GENETIC TRANSFORMATION STUDIES ON SORGHUM (*SORGHUM BICOLOR*) FOR SUCROSE ISOMERASE

A thesis submitted in Partial fulfillment of the requirement for the degree of Master of Science in Bio-Technology

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C E R T I F I C A T E

This is to certify that the dissertation entitled ***"GENETIC TRANSFORMATION STUDIES ON SORGHUM FOR SUCROSE ISOMERASE "***submitted by ***Mr. M.J SHYAM SUNDER*****,** bearing Roll No. ***11031G0346*** to the ***Centre for Biotechnology ,Institute of Science and Technology, Jawaharlal Nehru Technological University Hyderabad*** in partial fulfillment of the requirement for the award of degree in ***Master of Science in BIOTECHNOLOGY*** a bonafied work done during the period of JULY 2013 TO JANUARY 2014 under the guidance of ***Dr. POOJA BHATNAGAR MATUR***, Senior Scientist for pttc in(ICRISAT)Pantancheru, Hyderabad.

It is further certified that the dissertation or any part thereof has not been submitted elsewhere for any other degree or diploma.

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DECLARATION

I declare that the project work was done under the guidance of **Dr. Pooja Bhatnagar Mathur,** Senior Scientist for, Platform for Translational Research on Transgenic Crops (PTTC) at ICRISAT, Hyderabad and the dissertation entitled **"GENETIC TRANSFORMATION STUDIES IN SORGHUM BICOLAR FOR SUCROSE ISOMERASE"** was done for partial fulfillment of the requirements of the award of **Master of Science** degree in Biotechnology, for the academic period of 2011-2013. This is my original work and has not previously been submitted for the award of any other degree or diploma of any University.

M.J.SHYAM SUNDER

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ABBREVIATIONS

µM	micro molar
µl	micro litre
µg/l	microgram per litre
µg/ml	microgram per milli-litre
ºC	degree Celsius
BAP	benzyl amino purin
bp	base pair
Cef	cefotaxime
° C	degree Celsius
DNA	deoxyribonucleic acid
EDTA	ethylene diaminetetracetic acid
gm	grams
h	hour
mg	milli gram
mg/ml	milli gram/milliliter
min	minutes
ml	milliliter
mm	milli meter
nm	nano meter
MS	-Murashige and skoog medium

*npt*II	-neomycin phototransferase gene
*hpt*II	-hygromycin phototransferase gene
OD	optical density
kDa	kilo Dalton
pH	negative logarithm of hydrogen ion concentration
rpm	resolutions per minute
V/V	volume per volume
W/V	weight per volume
YEB	yeast extract broth
MS	Murashige and Skoog (1962) medium
SIM	shoot induction medium
SI	sucrose isomerase
SEM	shoot elongation medium
RIM	root induction medium
SAT	semi-arid tropics
Mm	millimetre
Bt	Bacillus thuringiensis
PCR	Polymerase Chain Reaction
PDSI	Pantoea dispersa

TITLE: "GENETIC TRANSFORMATION STUDIES IN SORGHUM BICOLAR FOR SUCROSE ISOMERASE"

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ABSTRACT

Seeds of sweet sorghum cultivars SSV74 and SSV84 were the primary explants used for the introduction pdSI genes(expression of Sucrose Isomerase enzyme) which converts into Isomaltulose(palatinose,α-D-glycopyranosyl-1,6-D-fructofuranose).Isomaltulose is an isomer of sucrose which has greater health advantages for diabetics and used as low calories sweetener, it is also a renewable starting material in industrial productions. The pdSI gene was under regulation of 35S promoter cloned into binary vector PMDC99. Both the vectors also contain hygromycin phosphotransferase gene (hptII) as plant selection marker against hygromycin.

A kill-curve experiment was performed to determine the optimal concentration at which the control explants is able to survive rate was high in 2.5 & 0.5 mg/L concentration whereas 7.5 mg/L, the survival rate of approximately 12-15%.

Agrobacterium cloned with pdSI gene was used for transfer of the genes into sweet sorghum cultivar. Pre-soaked imbibed seeds of sweet sorghum were punctured using sterilized needle and dipped in Agro culture (with pdSI gene) in YEB media for 15-20 minutes. The seeds were co-cultivated on MS media 48-72 hours. The explants were then transferred in to MS media augmented with cefotaxime and hygromycin. The selection marker strategy is as follows, During germination stage hygromycin concentration was 2.5mg/L, in elongation stage, 5mg/L and rooting was done at 7.5mg/L. plantlets which are showed rooting and survival at selection pressure of 7.5mg/L were then transferred to soil for acclimatization. Leaf sample of healthy, putative plants were collected and DNA was extracted using CTAB method for further molecular characterization.

INTRODUCTION

CHAPTER 1
INTRODUTION

Sorghum bicolor, commonly called **sorghum** and also known as ***durra***, **jowari**, or **milo**, is a grasss pecies cultivated for its grain, which is used for food, both for animals and humans, and for ethanol production. Sorghum originated in northern Africa, and is now cultivated widely in tropical and subtropical regions. *S. bicolor* is typically an annual, but some cultivars are perennial. It grows in clumps that may reach over 4 m high. The grain is small, ranging from 3 to 4 mm in diameter. Sweet sorghums are sorghum cultivars that are primarily grown for foliage, syrup production, and ethanol; they are taller than those grown for grain.
S. bicolor is the cultivated species of sorghum; its wild relatives make up the botanical genus *Sorghum*.

Sorghum (*sorghum bicolor L.*) is the fifth most important cereal crop world-wide (dogget 1988), a staple food for many people in Africa and Asia, and a major feed crop for domesticated animals. Common name for sorghum is jowar, the most common products made from whole grain are porridges, tortillas, couscous, rice substitute or just popped sorghum. Grain sorghum can be fed to domestic animals as a green fodder harvested in different stages of plant development. Grain sorghum is also used in beer and alcohol production.

Sorghum is a thermophile plant and tolerates drought. The crop adapts different soil types and toxicities such as alkalinity, acidity, aluminium toxicity, even standing water. Sorghum uses the C4 malate cycle, the most efficient form of photosynthesis and has greater water use efficiency than C3 plants. Sorghum may well offer the best opportunity to satisfy the doubling of meat (protein) in the developing world by 2020, as a food for the poor and an alternative feed to maize.

Diseases are also a major constraint to both production and quality of grain and forage sorghum. Downy mildew is a major limitation to grain yields in the United States as well as other parts of the world. Viral diseases such as Johnson grass Mosaic virus (JGMV) and Maize Dwarf Virus (MDMV) can be particularly damaging to yield and forage quality especially when the infection occurs early in the crop cycle. Integrated pest management (IPM) has historically

place greater hopes on host plant resistance. However, conventional host plant resistance involves quantitative traits of several loci, because of when the progress is slow and difficult to achieve. There are no sources of true resistance to heliothis, stem borer or shoot fly and some of the diseases.

With the advent of genetic transformation techniques, it has become possible to insert genes into the plant genome that confer resistance to insects. Genetic transformation of plants has become a source of agricultural innovation in plant biotechnology. Genetic engineering of plants is much easier than that of animals, because in plants there is a natural transformation system (*Agrobacterium tumefaciens*),plant tissue can be easily redifferentiated (a transformed piece of leaf may be regenerated to a whole plant) and the transformation and regeneration are relatively easy for a variety of plants. A number of genetically engineered plants have been developed during the course of time by using various methods like particle bombardment, electroporation and Agrobacterium-mediated transformation. Of which Agrobacterium-mediated transformation method becomes more popular due to the fact of itself being a natural genetic vector and due to high transformation frequency. Various genes conferring resistance to herbicide, insects, virus, fungi were identified from diverse species for integration in to plants. This ability to transcend species barrier brought worldwide interest in genetic engineering of plants for alleviation of biotic species. For which there is n background resistance in available germplasm. However for the effective application of this technology it is imperative to have the ability to transfer genes in to crop plants in an efficient and reliable manner.

A part from various chemical pesticides used in the pest management biological pesticides, bacteria such as *Bacillus thuringensis* (bt) and *Bacillus sphearicus* have been the most successful group of organisms identified for use in pest control on a commercial scale.Insecticidal genes such as bt toxins, trypsin inhibitors, and lectins, ribosomes inactivating proteins, secondary plant metabolites, vegetative insecticidal proteins and small RNA viruses can also be used alone or in combination with Bt genes for pest control. There are several subspecies of this bacterium, which are effective against lepidopteron, dipteran and coleopteran insects. Because of the crystalline nature of these proteins, the term cry is used in gene and protein nomenclature. The toxins genes have been classified in to 28 types based on insect specificity and sequence homology. Cry 1

type encodes proteins of 130 kDa, and are usually specific to lepidopteronlarvae; type 2 genes encode for 70 kDa protein that are specific to lepidopteron dipteran larvae; and type 3 genes encode for 70 kDa proteins that encode for proteins specific to coleopteran larvae. Type 2, 4, 10, 11, 16.19, 20and 21 proteins have specific toxicity to dipteran larvae.

- Sweet sorghum research at ICRISAT

  ICRISAT's two-pronged strategy developing improved hybrid parents and varieties technology by perspective entrepreneurs.

