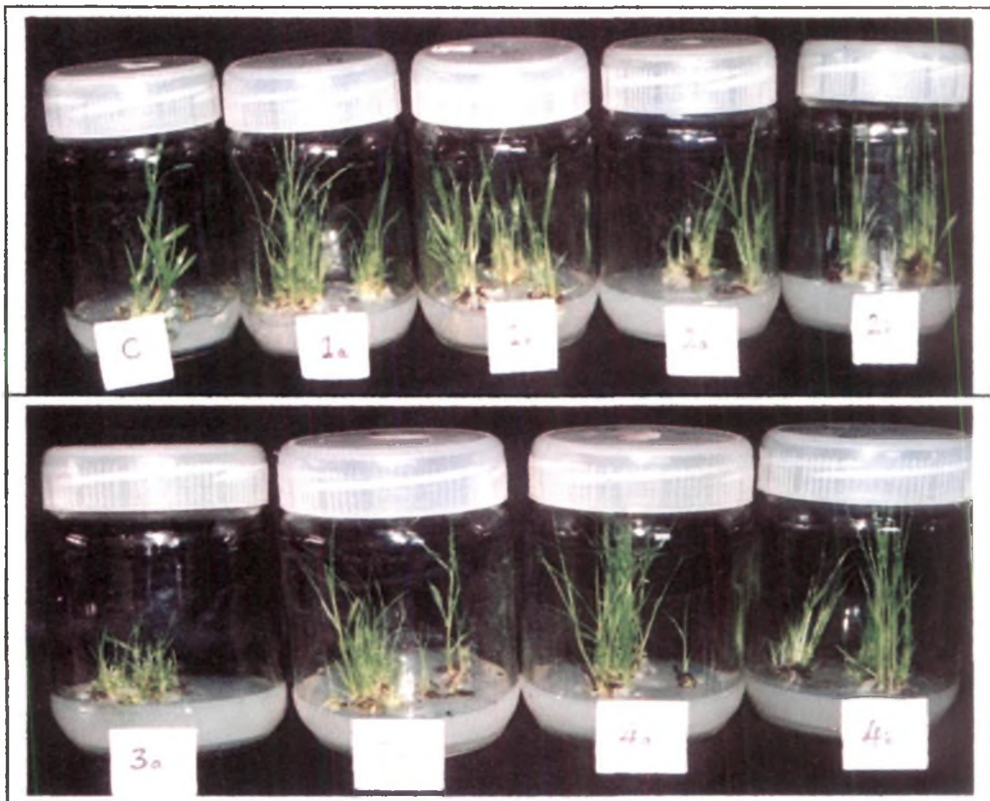


Plate 3.

Table- 4: Effect of hygromycin on percentage survivability of callus in finger millet.

Hygromycin Concentrations	% survivability callus
Control	90
10 μgm mL^{-1}	66
20 μgm mL^{-1}	40
30 μgm mL^{-1}	18
40 μgm mL^{-1}	**00**
50 μgm mL^{-1}	00

Explant (calli) used for regeneration and transformation was tested for Hygromycin sensitivity. Twenty days old callus was inoculated in different concentrations of hygromycin and the callus mortality was assessed based on callus blackening 15 days after treatment with hygromycin over regeneration media.

Table- 5: Effect of different concentrations of the bacterial culture in liquid callus induction media (for infection) and the duration of infection on the growth of bacteria after 48 hours of co-cultivation.

OD_{600}	Duration of infection (minutes)			
	4	**6**	**8**	**10**
Control	- - -	- - -	- - -	- - -
0.0-0.40	- - -	+	+	+ +
0.41-0.80	- - -	+	+ +	+ + +
0.81-1.20	+	+ +	+ +	+ + +
1.21-1.50	+ +	+ + +	+ + +	+ + +

Different concentrations of *Agrobacterium* culture were used to infect explant. The growth of bacteria was assessed by visual observation, after 48 hours of co-cultivation.