- **Progress in research**
- Research on the development of sweet sorghum cultivars was initiated in 1980 with the evaluation of 70 germplasm accessions.
- Two landrace lines, IS 6872 and IS 6896 with high stalk sugar content and biomass, were selected in 1981
- Later, several sweet sorghum lines were identified among Nigerian and Zimbabwean lines, and among advanced breeding progenies
- Sweet sorghum research discontinued in early 1990s due to changed focus driven by donors' perceptions and needs of national agricultural research systems (NARS).
- Research was renewed in 2002 to meet the increased demand for ethanol, driven by government policies to blend ethanol with petrol.
- The wide variability in germplasm and hybrid parents for the traits related to ethanol production, such as sugar content and high stalk yield, offers bright scope for the development of high stalk yielding sugar-rich varieties and hybrids.
- Promising varieties/restorer lines viz., NTJ 2, SPV 422, Seredo, ICSR 93034, S 35, ICSV 700, ICSV 93046, ICSV 25263, SP 4487-3, SP 4484-1, SP 4484-3, SP 4482-1, SP 4482-2 and SP 4481-1 have been identified.

PLANT ECOLOGY:

Sorghum bicolor (L),monech (2n=20) is a highly diverse species belonging to the genus sorghum of the tribe *Andropogonae*. The genus sorghum belongs to the class Monocotyledon and family is a Graminae (*poaceae)*.It is a native to Africa, from where it was taken to the warm and dry areas of all continents.

Sorghum is an annual herb growing to a height of 1-4 m.In habit it resembles maize. It has well developed root system including bunch of root hairs able to absorb plenty of water and nutrients. Beside sub-terranean root system forms strong aerial roots permeating through the soil and ensuring better stability in it stem is strong, hard and smooth divided by nodes and grain forms up to 1-1.5 m. sorghum leaves are 50-100 mm wide and 0.5-0.8m long. Leaves are covered with wax layer and stem likewise.

Inflorescence of sorghum is very characteristic part of the plant usually formed in different shapes and sizes. Panicle is erected; drooping spikelet's contain one flower and are gathered in 2, 3 or 4 on secondary branches. Sorghum is allagamous but their pollen is good enough to pollinate themselves.

Seeds of sorghum are round, oval or heart-shaped and can occur in white, creamy, yellow, brown or violet in colour. Like other cereals sorghum is predominantly starchy. The highest amount of starch and protein occur in endosperm. High fiber content in outer layer and poor digestibility of nutrients are characteristics of sorghum .The protein content of sorghum grain is significantly correlated with its weight and starch content, grain yield and protein content are in inverse correlation too.

Fig: 1 Sorghum plant**.**

Kingdom	Plantae
Sub-kingdom	Tranheobionta
Super-division	Spermatophyta
Division	Magnoliophyta
Class	Liliopsida
Sub-class	Commelinidae
Order	Cyperales
Family	*Poaceae*(grass)
Genus	Sorghum

Table no: 1 classification of sorghum plant.

IMPORTANCE:

Sorghum is commonly considered as a "poor man's crop" as it is mainly cultivated in the more agriculturally marginal areas, and areas of least economic development. Approximately 300 million people in sub-Saharan Africa and India rely on sorghum grain as a major staple food. In the past 50 years, the area planted to sorghum worldwide has increased 60%.Estimates suggest 51% of the crop goes for feed and 49% for food and other uses. India, although still the country with greatest area planted to sorghum, currently plants 10.5 million hectares compared to 16 million as recently as 1989.More than 50% of the area is in hybrids but yields are only 54%of world average.

USES:

Sorghum syrup and hot biscuits are a traditional breakfast in the Southern United States. Sorghum syrup is also used on pancakes, cornmeal mush, grits and other hot cereals. It can be

used as a cooking ingredient with a similar sweetening effect as molasses, despite the fact that the nutritional content of blackstrap molasses still has a higher nutritional value than sorghum syrup in most regards.

Sweet sorghum syrup is sometimes called "molasses" or "sorghum molasses" in some regions of the U.S., but the term *molasses* more properly refers to a different sweet syrup, made as a byproduct of sugarcane or sugar beet sugar extraction. In the U.S. since the 1950s, sorghum has been raised primarily for forage and silage, with sorghum cultivation for cattle feed concentrated in the Great Plains (Texas, Kansas, and Nebraska are the leading producers) where insufficient rainfall and high temperature make corn production unprofitable.

Grain sorghum has also been utilized by the ethanol industry for quite some time because it yields approximately the same amount of ethanol per bushel as corn. As new generation ethanol processes are studied and improved, sorghum's role may continue to expand. Texas A&M University is currently running trials to ascertain the best varieties for ethanol production from sorghum leaves and stalks in the USA. In India, and other places, sweet sorghum stalks are used for producing bio-fuel by squeezing the juice and then fermenting into ethanol.[4]

NUTRITIONAL QUALITY:

Sorghum mainly contains carbohydrates of which starch and dietary fiber are the main components. Starch is absorbed by the body and converted in to glucose. Glucose is taken up in the blood stream to provide the body with energy for essential functions of the body. Dietary fiber promotes healthy digestion and of the digestive tract.

Sorghum contributes largely to the total protein intake. It is remembered that the protein which occurs in grain lacks the essential cannot replace animal protein. For a high quality protein in the diet can be combined with soya or dry beans. Sorghum is rich in iron, potassium, calcium, and phosphorous. Minerals play an important role in body processes such as hormone functioning, tissue building and enzyme function. The sodium content (salt content) of sorghum is very low.

Calories	651	
Total fat	6.3 g	10%
Cholesterol	0 mg	0%
Sodium	12 mg	0%
Carbohydrates	143.3 g	48%
Dietary fiber	12.1 g	48%
Sugars	0.0 g	
Protein	27.1 g	
Calcium	5%	
Iron	47%	

Table no: 2 Nutritional values of sorghum. Serving per cup (192g)

PLANT INTRODUCTION

SEED VARIETY:

Sorghum seeds are different cultivars of belonging to different groups including as follows SSV 74 and SSV 84 (sweet sorghum), BTX -623M 35-1(grain sorghum).The both SSV74 and SSV84 seeds are used throughout the experiment.

Fig: 2 and 3 two variety seeds of sweet sorghum.

REVIEW OF LITRATURE

CHAPTER 2
REVIEW OF LITERATURE

2.1**SUCROSE ISOMERASE:**

Isomaltuose palatinose, α-D-glycopyranosyl-1,6-D-fructofuranose) is a naturally occurring isomer of sucrose (_α-D-glucopyranosyl-1,2-D-fructofuranose) that is valued as an a cariogenic sweetener. It is a nutritional sugar that is digested more slowly than sucrose, and it has health advantages for diabetics and non diabetics. Greater acid stability and lower hygroscopic tendency than sucrose are advantages for some food applications. Unlike sucrose, isomaltulose is a reducing sugar, which allows different chemical reactivity when it is used as an industrial precursor. Isomaltulose is currently used to manufacture sugar alcohols consumed as low-calorie sweeteners, and it is an attractive renewable starting material for the manufacture of biosurfactants and biocompatible polymers. It is produced industrially from sucrose by using immobilized cells of bacteria that produce an enzyme designated sucrose isomerase (SI), sucrose mutase, or isomaltulose synthase (EC 5.4.99.11).SIs have been purified from *Erwiniarhapontici* NCPPB 1578, *Protaminobacterrubrum*CBS574.77, *Serratiaplymuthica* ATCC 15928, *Agrobacterium radiobacter*, *Pseudomonasmesoacidophila*MX-45, *Klebsiella*sp., and the whitefly *Bemisiaargentifolii*. Several of the corresponding genes have been cloned, and the gene designation s*palI*, *smuA*, and *mutB* have been used. The crystal structure has been elucidated for the SI cloned from *Klebsiella* sp. Strain LX3, which confirmed that there is a TIM (triose phosphate Isomerase - like) barrel (_/_)8 core with active site architecture typical of glycoside hydrolase family 13.(Lu guang Wu and Robert G Birch 2004).

In addition to isomaltulose, SIs that have been described produce various proportions of the isomer trehalulose (_-Dglucopyranosyl-1,1-D-fructose) along with glucose and fructoseas by-products. Some purified SIs produce predominantly Isomaltulose (75 to 85%), and others produce predominantly trehalulose (90%) or only trehalulose in the case of the white fly enzyme. The ratio of the products obtained from bacterial SIs varies with the reaction conditions, particularly the temperature and pH, and under some conditions small quantities of other products, such as isomaltose and isomelezitose, may be formed. The formation of multiple

products lowers the yield and complicates the recovery of the desired isomer. Slow conversion of sucrose into isomaltulose and a narrow range of optimal reaction conditions also limit the industrial efficiency of isomaltulose production. Anideal SI for industrial use would exhibit high speed, complete conversion, high specificity, and a wide window of reaction conditions for isomaltulose production. Recently, we isolated multiple isomaltulose-producing bacteriaby functional screening and found that an isolate of *Pantoea dispersa* designated UQ68J is exceptionally efficient in sucrose isomerase activity. Here, we describe the cloning and characterization of four SIs from isomaltulose-producing bacteria and a comparison of the activities of the purified recombinant enzymes produced in *Escherichia coli*. The enzyme from *P. dispersa* proved to be exceptional among the characterized SIs and to have properties highly desirable for use in the industrial biosynthesis of Isomaltulose.

Sweet sorghum has been identified as a possible ethanol feedstock because of its biomass yield and high concentration of readily fermentable sugars. It has found limited use, however, because of poor post-harvest storage characteristics and short harvest window in cooler climates. Previous research (Bennett, A.S., Anex, R.P., 2008). Farm-gate production costs of sweet sorghum as a bioethanol feedstock. Transactions of the ASABE 51, indicates that fermentable carbohydrates (FC) can be produced at less expense from sweet sorghum than from corn grain. Previous research, however, did not include costs associated with off-farm transportation, storage, or capital costs associated with milling and energy recovery equipment that are required to provide FC suitable for biological conversion. This study includes these additional costs and reevaluates sweet sorghum as a biocommodity feedstock.