- - -	No growth
+	Less growth
+ +	Optimum growth
+ + +	**More growth**

OD_{600} of Agrobacterium culture (for infection) and the duration of infection

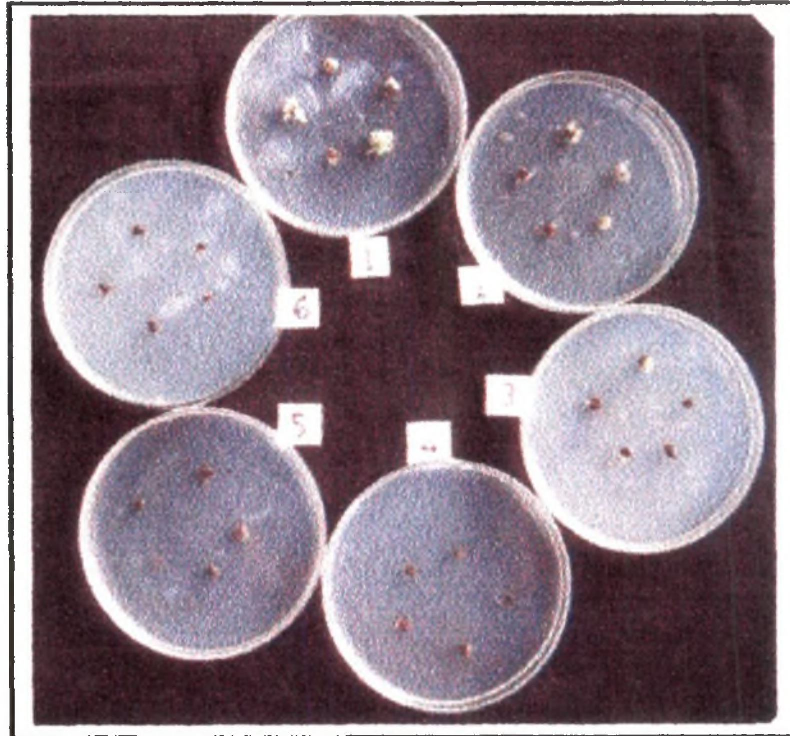


Plate 4: Sensitivity of callus to different concentrations of hygromycin.

1. Control (MS morphogenic media with out hygromycin)
2. 10 $\mu g.mL^{-1}$ hygromycin
3. 20 $\mu g.mL^{-1}$ hygromycin
4. 30 $\mu g.mL^{-1}$ hygromycin
5. **40 $\mu g.mL^{-1}$ hygromycin**
6. 50 $\mu g.mL^{-1}$ hygromycin

Transformation efficiency is also influenced by Agrobacterium culture concentration and duration of infection with explant. Therefore experiment was conducted to arrive at optimum OD_{600} Agrobacterium for optimum duration of infection. It was observed that the bacterial culture with a range of OD_{600} of 0.81 to 1.50, when infected to the callus for duration of 5 to 6 minutes resulted in the optimum growth of bacteria around the callus after 48 hours of co-cultivation. Almost all the concentration tested showed very high growth of the *Agrobacterium* when the co-cultivation was prolonged to 72 hours (Table 5).

Effect of acetosyringone in pre-induction and co-cultivation media.

The presence of acetosyringone is appeared to be most crucial. Absence of acetosyringone from co-cultivation medium as well as in the pre-induction media resulted in the complete absence of transformed calli. Acetosyringone concentration of 100µM was found to be optimum for the bacterial transformation (Table 6).

Effect of cefotaxime on the growth of Agrobacterium on selection medium.

Cefotaxime antibiotic was tested at different concentrations for their ability to inhibit the growth of *Agrobacterium* upto 20 days after inoculating the explants on the selection medium. There was complete inhibition of *Agrobacterium* at 400µg/mL of cefotaxime (Table 7).

B. Production of stable transformed calli

Calli explants derived from the scutellar portion of the seeds of finger millet were co cultivated for 2 days with *Agrobacterium* binary vector pCAMBIA1380, pre-induced with acetosyringone (100µM). After co-cultivation calli were transferred to the selection

Table-6: Standardization of the concentration of Acetosyringone in the co-cultivation medium.

Concentration of Acetosyringone	% Transformed calli
0 µM	6.6
50 µM	48.0
100 µM	**67**
150 µM	58

Twenty day old callus was infected with Agrobacterium culture having OD_{600} 0.81-1.20 for a duration of 5 minutes and they were co-cultivated in the media supplemented with different concentrations of Acetsyringone. The calli were then transferred to the selection media. The effect was assessed based on the number of calli remaining green after 15 days of inoculation in the selection media. That calli which remain green were considered as transformants (putative).

Table – 7: Effect of different concentrations of cefotaxime on the growth of Agrobacterium on the selection medium.

Cefotaxime (μgm mL^{-1})	Days after selection			
	5	**10**	**15**	**20**
100	+ +	+ +	+ +	+ +
200	+	+ +	+ +	+ +
300	- - -	+	+	+
400	- - -	- - -	- - -	- - -
500	- - -	- - -	- - -	- - -

Different concentrations of Cefotaxime were used to inhibit the growth of the bacteria in the selection medium. Effect of Cefotaxime on growth of the bacteria was assessed by visual observation.

- - - No growth
+ Growth
+ + More growth

medium (regeneration media +hygromycin, 40µg mL^{-1}+cefotaxime, 400µg mL^{-1}), 15 days after inoculation, observation was taken for transformed and non-transformed calli. The transformed calli remained green and the non-transformed calli turned black (Plate 5). The transformed calli were then placed on elongation media containing cefotaxime (400µg mL^{-1}).

Regeneration of shoots from the transformed callus

Calli explants were co-cultivated for two days with *Agrobacterium* culture EHA 105*(pCAMBIA1380)* and they were put into selection medium containing hygromycin (40µg mL^{-1}) and cefotaxime (400µg mL^{-1}). Observation was taken for transformed and non-transformed calli, 15 days after inoculation. The percent of transformed calli (the calli remaining green on the selection medium) was 13 (Table 8). The percent regeneration of transformed calli that were obtained from the selection media was 42.9 and the percent regeneration of the control calli i.e. regeneration from the callus which were not infected by *Agrobacterium was* 88% (Table 9).

III. Development of transgenic finger millet plants overproducing mannitol.

Genetic analysis of putative transformants.

Transformation studies was done for fingermillet (GPU-28) plants carrying the binary vector pCAMBIA1380, which contains the gene mtlD under the regulation of the constitutive promoter (35s CAMV), and hygromycin resistant gene *hpt II* as the selection marker. Putative transformants and non-transformants (control plants) were developed (Plate 6) and were grown under greenhouse conditions. DNA was extracted from both putative transformants and control plants. To study the stable integration of the construct in these plants, PCR analysis was done using specific primers for *hpt.*

Table 8: Transformation efficiency of pCAMBIA1380 based on number of transformed (putative) calli on selection media.

Treatment	Av.number of callus/plate	Av.no of transformed callus/plate	% Transformed Calli
Uninfected callli (control calli)	25	0	0
Infected calli	30	4	13

{Infected calli refers to the *Agrobacterium* (pCAMBIA 1380) treated calli and the uninfected calli refers to the calli that are not treated with *Agrobacterium*}.

Twenty day old callus was infected with *Agrobacterium* culture having OD_{600} of 0.81-1.50 for duration of five minutes and were co-cultivated for two days and then put for selection. After 15 days of inoculation in selection media, observation was taken for the number of calli remaining green (considered as putative transformants).

Table 9: Percent regeneration of shoots from the transformed (putative) calli.

Treatment	Number of callus/plate	Number of callus Regenerated	% Regeneration
Untreated calli	25	22	88
Transformed calli	28	12	42.9

{Untreated calli - calli that are not treated with *Agrobacterium* (pCAMBIA 1380)
Transformed calli - the green / transformed calli obtained on the selection media, 15 days after inoculation}

Both the uninfected calli and the transformed /green/regenerating calli (Fifteen days after inoculation in the selection media) were transferred on to elongation media and regeneration efficiency was assessed after 30 days of inoculation.

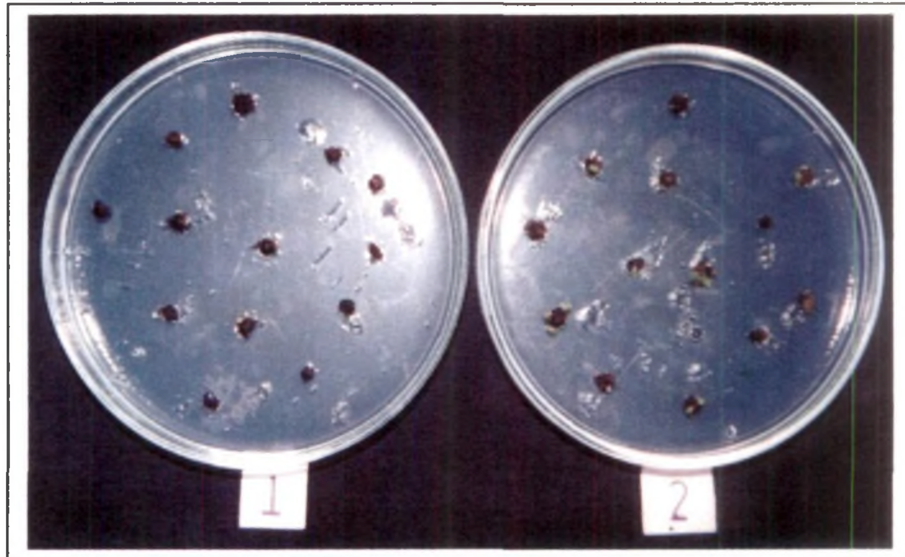


Plate 5 : Control and the Agrobacterium infected calli on the selection media, 15 days after inoculation.