A total of eight harvest-transport-processing options are modeled, including 4-row self-propelled and 2-row tractor-pulled forage harvesters, two different modes of in-field transport, fresh processing, on-farm ensilage and at-plant ensilage. Monte Carlo simulation and sensitivity analysis are used to account for system variability and compare scenarios. Transportation costs are found to be significant ranging from \$33 to \$71 Mg^{-1} FC, with highest costs associated with at-plant ensilage scenarios. Economies of scale benefit larger milling equipment and boiler systems reducing FC costs by more than 50% when increasing annual plant capacity from 37.9 to 379 million liters. Ensiled storage of high moisture sweet sorghum in bunkers can lead to significant losses of FC (>20%) and result in systems with net FC costs well above those of corn-derived FC. Despite relatively high transport costs, seasonal,

fresh processed sweet sorghum is found to produce FC at costs competitive with corn grain derive.

2.2Characterization of *Pantoea dispersa* UQ68J: producer of a highly efficient sucrose isomerase for isomaltulose biosynthesis.

Isomaltulose (*α*-d-glucopyranosyl-1,6-d-fructofuranose, also called palatinose) is a structural isomer of sucrose (*α*-d-glucosyl-1,2-d-fructose), found as a natural constituent of honey and other sugar-rich fluids (Stodola *et al.* 1956; Weidenhagen and Lorenz 1957; Siddique and Furgala 1967). It is a nutritive disaccharide, with sweetness and bulk similar to sucrose, and several characteristics that are advantageous over sucrose for some applications in the food industry. Isomaltulose is non cariogenic (not causing dental decay); has a low glycemic index (useful for diabetics); selectively promotes growth of beneficial bifido bacteria among human intestinal microflora; has greater stability than sucrose in some foods and beverages (due to greater acid stability and resistance to metabolism by many microbes); is less hygroscopic; and can be simply converted into sugar alcohols with other useful properties as foods (Takazoe 1989; Schiweck *et al.* 1991). The safety of isomaltulose has been comprehensively verified, resulting in unqualified approval as human food, and it is widely used commercially as a sucrose substitute in foods, soft drinks and medicines (Lina *et al.* 2002).

Furthermore, because isomaltulose has an accessible carbonyl group, it has attracted attention as a renewable starting material for the manufacture of bioproducts such as polymers and surfactants with potential advantages over substances manufactured from petroleum (Schiweck*et al.* 1991; Cartarius*et al.* 2001; Lichtenthaler 2002)

Commercial isomaltulose is produced from food-grade sucrose by enzymatic rearrangement from a (1,2)-fructoside to a (1,6)-fructoside followed by crystallization. Sucrose isomerase (SI) enzymes able to convert sucrose to isomaltulose; typically among other isomers such as trehalulose (*α*-d-glucosyl-1,1-d-fructose) or leucrose (*α*-d-glucosyl-1,5-d-fructose); have been demonstrated in *Protaminobacterrubrum*, *Erwiniarhapontici*, *Erw. carotovora* var. *atroseptica*, *Serratiaplymuthica*, *Ser. marcesens*, *Pseudomonas mesoacidophila*, *Leuconostocmesenteroides*, *Klebsiella* spp., *Agrobacterium* sp., haploid yeast and *Enterobacter* sp. Isomaltulose is currently produced in industrial scale column reactors containing immobilized bacterial cells. Incomplete substrate conversion, slow isomaltulose production and multiple by-products are the main factors

limiting commercial efficiency (Fujii*et al.* 1983; Schiweck*et al.* 1991; Véronèse and Perlot 1999).

We devised an enrichment and screening method for bacteria with high SI activity and applied it to samples from various environments likely to favour organisms able to convert sucrose to storage isomers such as isomaltulose. In this paper, we describe a bacterium capable of highly efficient conversion of sucrose into isomaltulose.

Doubled sugar content in sugarcane plants modified to produce a sucrose isomer.

Sucrose is the feedstock for more than half of the world's fuel ethanol production and a major human food. It is harvested primarily from sugarcane and beet. Despite attempts through conventional and molecular breeding, the stored sugar concentration in elite sugarcane cultivars has not been increased for several decades. Recently, genes have been cloned for bacterial isomerase enzymes that convert sucrose into sugars which are not metabolized by plants, but which are digested by humans, with health benefits over sucrose. We hypothesized that an appropriate sucrose isomerase (SI) expression pattern might simultaneously provide a valuable source of beneficial sugars and overcome the sugar yield ceiling in plants. The introduction of an SI gene tailored for vacuolar compartmentation resulted in sugarcane lines with remarkable increases in total stored sugar levels. The high-value sugar isomaltulose was accumulated in storage tissues without any decrease in stored sucrose concentration, resulting in up to doubled total sugar concentrations in harvested juice. The lines with enhanced sugar accumulation also showed increased photosynthesis, sucrose transport and sink strength. This remarkable step above the former ceiling in stored sugar concentration provides a new perspective into plant source-sink relationships, and has substantial potential for enhanced food and biofuel production (Wu L, Birch RG 2007).

2.3 Plant biotechnology and its scope in crop improvement:

Biotechnology offers a wide potential for application of molecular biology techniques for human welfare. Plant biotechnology is an ever-emerging highly rewarding technology with large potential applications in crop improvement. Plant biotechnology has made rapid progress, which

resulted from an increase in the understanding of how cells work at molecular, biochemical and physiological levels. It has steadily grown from the development of techniques, which allow the transfer of genes from one plant species to another or from other organisms such as bacteria. The development of improved crops includes those conferring resistance to fungal pathogens,viruses and the nutritional improvement like the golden rice (Burkhardt, 1997).

The objective of plant biotechnology is to identify the agricultural problems that need to be solved by complementing classical breeding and thus reducing the time scale required to produce a genetically enhanced germplasm. Plant biotechnology when included with traditional crop improvement programs enables a more efficient environmentally compatible and ultimately cost effective utilization of resources for improved agricultural production. The tools of biotechnology when provided to plant breeders present many opportunities for increased reliability in crop production while ensuring increased profitability and environmental compatibility (Sharma and Ortiz, 2000).

The development of transgenic plants depend on plant transformation efficiency, which relies on the introduction of plasmid construct or segment of plasmid construct into the genome of plant cell that confers resistance to diseases with the target gene of interest. Transgenic plants are regenerated from transformed cells, as most of the plant cells are totipotent they possess the ability to regenerate the whole plant from the single cell.

Only a limited number of reports outlining sorghum transformation systems using biolistics have been published to date, with all reporting very low transformation frequencies (Casas, 1993; Zhu, 2001). Progress in sorghum transformation has been hampered by difficulties associated with tissue culture, such as accumulation of phenolic pigments and low regeneration of putative transgenic plants often has hampered optimization of conditions for sorghum transformation. Zhu (1998) transferred a rice chitinase gene in to sorghum by biolistic-mediated transformation. Zhao (2000) reported a successful protocol for Agrobacterium mediated transformation from immature inflorescence calli. Emani (2002) reported transgene inactivation due to gene silencing in sorghum, on biolistic-mediated transformation.

2.4 **Tissue culture - a pre-requisite for genetic transformation**

A tissue culture stage is required in most current transformation protocols to ultimately recover. The plants; indeed, it is the totipotency of plant cells that underlies most plant transformation systems. Plants are regenerated from cell culture via two methods, somatic

embryogenesis and organogenesis. Both are controlled by plant hormones and other factors added to the culture medium. Somatic embryogenesis is the generation of embryos from somatic tissues, such as embryos, microscopes or leaves. Proliferating somatic embryos in liquid culture or solid medium are suitable targets for transformation because the origin of proliferating embryogenic tissues is at or near the surface of the older embryos and are readily accessible to DNA delivery. Embryogenic tissues are, in general, very prolific and a low recovery of many transformants that are, in most cases, non-chimeric, because of the assumed single cell origin of the somatic embryos (Maheswari,1995),it is the tissue culture approach generally chosen for monocots because callus was easily initiated from scutellum of immature embryos after exposure to auxin. The concentration and the choice of auxinare to large extent dictated by the genotype and species.

2.5Transformation methods:

Successful transformation of plants demands certain criteria to be met. Among the requirements for transformation the following are important:

There are two basic approaches of DNA transfer in plants

I. Artificial methods
   a. physical methods
   b. chemical methods
II. Natural methods

2.5.1 I. ARTIFICIAL METHOD OF DNA TRANSFER

Physical Methods:

Various methods of DNA transfer by physical methods include.

1. Microinjection

In this method, DNA may be introduced into cells or protoplasts with use of very fine needles of 0.5- 10 m diameter. Some of the DNA injected may be taken up by nucleus. Microinjected DNA may become stably integrated into chromosomes of host cell through recombination and may be inherited in a Mendelian manner. Transformation frequencies are

considerably high and due to some specific advantages and application, this method is used for artificial gene transfer in certain fields. Microinjection of DNA into plant nuclei (in protoplasts to tissue) is more difficult than for animal cells but now it is bitterly developed.

2. Macroinjection

Macroinjection is the method tried for artificial DNA transfer. In this method needles used for injecting DNA are with diameters greater than the cell diameter. DNA is injected with conventional syringe into the region of plant, which will develop into floral tillers. Macroinjection method was found to be successful with rye plants. It is also being attempted for other cereal plants.

3. Electroporation

Electroporation can be used to introduce exogenous DNA to plant protoplasts (dicot and monocot). Fromm (1985) first reported gene transfer in to maize protoplast by electroporation and Langridge (1985) first reported the stable transformation of carrot protoplast with DNA by electroporation. Electroporation can be used to increase efficiency of transformation or transfection of bacterial cell.