1. Selection media + control (untreated) calli.

2. Selection media + infected (treated) calli.

Selection media : MS media + 0.75 mg L^{-1} TDZ + 40 µgm ml^{-1} hygromycin + 400 µgm ml^{-1} cefotaxime.
Untreated calli : The calli that are not infected with Agrobacterium.
Treated calli : The calli infected with *Agrobacterium*

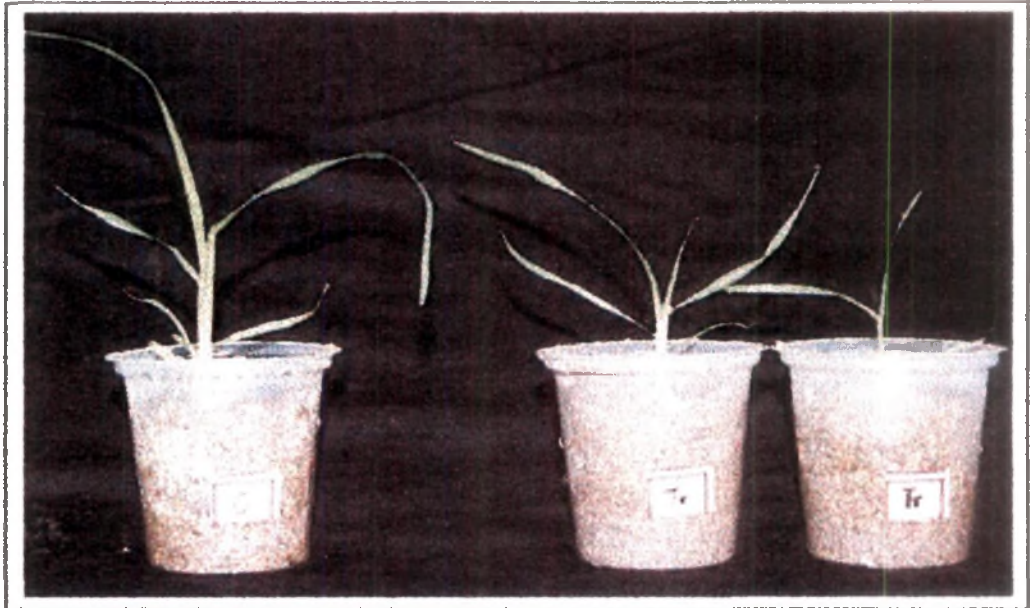


Plate 6: Control and the transgenic plants kept for hardening.

DNA extraction.

Plant DNA was extracted from both transformed and non-transformed finger millet plants by C-TAB method. Plasmid DNA of pCAMBIA1380 was also isolated. The quality of the DNA was checked both by spectrophotometric analysis and agarose gel electrophoresis. Further the DNA was used for PCR analysis.

PCR analysis.

PCR analysis was done using *hpt* primers, which would amplify a band of 350bp. Plasmid DNA was used as the positive control in PCR analysis. PCR was performed for 30 cycles, where the DNA (both plasmid and plant DNA) was denatured at 95°C for 5min; annealing reaction was done at 62°C for 1min per cycle and the extension step was carried at 72°C for 2 min in each cycle. The PCR results showed the expected band of 350bp clearly indicating that the plants are transformed (Fig. 2).

Southern analysis

To assess the stable integration of gene of our interest, southern analysis was performed for h*pt II* PCR-product. The genomic DNA was isolated from PCR-positive plants and non-transformed plants. PCR analysis was carried out for these plants using h*pt II* specific forward and reverse primers. Annealing and the extension of the strands was carried out by Taq Polymerase. The PCR product was analyzed on 1.5% agarose gel. The PCR product was transferred on to a nitrocellulose membrane and probed with labeled *hpt II*. All the PCR-positives showed a strong hybridization with labeled *hpt II* indicating the stable integration of the gene (Fig. 3)

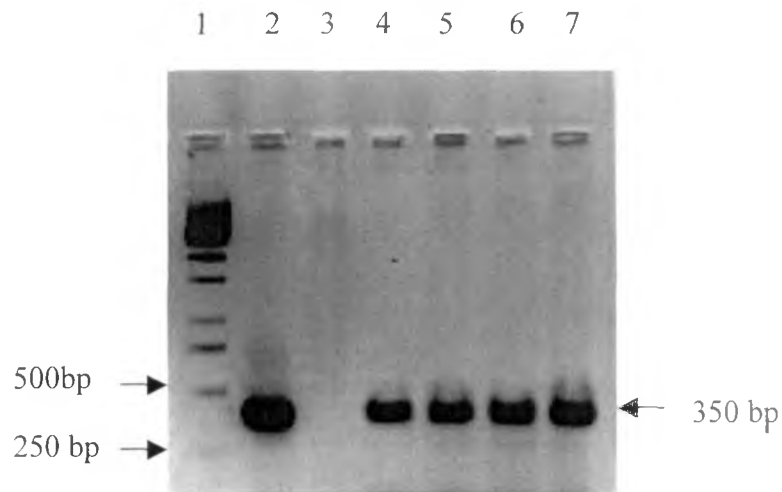


Fig 2: 1.5% Agarose gel showing the PCR amplified products for *hptII*

Lane 1: 1 kb gene ruler
Lane2 : pCAMBIA 1380
Lane 3 : Untransformed plant
Lane 4 to 7: Transformed plants