4. Sonication

In the process of Sonication low frequency ultrasonic waves ranging between 1.0 MHz – 1.5 MHz have the capacity to produce small pores in the cell which facilitate the entry of plasmid containing the desired gene.

5. Biolistics

Among the physical methods for artificial transfer of exogenous DNA, biolistic transformation is relatively novel method. The term 'biolistic' (biological ballistics) was coined to describe the transfer of foreign DNA into living cells or tissues through bombardments with particle gun. The method was developed to overcome the limitations of DNA delivery in other methods. It avoids the need of protoplasts and has better efficiency. The method can be used for any plant cells, leaves, root sections, embryos, seeds, and pollen. The method involves bombardment of particles carrying DNA of interest onto target cells using high velocity transfer mechanism. Sanford first developed the method in 1987 and in their model system onion epidermal cells were used. Klein in 1987 transferred genomic RNA of Tobacco Mosaic Virus (TMV) using this method and 30-40% of the epidermal cells of onion showed the signs of virus replication. The technique is finding universal application in the direct transformation of whole

cells in culture, in tissue and in sub cellular organelles such as mitochondria and chloroplasts. In this method DNA of interest is coated on heavy microparticles of tungsten or gold. DNA used for coating the particles is first precipitated with $CaCl_2$, spermidine and polyethylene glycol. They are carried by nylon macro projectile is withheld in the barrel after a short while by the stopping plate while only DNA coated macroprojectile enters the aperture of stopping plate and continues to accelerates towards target cell and strikes it. This causes penetration of exogenous DNA through cell wall. For stable transformation to occur, the amount of DNA reaching the cells, thickness for the tissue being penetrated and potential of the target tissue or cell to regenerate into plant are the important factors. Biolistic transformation technique has been shown to be successful with papaya, sugarcane, soybean, tobacco etc. Genomes of sub cellular organelles have been made accessible to genetic manipulations by this method. The particle gun has also been used with pollen, early-stage embryoids, somatic embryos and meristems. Some target tissues such as embryogenic suspension cultures (Fromm, 1990) and meristematic tissue (McCabe, 1988) have proven to be transformable and able to give rise to transgenic plants. When the biolistic process is applied to plant tissues, plants regenerated from such tissues are usually chimeric in terms of introduced in terms of introduced foreign gene due to random bombardment of a small number of cells in a multiple system.

Chemical methods

Chemical methods of DNA transfer involves

1. **Use of polyethylene Glycol (PEG)**

Chemical agents such as PEG (Negrutiu, 1987) increase the permeability of the cell membrane thereby causing the transformation ability to the plant cells. This method has been successfully applied to *petunia, Nicotiana* and other plant systems like maize, rice, etc.

2. **Transfection using calcium phosphate**

The process of transfection involves the mixture of isolated DNA (10-100 μg) with solution of calcium chloride and potassium phosphate under conditions, which allow fine precipitation of calcium phosphate to be formed. Cells are then incubated with precipitated DNA either in

solution or in tissue culture dish. A fraction of cells will take up the calcium phosphate –DNA precipitate by endocytosis.

2.5.1 II. Natural method of DNA transfer

***Agrobacterium-mediated* DNA transfer**

Transformation of plants by Agrobacterium-mediated DNA transfer is currently the most commonly used means of accomplishing plant gene transfer. The nature's genetic engineer contributes a lot to the rapid development of research through this mode of DNA transfer. *An Agrobacterium-mediated DNA transfer employs* the transfer of a foreign gene (DNA) into the nucleus of the plant cell. Genetic engineers have developed vectors based on this natural process. The desired genes are inserted into this vector by standard molecular cloning techniques, re-introduced into the bacterium, which is then incubated with the plant tissue to be transformed. The specific piece of DNA containing the gene is then transferred to the plant cell nucleus and integrated into the chromosome. This system has worked out in a very broad range of species including a larger number of crop plants.

Molecular basis of *Agrobacterium* transformation

*Agrobacterium tumefaciens*is a gram-negative soil bacterium. *Agrobacterium tumefaciens* induces crown gall tumors (Smith and Townsend, 1907).Series of the classic experiments by Braun and coworkers demonstrated that once tumor formation has been initiated, the further presence of *Agrobacterium* is not required for subsequent tumor proliferation (Braun and Stanier, 1958). The molecular studies on *Agrobacterium* and subsequent finding of kerr (1971) were very useful to establish the central role of *Agrobacterium* plasmids in crown gall development.

Plant tumors resulting from *Agrobacterium* infection synthesize a variety of unusual amino acid derivatives called opines (Petit, 1970) due to the expression of T-DNA genes encoding opine synthase enzymes and nopaline synthase enzymes. Ti plasmids and the *Agrobacterium tumefaciens* strains harboring them can be classified according to the type of opines produced. The three best studied opines are octopine, nopaline and agropine. The

generation of tumors producing specific opines catabolize only by the inciting *Agrobacterium* strain is a central feature of the pathogenic relationship between *Agrobacterium* and plant. Some of the plants regenerated from nopaline containing tumour tissue continue to synthesize nopaline. The strains that utilize nopaline induce tumors that synthesize only nopaline. Plasmids in the octopine group have shown to be closely related while those in the nopaline group are in a diverse way. Transformation using disarmed (non-tumorigenic) *Agrobacterium tumefaciens*plasmid vectors can result in transgenic plants of normal phenotype, which express the introduced genes. The methods of transformation of intact cells or tissues with *Agrobacterium Tumefaciens* have been developed using excised tissue of *Nicotiana* and *petunia* species (Horsch, 1985; Rogers, 1986). Nucleic acid hybridization studies have shown that octopine and nopaline plasmids are closely related having a wide stretch of homology in the T-DNA region (Willmitzer, 1983).

The genetic elements encoded by *Agrobacterium,* which are essential for T-DNA transfer, are the T0dna border sequences and the chromosomal virulence genes present on the Ti plasmid outside the T-DNA. The attachment of *Agrobacterium* to the plant cell is mediated by the chromosomal virulence genes (Douglas, 1985; Thomashow, 1987), which are constitutively expressed. The 25 base pair direct repeats flanking the T-DNA (Yadav. 1982; Zambryski, 1982) are the only part of T-DNA important for transfer. The right border repeats is an essential cis acting element for transfer whereas the left border repeat is thought to merely signal where the transfer of DNA normally ends. Subsequent steps in the T-DNA transfer require the proteins encoded by the *vir* region (*vira, virb, virc, virdvire, and virg*).

Agrobacterium tumefaciens infects only wounded actively dividing plant cells. The cells secrete wound specific compounds such as acetosyringone and α- hydroxyl acetosyringone. These phenolic compounds act as chemo attractants for *Agrobacterium* (Ashby, 1987) and inducers of the *vir* genes. Both processes are proposed to be mediated by the gene products of *vira* and *virg*(Stachel and Zambryski, 1986).*Virg* trans fectionally activates the *virb, virc, vird,Vire,* and *virg* loci. A number of sugars act synergistically with phenolic compounds to enhance the *vir* gene expression. The genes on the Ti-plasmid and their functions were identified by transposon insertion and deletion mutagenic methods. The Ti-plasmid contains a well-defined T-DNA region encoding a series of genes responsible for the synthesis of auxins and cytokines in transformed plant cells (Akiyoshi and Inze,1984), which causes over production of phytohormones and that cause tumor proliferation.

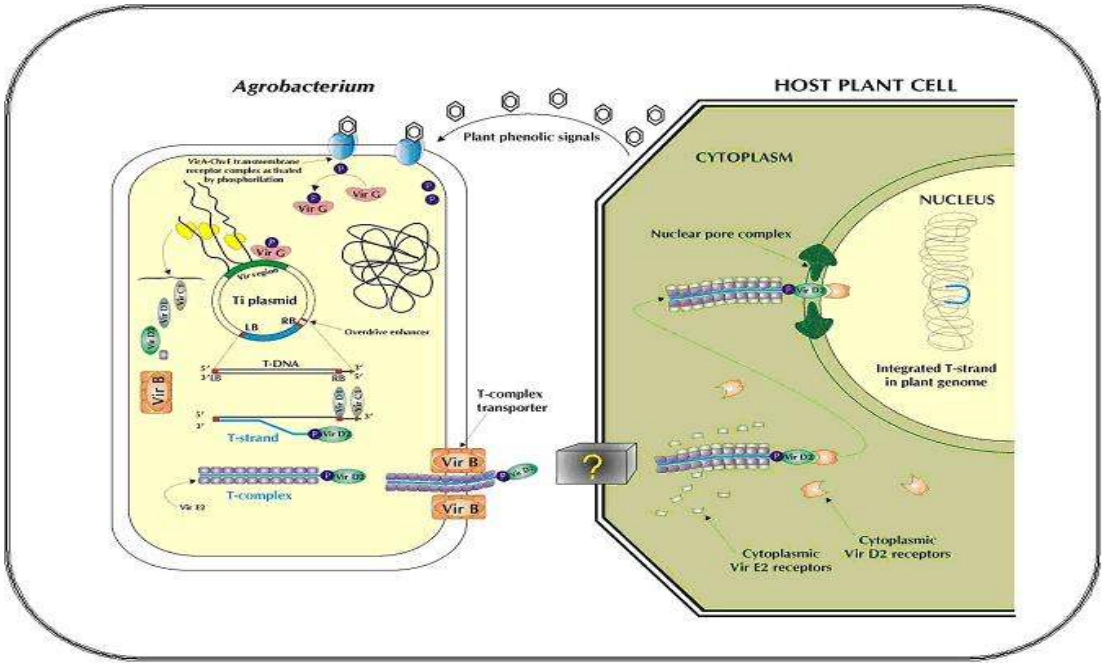

Fig:4 Agrobacterium Transformation.