The genomic DNA isolated from control and the transgenic Finger millet plants were subjected for PCR analysis using *hpt II* specific primers. The PCR products were analysed on 1.5% agarose gel. The amplified product of 350bp was obtained in plasmid(lane:1) and transgenic plants(lane:4 to 7) but was not seen in control(lane:3)

Fig.3: Southern analysis of *hpt II* PCR product

Lane 1: Gene ruler 1 kb ladder DNA
Lane 2 & 3: Genomic DNA from Untransformed Finger millet plants
Lane 4 to 7: Genomic DNA from Finger millet transgenic plants over expressing *mtlD*
Lane 8: pCAMBIA 1380 plasmid DNA

The genomic DNA was isolated from control and transgenic Finger millet plants. PCR was performed using *hpt II* primers. PCR products were electrophoresed and transferred on to the nitrocellulose membrane. Southern blot was performed using the labeled hpt *II.* All the PCR positive plants showed a strong hybridization (lane 4 to 7).

DISCUSSION

V. DISCUSSION

Plant productivity is greatly influenced by environmental stresses, such as freezing, drought, salinity and flooding. Among these drought and salinity are the more important factors limiting plant growth and productivity. One of the plant responses that aids in acclimation to water deficit and high salinity is the accumulation of compatible solutes or osmolytes, such as quaternary amines (glycine betaine), amino acids (proline) and sugar alcohols (mannitol). Accumulation of compatible osmolytes is one of the most important dehydration tolerance mechanisms adopted by the plants under stress. The osmolytes help in osmoregulation, considered being one of the best mechanisms of drought resistance for crops exposed to dehydration stress. The accumulation of osmoprotectant can be achieved through plant genetic engineering.

Mannitol is the most widely distributed non-cyclic sugar alcohol in nature and has been reported in more than hundred species of vascular plants. Apart from plants it is also known to accumulate in bacteria, algae, fungi, insects and mammals. Suggested physiological role includes osmoregulation (Lewis and Smith, 1967; Hellebust, 1976), service as compatible solutes (Yancey et al., 1982; Brown and Simpson, 1972), storage of reduced carbon energy (Lewis and Smith, 1967; Loecher, 1987) and neutralization of hydroxyl radicals (Smirnoff and Cumbes, 1989).

In higher plants mannitol synthesis occurs simultaneously with either sucrose synthesis, as in celery (Rumpho et al., 1983) or with raffinose saccharide synthesis as in olive (Flora and Madroe, 1993). So mannitol is the major photosynthetic product in these plants. Biosynthetic pathway has been characterized in celery, where mannitol is synthesized from DHAP (dihydroxy acetone phosphate). The reactions involve the enzymes mannose-6-P isomerase, mannose-6-p reductase and mannitol-1-P phosphatase. But in the case of transgenic tobacco, mtlD (gene coding for mannitol-1-P dehydrogenase) expression leads to mannitol biosynthesis, wherein cytoplasmic fructose-6-P and NADH are utilized by mtlD to form mannitol-1-P and NAD. A non-specific phosphatase enzyme then dephosphorylates mannitol-1-P. Thus mannitol biosynthesis in celery proceeds via a different pathway which requires an additional enzyme catalyzed step than that in transgenic Tobacco (Rumpho et al., 1983).

Thus mannitol-1-P dehydrogenase is the rate-limiting enzyme for mannitol biosynthesis, in case of transgenic plants. Attempts to enhance osmotolerance by increasing mannitol accumulation should be centered on this enzyme. Hence overproduction of mannitol can be achieved through genetic engineering of mtlD gene.

There are different methods of gene transfer like protoplast transformation, biolistics or microprojectile bombardment and *Agrobacterium* mediated transformation. *Agrobacterium* mediated gene transfer constitute an excellent method for introduced genes in plant cells because DNA can be introduced into whole plant tissues, the integration of DNA is a relatively precise process, the stability of expression of most genes that are introduced by *Agrobacterium* appears to be excellent.