2.4.3 The transformation strategy:

The key systems involved in the design of transformation strategy involve Selection of transformed cells from the non-transformed ones using selectable markers

1. Selectable markers:

Many genes conferring resistance to specific antibiotic and herbicide genes can be used as selectable markers for the transformation of both dicotyledonous and monocotyledonous plants, Selectable markers are usually necessary for efficient production of transgenic cells and plants. After gene transfer, transformed cells are greatly outnumbered by non-transformed cells. A selectable marker gene allows the preferential growth of transformed cells in the presence of corresponding selective agent.

a. Amino glycosides.

The neomycin phosphotransferase, type II (hptII) gene has been for the transformation of more plant species than any other selectable marker. The coding sequence was originally isolated from the bacterial transposon Tn5. hptII provides resistance to certain amino glycoside antibiotics such as kanamycin, paromycin and geneticin. Kanamycin selection has proved to be the most widely applicable, and has been used in the majority of transformation procedures for

dicotyledonous plants. The npt II coding sequence has been fused to constitutively regulated promoters, such as the nopaline synthase (NOS) and the Cauliflower Mosaic Virus (CaMV) 35 S regulatory sequence (fraley, 1983) and cloned into plant transformation vectors by many researchers (Hinchee, 1994). The different constitutive promoters confer different selection efficiens.

b. Hygromycin

Hygromycin selection is based on the use of E.coli bacterial gene, apf IV that encodes for hygromycin phosphotransferase. This enzyme inactivates hygromycin and confers a resistant phenotype on the plant cells expressing the gene. Hygromycin is highly toxic to most plant species and the frequency of transformation after hygromycin selection in many species is lower than with kanamycin selection (Hpt-II).

Resistance to hygromycin is conferred by the selectable marker gene hygromycin phosphotransferase (hpt) first isolated from steptomyces hygroscopicus, which is widely used as a selectable marker gene in genetic transformation experiments. It is used fot the selection and maintenance of prokaryotic and eukaryotic cells transfected with the hygromycin resistance gene. Hygromycin kills bacteria, fungi, and higher eukaryotic cells by inhibiting protein synthesis. Hygromycin prevents polypeptide elongation by interfering with aminoacyl t-RNA recognition and ribosomal A-site occupation (cabanas, 1978). Hygromycin can lead to misreading during translation in vitro. However this effect was not duplicated in vivo. It has been reported to interfere with translocation and to cause mistranslation. The resistant gene, hpt IV codes for a kinase enzyme that inactivates hygromycin through phosphorylation. Cloning of the resistant gene and fusion with eukaryotic promoters has resulted in the development of vectors that permits selection for resistance to hygromycin in both prokaryotic and eukaryotic cells.

c.cefotaxime:

cefotaxime is an inhibitor of bacterial cell wall synthesis. It inhibits the cross-linking of peptidoglycan by binding and inactivating of transpeptidases. Since this is non-toxic to plant cells and highly effective against gram-negative bacteria, it is widely used for the elimination of *Agrobacterium* species after inoculation

MATERIALS & METHODS

CHAPTER 3
METERIALS AND METHOD

3.1 Plant material

Sorghum genotype SSV84 and SSV74, are the popular sweet sorghum seeds for the production sucrose Isomerase, these are the high yielding varieties and short duration crop of 90 to 120 days available in gene bank of the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT) was used as the plant material throughout the experiment.

3.2*Agrobacterium*strain and binary vector:

Gene construct: pdSI gene
Plant selectable marker: Hygromycin resistance gene (hptII).
Bacterial selection: kanamycin resistant gene (nptII)

*A.tumefaciens*strain EHA105 was used as the natural transformation vehicle. Thebinaryvectorwas inserted with the *pdSI* gene isolated from *Pantoea dispersa*. The vector PMDC99 (fig) contains multiple cloning sites and it is also possible to screen blue/white colonies of β– galactosidase and also has high copy plasmid replication in *E.coli.* The gene construct has nptII gene as bacterial selection marker and hptII as plant selectable marker.

pMDC99:35S:PdSI

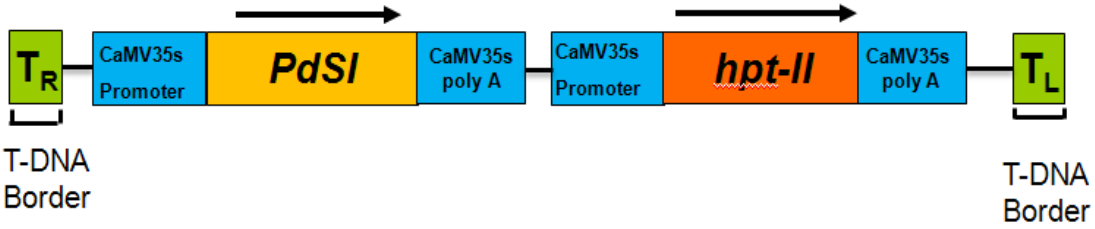

Fig: 5 Gene construct of pdSI.

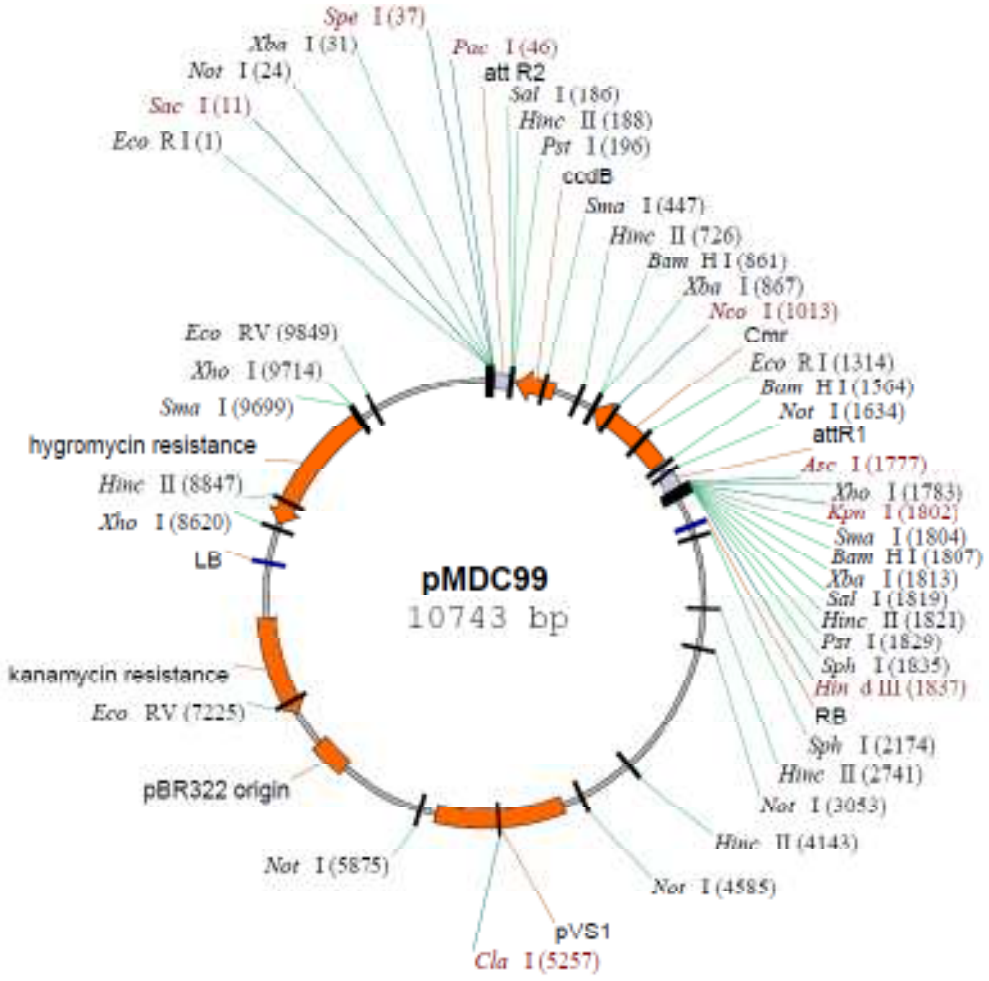

Fig: 6 PMDC 99 VECTOR

3.3 Culture media

The culture medium used was mainly the basal Murashige and Skoog medium (Appendix Table 7:1) with 3% sucrose and 0.7% agar for the *in vitro* germination of seeds. The pH of the medium was adjusted to 5.8 using 0.1N NaOH. The medium was then sterilized in an autoclave at 121ºC, 15 lbs. pressure for 20 minutes. The basal medium with cefotaxime concentration 25mg/L+ hygromycin concentration ranging from 2.5, 5.0, 7.5 mg/L were used for antibiotic selection of infected explants. Cefotaxime is a bacteriostatin which controls the growth and virulence of *Agrobacterium.*

3.4 Screening of seeds

Seeds of sweet sorghum SSV74 & SSV84 are screened before they are used for infection. This is done in order to select healthy seeds.

3.5 Sterilization of seeds

The chosen healthy seeds were sterilized by shaking with 70% ethanol for 1-2 minutes. The seeds are then rinsed with sterile distilled water and shaken with 0.1% $Hgcl_2$ (mercuric chloride) for 6-7 minutes. After that, seeds were rinsed with sterile distilled water for 3-5 times.