Engineering of mannitol over production in model plants like tobacco and *Arabidopsis* is successful in increasing osmotolerance. In the present study attempts were made to over produce mannitol in finger millet (var. GPU-28) by transferring mtlD gene (coding for mannitol-1-P dehydrogenase), so that plants are capable of tolerating increasing levels of drought stress.

Standardization of Regeneration protocol:

The success of any transformation experiment in a plant depends on availability of a reliable invitro regeneration protocol.

The most widely used explant for the regeneration of monocots is the callus derived from scutellum portion of the seed. However, various other explants like coleoptile (Godwin *et al.,* 1994), shoot apex (Sunghun e*t al.,* 1996) and florets (Langridge e*t al.*, 1992) have been successfully employed to regenerate shoots.

In the present study, the scutellum-induced callus was used as the explant for the production of multiple shoots. Initially an efficient medium for the production of active and healthy callus was standardized. The callus weight was highest when seeds of finger millet was plated on MS medium supplemented with 0.25mg L^{-1} kinetin and

2.5mg L^{-1} 2,4-D.Maximum shoots per explant were obtained from 0.75mg L^{-1} TDZ and maximum rooting and shoot elongation was obtained from 1mg L^{-1} GA. Concentration of TDZ and GA affected overall multiple shoot production. Even using 2,4-D and BA in combinations can obtain desirable callus growth and regeneration obtained in finger millet (Shivakumar, 2000; Hema, 2001).

Hygromycin sensitivity test:

The concentration of hygromycin that has to be used in selection medium is one of the most critical factors in selecting transformed plants. Therefore the sensitivity test to hygromycin was carried out. The callus explants were tested on MS medium supplemented with 0.75mg L^{-1} TDZ and also different concentration of hygromycin. Calli became black and then died at 40μg mL^{-1} and beyond. Similar effects of hygromycin were observed in the studies conducted by Hiei et al. (1997) and Kumaria et al. (2001), in their work on rice.

Standardization of Agrobacterium mediated transformation in Finger millet:

To standardize the gene transfer procedure in Finger millet, plasmid EHA105 (pCAMBIA 1380) was used. The plasmid has *hpt II* as a selectable marker. Hence transfer of pCAMBIA 1380 results in hygromycin resistance in the plants. The choice of a suitable explant source as starting material for infection of *Agrobacterium* is one of the most important factors in developing transformation protocol. In this study 20 days old callus initiated from scutella of mature seeds was used, because previous reports found that scutellum derived calli from mature seeds were excellent starting material for transformation of rice by *Agrobacterium* (Hiei *et al.,* 1994; Rashid *et al.,* 1996; Toki, 1997).

Initially experiments were conducted to standardize OD_{600} of *Agrobacterium* culture (for infection) and duration of infection. The optimum OD_{600} and optimum duration of infection were selected based on the growth of bacteria around the callus after 48 hours of co-cultivation. Optimum growth of bacteria around the callus was observed

when the explants are infected for duration of 5 minutes with the *Agrobacterium* culture of OD_{600} ranging from 0.81 to 1.50. Upadhaya et al. (2000), reported similar type of results.

Acetosyringone has been found to induce vir genes and to extend the host range of some *Agrobacterium tumefaciens* strains (Boulton *et.al.*, 1989; Godwin *et.al.*, 1991). Use of Acetosyringone has enhanced transformation efficiency in certain dicots and has been found to be one of the factors affecting transformation in monocots, such as corn (Gould *et al.*, 1991; Ishida *et al.*, 1996), rice (Aldemita and Hodges, 1996; Chan *et al.*, 1992), wheat (Cheng *et al.*, 1997) though it is not found to be necessary in barley (Tingay *et al.*, 1997). In the present study, incorporation of acetosyringone in the co-cultivation medium was found to provide the main boost to transformation efficiency. Incorporation of acetosyringone (100μM) in the cocultivation medium gave positive effect and high transformation efficiency. Because acetosyringone in the pre induction medium is known to enhance T- DNA strand synthesis (Li *et al.*, 1992) and T- DNA strand production is reported to peak 12- 24 hours after acetosyringone mediated induction (Cultianez- Macia and Hepburn, 1988), induction of vir genes prior to co-cultivation and efficient release of T- DNA may be the cause of high frequency transformation with pre induced EHA 105 (pCAMBIA 1380) (Aldemita and Hodges, 1996).

In the present study, regeneration media was used for co-cultivation and selection. Co-cultivation and selection on regeneration medium is found to be better than callus induction medium, as calli on the regeneration medium were more embryonic in nature (Kumaria et al., 2001).