3.6 Kill curve experiment

The first critical step for antibiotic selection of co-cultivated explants is determining the optimal antibiotic concentration at which the control explants is able to survive. A kill curve is a dose response experiment where the expants are subjected to increasing amounts of antibiotic to determine minimum antibiotic concentration needed to kill all the cells over the course of one week

The plant selectable marker used with this particular construct is *hpt*II gene (hygromycin resistant). MS basal media with varying concentrations of hygromycin (0, 2.5, 5.0, 75, 10.0 mg/L) was prepared. Control seeds were inoculated in the media and kept for one week.

3.7 Culture preparation

3.7.1 Bacterial culture with gene of intrest was streaked on to yeb medium(aapendix) from glycerol stock and kept in incubator for 48hours at 28°C

3.7.2. Agro – infection (co-cultivation):

Agrobacterium cultures carrying the plasmids were grown overnight at 27 ^{0} C in YEB broth. 25 ml of bacterial suspension was centrifuged in a sterile centrifuge tube for 10 minutes at 5000 rpm and then supernatant was decanted. The bacterial pellet was resuspended in the same volume (25 ml) of MS basal medium broth (Half strength MS). The suspension was transferred to a sterile petriplate under the laminar flow.

The sterilized pre-soaked sorghum seeds (SSV74 & SSV48) were punctured on the embryo side with the help of sterile syringe and were immersed in the bacterial suspension that has been mentioned above for 30 minutes, After 30 minutes of incubation these punctured seeds placed in a petriplate having basal MS medium. The seeds were placed with a density of 25-30 seeds per petriplate, and sealed with paraflim. Then they were left in the culture room for 48-72 hours.

3.7.3 Sub-culturing and selection:

After 2 days of co-cultivation, the germinated explants were transferred in to MS media containing 250 mg/l cefotaxime to arrest the growth of Agrobacterium, with the selection pressure of 50 mg/l (H 2.5 conc) and cultured for 7 days in the culture room for further development of shoots.

3.7.4 Elongation of shoots:

The shoots which were regenerated after 7 days from the (H-2.5) were further transferred to the MS medium consisting of cefotaxime (2500 ul/L) and with the selection pressure of 50 mg/l (H 5.0 conc) and cultured for 7 days in the culture room for further elongation of shoots.

3.7.5 Rooting:

After 7 days of incubations the elongated shoots were transferred to rooting media supplemented with cefotaxime 2500 ul/l and with the selection pressure of 50mg/l (H-7.5 conc) and were cultured in the culture room for 7 days for the development of roots.

3.7.6 Hardening /acclimatization:

The rooted explants after 7 days of rooting stage were transferred to Jiffy cups filled with autoclaved sand and kept in invitro conditions for proper acclimatization for prior to transfer to glass house and they were covered with plastic covers to maintain humidity levels for 2 days in the incubator.

Then the acclimatized plantlets were transferred to glasshouse for hardening.

MOLECULAR CHARACTERIZATION

CHAPTER 4
MOLECULAR CHARACTERIZATION

4. Molecular characterization of the putative Transgenics.

Various molecular biology techniques are used to check whether transformation has occurred or not. The plant is tested at transcriptional and translational level; to test the presence of transgenes.

4.1 Isolation of genomic DNA from putative transgenic plants.

Principle:

Genomic DNA from the putative plants was extracted by using CTAB method. The principle behind isolation of DNA by CTAB extraction buffer is that the non-ionic detergent **Cetyl Trimethyl Ammonium Bromide (CTAB)** is used to liberate and complex with total cellular nucleic acids. CTAB forms an insoluble complex with nucleic acids when the initial NaCl concentration is lowered to ~0.5 M (*Rogers et al.,*). Polysaccharides, phenolic compounds and other enzyme inhibiting contaminants found in plant cells are efficiently removed through phenol chloroform addition. The nucleic acid–CTAB complex is only soluble in high salt concentration like NaCl which is used to remove the CTAB from the nucleic acid–CTAB complex. The residual CTAB is removed by washing the nucleic acid pellet with 80% ethanol; CTAB is more soluble in ethanol and is discarded with the wash solution CTAB method is highly versatile as even little amount (mg) of tissue can be used when sample size is limiting. The protocol is relatively simple, fast and easy.

Reagents Required:

CTAB extraction buffer. (Appendix no.)

- Chloroform: isoamyl alcohol 24:1 (v/v)
- Iso-proponal (ice cold).
- 70% etanol
- 5 M NaCl

- TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0
- RNase A: 10 mg/mL
- Proteinase K: 1 mg/mL (Made fresh before use)
- Extraction buffer: 100 mM Tris, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 2% CTAB, 0.3% β-mercaptoethanol (Add just before use) (appendix)
-

Method:

- Young leaves were collected from the putative transformants. Leaf tissue was frozen in liquid nitrogen and was ground to fine powder in a mortar and pestle and then transformed into eppendorf tube containing 700µl – 1 ml of pre warmed CTAB extraction buffer.
- The contents were mixed well and incubated at 65°C for 45 minutes – 1 hour.
- To the incubated mixture, equal volume of chloroform: isoamyl alcohol was added and mixed thoroughly by shaking the tubes.
- The mixture was centrifuged at 12,000 rpm for 15 minutes.
- The aqueous phase was collected in a new tube, without disturbing the organic phase, if the aqueous phase is not clear, add (chl: IAA) and centrifuge again.
- After the aqueous phase was collected, ice-cold iso-propanol was added (0.6 times or 2/3rd volume of the aqueous phase) for precipitation. The mixture was inverted gently 4-5 times and incubated for 15-20 minutes at -20° C.
- After incubation, the solution was centrifuged at 12,000 rpm for 20 minutes.
- The pellet was washed with 70% ice- cold ethanol and centrifuged at 12,000 rpm for 20 minutes.
- The supernatant is discarded carefully as the pellet is loose. The pellets are then air - dried till the last traces of ethanol is removed.
- The pellet is then dissolved in TE buffer (100 µl). Appendix

4.2 DNA purification.

- 5 µl of RNase A (10 mg/mL) was added and incubated in 37 °C water bath for approximately 1 hour.
- 5 µl of proteinase K (1mg/mL) was added and incubation at 37 °C was continued for another 15 to 30 minutes.

- Add 250 µL of phenol and 250 µL of chloroform to each Eppendorf tube.
- Vortexed briefly, and centrifuged at 14,000 rpm for 10 to 15 minutes. Upper layer was collected in new 1.5-tube.
- Repeat the extraction step with equal volume of chloroform: octonal
- To the collected aqueous phase, 1/10 vol. 3M Na acetate and 2 vol. absolute ethanol were added and mixed.
- Kept in freezer (-20 °C) for 1 hour. Centrifuged at 14,000 rpm for 10-20 minutes. Drain and wash with 70% v/v EtOH. Remove EtOH.
- Vacuum-dried the tubes containing DNA pellet. Add 100 to 200 µL TE. Allow time for complete resuspension.

To test the quality of DNA, the samples were run on 0.8% agarose gel using 1 X TAE running buffer.

4.3Agarose Gel Electrophoresis:

Agarose gel electrophoresis is a simple and highly effective method for separating and identifying 0.05 kb to 25 kb DNA fragments. Most of the biological macromolecules exist either as ions in solution or can be modified to have ionic molecules associated with them, which makes them to move in an electric field. The speed at which they pass through the matrix in the presence of the electric field is called their electrophoretic mobility. DNA molecules exposed to this electric field migrate towards the anode due to the negatively charged phosphates along the DNA backbone.

Reagents Required:

TAE buffer (50X)

EDTA

57.1 ml glacial acetic acid

Volume made up to 1liter by adding distilled water

pH adjusted to 8.5

Gel loading buffer (6X)

0.25% (w/v) bromophenol blue

30% glycerol

Method

To prepare 1% (w/v) Agarose in TAE, 1 gm of Agarose (electrophoresis grade) was added in 100 ml 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and melted by heating a microwave oven until the solution is clear. On cooling to 55–60°C, 5 µl of 10 mg/ml syber green was then added. Gel tray was prepared by sealing ends with tape and placing a comb containing desired teeth. Agarose solution was poured in to the gel tray at about 50 °C and allowed to solidify by cooling. To run the gel the comb was gently removed and the tray was placed in the electrophoretic chamber and covered with electrophoresis buffer (1X TAE buffer) until wells were submerged.

Samples for electrophoresis were prepared by mixing 1 µl of gel loading dye for every 5 µl of DNA solution and mixed well. The samples were then electrophoresed at 60V till the dye migrated to appropriate distance. The gel was then viewed using Gel Doc Apparatus.

4.4 Isolation of Plasmid DNA.

Plasmid DNA was isolated according to the procedure reported by Sambrook et al., (1989).