To overcome the problem of elimination of *Agrobacterium* after transfer of genetic information, cefotaxime was used. There was complete inhibition of the *Agrobacterium* at 400μg mL^{-1} of Cefotaxime. The calli that remains green on the selection medium even after 15 days of inoculation were considered as transformed calli. About 13 percent of the calli remained green and were taken for further regeneration. The transformation efficiency of pCAMBIA 1380 was considered based

on the percent of calli that remains green (the calli in the state of regeneration, after 15 days of inoculation in the selection media).

Development of putative transformants

Putative transformants were developed from the hygromycin resistant calli. The calli that remain green and regenerate on the selection media were selected. Good regeneration and rooting of the plants were obtained on the elongation media. The percent regeneration of transformed calli was 42.9, but there was 88 percent regeneration from the control (uninfected) calli. Reduction in the regeneration of transformed calli is because the calli undergo a loss in regeneration potential in long-term cultures (Khanna and Raina, 1997). However, plant regeneration potential in the transformed calli of the present study though appreciable, was lower when compared to the control calli, probably due to adverse effect of long period of eliminating bacterial growth and long-term exposure to cefotaxime. Another strong reason could be that, selection agent hygromycin comes in the way of regeneration, as concluded by Hiei et al. (1997); Nuccio et al. (1998) and Kumaria et al. (2001). It has been reported that, in general, the regeneration frequency is lower with transformed calli (Hiei et al., 1997; Khana and Raina, 1999).

Genetic analysis of the transgenic plants.

To study the stable integration of the gene into host genomic DNA, several analysis could be followed. Most simple and a reliable method is Gus assay (Wilmink and Dons, 1993). The other important methods are PCR (Polymerase chain reaction) analysis where the thermocyclers are used to amplify the required DNA fragment by using specific primers; and southern blot analysis, which gives the perfect confirmation for T-DNA integration into host genomic DNA.

In the present study PCR analysis and southern blotting was done to confirm the stable integration of the gene in the Finger millet plants. The specific primer for *hpt II* was used. The PCR results showed the expected band of the size 350bp, as shown by the plasmid, which was used as the positive control. As expected, the control plants did not show any amplification for *hpt II*. Southern blotting of *hptII* PCR products

showed a strong hybridization with the labeled *hpt II*. The results clearly indicate that the plants are transgenic.

Genetic engineering of mannitol in higher plants

Due to the importance of creating drought tolerant crops- various strategies are being persued to genetically engineer increased osmoprotection in plants. There is a potential for increasing osmotolerance by genetic manipulation of proline synthesis.

It has been proposed that transcriptional control of the mtlD gene, which encodes mannitol-1-P dehydrogenase, an essential enzyme for mannitol synthesis, is important for the regulation and accumulation of this sugar alcohol during osmotic stress in transgenic plants especially in tobacco and *Arabidopsis*. The increase in the mannitol levels was accompanied by coordinate increase in the mtlD transcript levels. Therefore to enhance osmotolerance by increasing mannitol accumulation should be centered on this enzyme.

Putative transformants and control plants were subjected to PCR analysis. The putative transformants showed the band of expected size, thereby confirming that the plants are transgenic for mannitol overproduction.

Transformation efficiency depends upon number of factors like choice of explant, super virulence of binary vector, selectable agent, inclusion of acetosyringone during co-cultivation, period and temperature of co-cultivation and pH of the medium (Hiei et al. (1994); Aldemita and Hodges (1996); Rashid et al. (1996); Dong et al. (1996); Ishida et al. (1996) and Mohanty et al. (1999). Small, hard calli of GPU-28 was more congenial for transformation. Hygromycin selection has been used routinely for selection after transforming (Ayres and Park, 1994). It allows better discrimination between transformed and non-transformed tissue and does not induce albinism or fertility problems. The transformation efficiency of binary vector pCAMBIA1380 was low, probably because it does not contain extra *vir* gene. Extra *vir* region is known to increase the virulence.

Earlier transformation work on finger millet (Indaf 9) has shown that regeneration efficiency of the transformed tissue was very less (8%) (Hema, 2001). In the present study regeneration efficiency of the transformed tissue was much higher (almost 43 %). Probable reasons for this may be due to the growth hormones used, and the standardized protocol for both regeneration and callus induction in the present study, were more conducive. The OD_{600} of the bacterial culture, infection time and the binary vector were more effective in transforming the callus. More over the calli obtained were harder, which is very necessary for the transformation to occur. One more important reason for good regeneration could be that, after co-cultivation the calli were not continuously exposed to the selection media. They were inoculated in selection media only for15 days after co-cultivation. The continuous exposure of plants to selection media is likely to result in loss of regeneration efficiency and stunted growth (Hiei et al., 1997; Nuccio et al., 1998; Kumaria et al., 2001). Another reason may be the variety used in the present study, GPU –28. This variety seems to have more capacity to regenerate and is more likely to get transformed.