Reagents required

- GTE buffer: 50 mM Glucose, 25 mMTris-HCL (pH 8.0), 10 mM EDTA (pH 8.0). Autoclave and store at room temperature.
- Lysozyme (50mg/ml),
- Lysis buffer: 0.2 N NaOH and 1% (w/v) SDS (Freshly prepared)

- 5 M Potassium acetate, pH 4.8,
- 3M Sodium acetate , pH 5.2,
- RNAseA(10 mg/ml).
- TE saturated phenol,
- Chloroform: isoamylalcohol (24:1),
- Isopropanol,
- 80% ethanol,
- TE buffer: 10 mM Tris (pH 8.0) and 1 mM EDTA (pH 8.0)

M**ethod**

- *Agrobacterium* strain EHA 105 carrying plasmid construct was grown on YEB agar plates containing 100 µg/ml kanamycin.
- Single isolated colony was inoculated in 20 ml of LB medium (Appendix) with kanamycin and grown overnight at 28°C on a rotary shaker at 220 rpm.
- 10 ml overnight grown bacterial culture was pelleted by centrifuging for 10 min at 6000 rpm.
- The pellet was suspended in 600µl of GTE and 30µl of lysozyme is added,incubate for 5 min at room temperature (can be placed even in ice), in order to maintain the osmoticum.
- To the resuspended bacterial solution, 1200 µl of freshly prepared lysis buffer was added and the samples were placed on ice for 5 min.
- After 5 min 900 µl of 5 M potassium acetate (pH 4.8) was added to the lysed bacterial solution and the samples were mixed well by inverting the tubes slowly and the mixture was placed back on ice for 5 min.
- The solution was centrifuged for 20 min at 10,000 rpm and the supernatant was transferred to fresh tubes to which 3 to 5 µl of RNase A (10 mg/ml) was added, to remove RNA, and incubated at 37 °C for 30 min.

- Equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1) was added to remove proteins present in the DNA mixture and centrifuged at 10,000 rpm for 20min.
- Aqueous phase was collected in fresh tube and equal volume of chloroform: isoamyl alcohol (24:1) was added and the sample solution was centrifuged for 20min at 10,000 rpm.
- The top aqueous phase was collected into fresh tubes and to this 0.1 volumes of sodium acetate 3M (pH 5.2) and -3 volumes of chilled ethanol is added and keep it at -20 °C for 1 hour to precipitate nucleic acids.
- The sample was centrifuged for 15 min at 10,000 rpm / 4 °C and the pellet was washed with 80% ethanol and air-dried.
- The dried plasmid DNA pellet was finally dissolved in 100 µl of TE.

4.5 PCR - POLYMERASE CHAIN REACTION

Putative transformants were screened by polymerase chain reaction (PCR) for the presence of Gene for routine analysis for transgene detection. The 1.23kb region of pdSIgene was amplified using 22 mer and 24 meroligonucletide primers (CGG CTT TAG AGA GAT TTG AGA G & TCT TCG TCT TGA GCA GGG TGG AGC). Each reaction was performed in 25 µl (final volume) of reaction mixture

PCR requirements

1. Two oligonucleotide primers

2. Template DNA to be amplified

3.A Thermostable DNA polymerase

4. Magnesium ions

5. Four Deoxyribonucleotides.

6. Buffer [10x]

PCR methodology:

PCR was performed using β-lyc transgene specific primers at appropriate reaction conditions using the Eppendorf PCR Master Cycler Gradient apparatus. The following ingredients were added in PCR tube, which was kept on ice

PCR reaction mixture

10X PCR buffer	:	2.50 µl
50 mM $MgCl_2$	:	0.75 µl
dNTPs (10 mM each)	:	0.50 µl
F Primer (10 pmol/ µl)	:	0.50 µl
R Primer (10 pmol/ µl)	:	0.50 µl
Taq polymerase (5 U/µl)	:	0.20 µl
Water	:	18.75 µl
DNA (100 ng/µl)	:	1.0 µl
Total reaction volume	:	25.0 µl

By using a thermocycler set the program under the following conditions for 35 cycles.

Step 1.Initial denaturation temperature at 95 °C for 5 min

2. Denaturation temperature at 95 °C for 1 min

3. Annealing temperature at 58.5 °C for 1 min

4. Extension temperature at 72 °C for 5 min

Step 2 to 4 were repeated 35 times

5. Final Extension temperature at 72 °C for 10 min

6. Samples were maintained at 4^oC

After the completion of the 35 cycles, the samples are loaded with a loading dye on to a 1.2% agarose gel and are used for gel electrophoresis. The amplified products are visualized using gel dock.

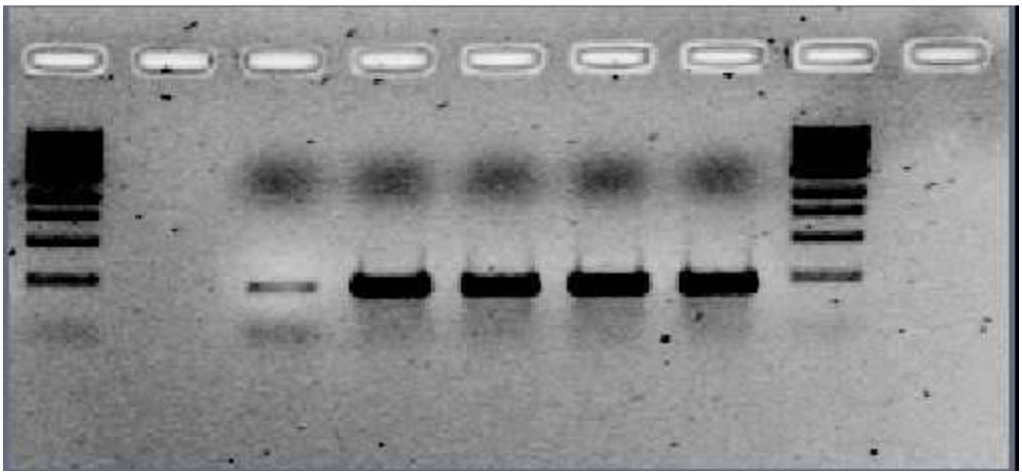

Fig: 7 plasmid pcr for pdSI

RESULTS
&
DISCUSSION

CHAPTER 5
RESULTS AND DISCUSSION

Agrobacterium –mediated transformation in sorghum using the seeds as explants for the production of transgenic plants was found to be an efficient method for the production of sucrose Isomerase from pdSI gene. Various factors influenced the efficiency of both the regeneration and transformation system from the germination till the hardening stage.

Co-cultivation:

Out of 25-30 seeds in the plate, on an average 20-25 seeds are germinated. The explant preparation was found to be effective for the transformation. For efficiency of explant preparation O.D. of bacterial culture was found to be between 0.5-0.6 for sorghum, at which the transformation efficiency was maximum.

The explants without any overgrowth were transferred for sub-culturing and selection.

Sub-culturing and selection:

The healthy explants with germination of shoots were transferred and were exposed with varying concentration of hygromycin for selection. Most of the explants show positive response in the initial stages of the selection.(H -2.5 mg/L).

Elongation of shoots:

After a week of incubation the sub-cultured explants of selection pressure were transferred in to the next stage of selection (H-5.0 mg/L).

Rooting and hardening:

The healthy plants selected under hygromycin pressure from sub-culturing and selections were transferred in to tube (H-7.5 mg/L) for rooting. Explants showing prominent roots were observed from the plants. These plants were transferred for hardening .overall the complete regeneration of putative transgenic plants took about 90-120 days.

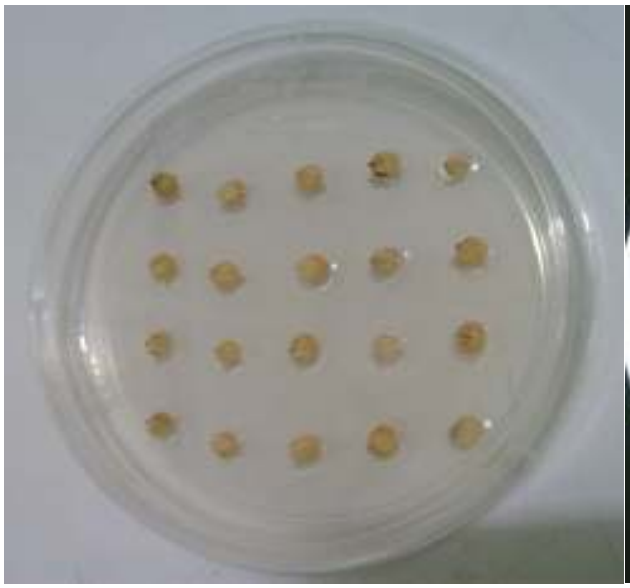

Fig: 8 co-cultivation.

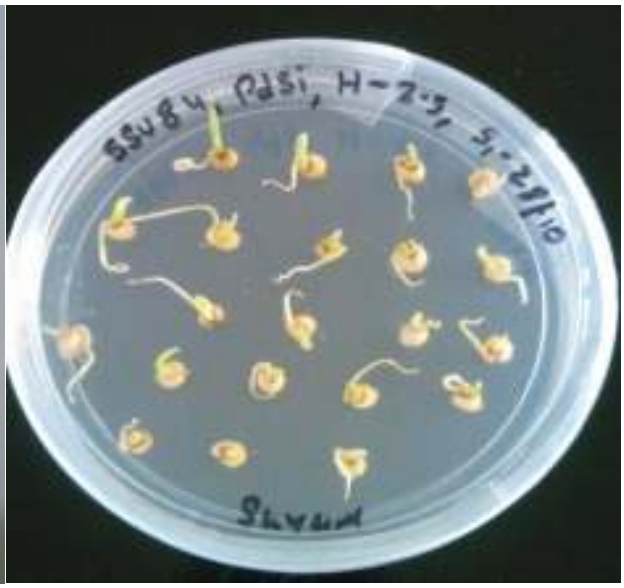

Fig: 9 germination of seeds.

Fig: 10germination of seeds.

Fig: 11 sub-culturing.

Fig: 12 Sub-culturing and selection.

Fig: 13 Elongation of shoots.