SUMMARY

VI. SUMMARY

Genetic engineering has opened new avenues to modify crops and provide new solutions to solve specific needs. Through this technology genes can be introduced from distinct organisms into crop plants.

Plants accumulate mannitol in response to moisture stress and osmotic stress. Various studies have focused on the ability of mannitol as a compatible solute involved in osmotolerance. It has been reported that mannitol is synthesized from fructose-6-P catalyzed by a key enzyme mannitol-1-P dehydrogenase in the transgenic plants. Therefore in the present study the gene mtlD coding for this key enzyme was used to transform Finger millet. Moisture stress tolerance of plants can be achieved through overproducing mannitol by genetic engineering. The main approach therefore has been initially to standardize the regeneration and transformation protocol in Finger millet. Subsequently attempts were made to obtain transformed callus overexpressing mtlD and to achieve regeneration.

Initially an efficient regeneration protocol was standardized and it involves callus induction, callus growth and regeneration. Optimum growth of the callus were obtained at 2.5 mg L^{-1} 2,4-D and 0.25mg L^{-1} kinetin and maximum number of shoots were produced from 20 days old callus explant when they were plated on MS medium supplemented with 0.75mg L^{-1} TDZ.

The transformation protocol was standardized using the construct EHA105 (pCAMBIA1380) harboring genes for mannitol biosynthesis (mtlD) and for hygromycin resistance (hpt II). Experimental conditions were developed initially by treating callus with *Agrobacterium* culture having OD_{600} ranging from 0.81 to 1.50 for 5 minutes and then this callus were co-cultivated in co-cultivation medium having acetosyringone 100μM, for 2 days and then transferred to selection medium containing hygromycin (40μg mL^{-1}). We have obtained transformation efficiency of 13% for transformed callus and the regeneration efficiency of the transformed callus was 42.9%.. Low transformation and regeneration efficiency may be due to many

factors that influence *Agrobacterium* mediated transformation, like *Agrobacterium* strain, the binary vector, explant and selectable agent.

Both the control plants and the putative transformants were grown in green house conditions. DNA was extracted through standard procedures and subjected to PCR analysis using specific primers for hpt. The analysis showed the expected band size of 350 bp in transformed lines where as the control plants did not show any amplification as expected. The southern blotting done for PCR amplified products showed a strong hybridization with the labeled *hptII.* Thus the results clearly indicate that the plants are transgenic.

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APPENDICES

VIII. APPENDIX

Composition of MS media

Ingradients	Salt concentration stock solution (g/L)	Aliqutos taken for one litre of medium (mL)	Final concentration of salt of medium (mg/L)	Group
Inorganic salts				
NH_4NO_3	66.00	25	1650	I
KNO_3	76.00		1900	
$MgSO_4. 7H_2O$	14.80		370	
KH_2PO_4	6.80		170	
$CaCl_2$	4.40	10	440	II
Na_2 EDTA	7.45	5	37.2	III
$FeSO_4 7H_2O$	5.57		27.2	
Micro nutrients				
H_3BO_4	0.62	10	6.2	iv
$ZnSO_4. 4H_2O$	0.86		8.6	
$MnSO_4 4H_2O$	2.23		22.3	
KI	0.083		0.25	
$Na_2Mo_4. 2H_2O$	0.025		0.025	
$CuSO_4. 5H_2O$	0.0025		0.025	
$CoCl_2 6H_2O$	0.0025			
Organic salts				
Glycine	0.20	10	2.0	v
Myoinositol	10.00		100.00	
Thiamine HCl	0.01		0.10	
Pyridoxine HCl	0.05		0.50	
Nicotinic acid	0.05		0.05	
Biotin	0.05			
Sucrose			30000.00	
Agar			8000.00	
PH			5.8	

Constituents required for AB medium .

AB medium (per litre)

AB buffer- Solution I
KH_2PO_4 – 60g
NaH_2PO_4 – 20g
PH should be around 7.0

AB- salts- solution II
NH_4Cl - 20g
$MgSO_4.7H_2O$ - 6g
KCl – 3g
$CaCl_2$ – 3g
$FeSO_4$. $7H_2O$ – 50mg

100mL of both these solutions are autoclaved separately and stored

Constituents required for YEM medium (Per 100ml).

Yeast extract	- 0.04 g
Mannitol	- 1g
NaCl	- 0.01g
$MgSO_4.7H_2O$	- 0.02g
K_2HPO_4	- 0.05g
Agar	- 1.5%
PH	- 6.8-7