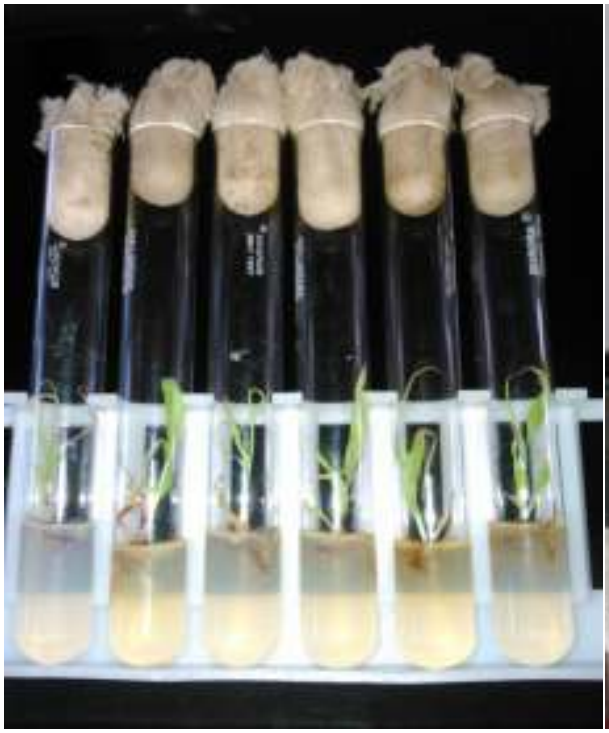

Fig: 14 Rooting stage

Fig:15 Acclimatization in Jiffy cups.

Fig: 16 and 17 hardening at glass house.

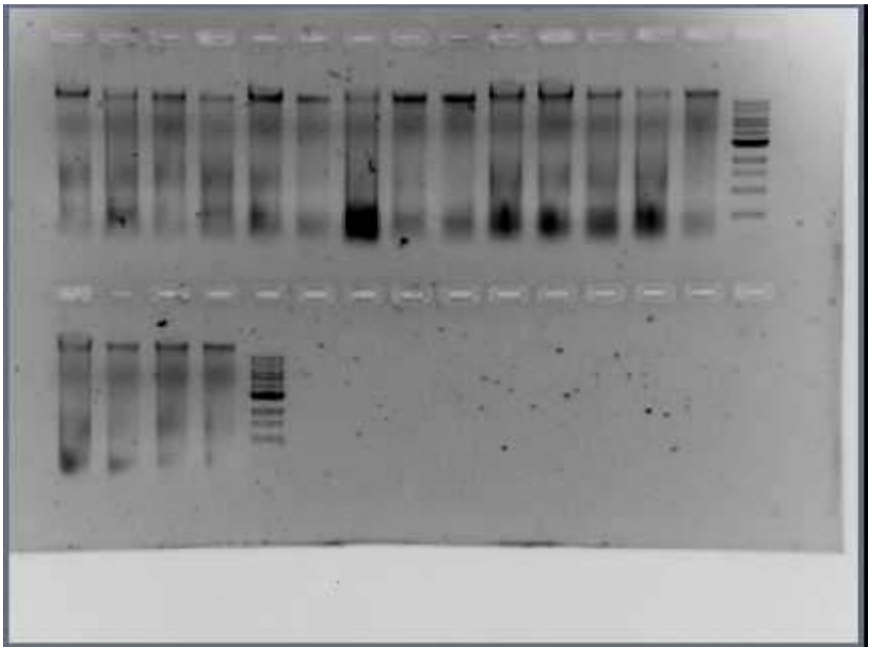

Fig: 18 Isolation of genomic DNA from sorghum putative transgenic plants.

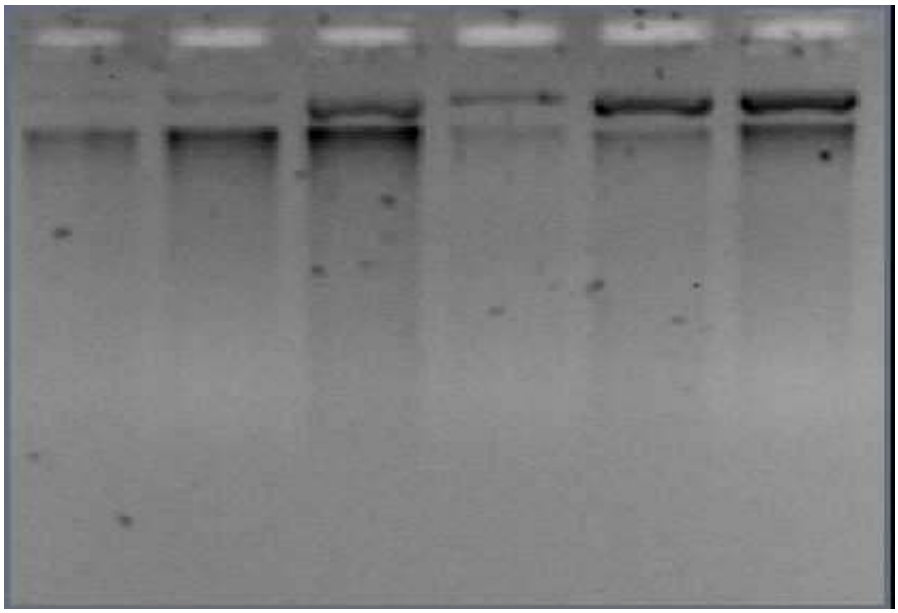

Fig: 19 Isolation of plasmid DNA for pdSI.

DISCUSSION

Chapter 6

DISCUSSION

The transgenic plants were produced by Agrobacterium-mediated transformation. The transformation efficiency and seed germination after co-cultivation was found to be high with Agrobacterium-mediated transformation when the optimum O.D value of bacterial culture is between 0.5-0.6, and a large numbers of shoots have been originated from the explant seeds used. The transformation is done by using puncture method of seed explant, where we can produce large number of shoots and the time of regeneration of plants takes less time compared to other explant material preferred.

A part from seed puncture method, shoot meristems are also used as explant material, the shoot meristems can also produce a large number of shoots, but the regeneration of plants takes some time when compared to puncture method. The antibiotic hygromycin is very effective plant selectable marker for producing insect resistance plants. An antibiotic assay is performed to know the efficient level of hygromycin concentration for selection is 7.5 mg/L. The putatively transformed explants were assayed for resistance to hygromycin on the tissue culture medium. Molecular characterization techniques for the analysis of the putative transgenics were also observed.

REFERENCE

REFERENCE

Ashby, A.M., Watson, M.D., Loake, G.J. and Shaw, C.H. (1987). Ti plasmid – Specified chemotaxis of *Agrobacterium tumfacien*s C58 C1 towards Vir-including phenolic compound and soluble factor from monocotyledonous and dicotyledonous plants. *Journal of Bacteriology*, PP: 4181-4187.

Akiyoshi, D.E., Klee, H., Amasino, R.M., Nester ,W.E., and Gordan. (1984). M.P. T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. *Cross Mark.* PP: 5994-5998.

Bennett, A.S., Anex, R.P. (2008). Production transportation and milling costs of sorghum as feedstock for centralized bioethanol production in upper midwest. *Bioresearch Technology.* PP: 1595-1607

Burkhardt. (1997). Improving the nutritional value of golden rice through increased pro-vitamin a content. *Nature Biotechnology.* PP: 482-487

Casas., Zhu. (2001).The investigation of optimal bombardment parameters for transient and stable transgenic expression in sorghum. *Invitro Cellular and Development biology.* PP: 341-348

Cabanasa, M.J., David, Vazgueza, B., Jaun, Modolella, B. (1978). Inhibition of ribosomal translocation by aminoglycoside antibiotics. *Biological and Biophysical Research Communicatio.* PP: 991-997.

Doggett, H. (1988).Tropical agriculture series. *Sorghum.* PP: 529-530**;**

Douglas., Thomoshow. (1987). Agrobacterium to the plant cell is mediated by the chromosomal virulence genes. *The EMBO* . PP: 857-863.

Fromm. (1985). Gene transfer and genetic transformation*. Transgenic plants.* PP: 125-135.

Fraley, R.T., Roger, S.G., Horsch, R.B., sander, P.R., Flick, J.S., Adams, S.P. (1983). Expression of Bacterial genes in plant cell. *Cross Mark.* PP: 4803-4807.

Fuji., schiweck., Veronese., and Perlot. (2002). Isomaltulose (palatinose): a review of biological and toxicological Studies. *Food and Chemical Toxicology.* PP: 1375-1381.

Hinchee, M.A.W., Corbin, D.R., Armstrong, Ch. L., Fry, J.E., Sato,S.S., De Boev ,D.L., Peterson,W.L. (1994). Plant Transformation. *Plant Tissue Culture;* PP: 231-270.

Horsch, R.B., Roger, S.G. (1985).A simple and general method for transferring genes into plants. *NASA Astrophysics data system.* PP: 1229-1231.

Kerr, A., Ellis, J.G. (1907). *Agrobacterium tumefaciens*: a natural tool for plant transformation. *electroni journal of Biotechnology;* PP: 0717-3458 .

Landridge, William, H.R., Baoj, Li., Aladar, A. Scale. (1985). Electric field mediated stable transformation of carrot protoplasts with naked DNA. *Plant cell reports.* PP: 355-359.

Lina , B.A.R. (2002). Isomaltulose (palatinose): a review of biological and toxicological studies. *Food and Chemical Toxicology.* PP: 1375-1381.

Lu, guang ,Wu, and Robert, G.B. (2004).Characterization of the high efficient sucrose isomerase from *Pantoea dispersa* UQ68J and cloning of the sucrose isomerase gene. *Applied environmental Microbiology.* PP: 1581-1590.

Maheshwari, N. (1995). Invitro culture of wheat and genetic transformation-retrospect and prospect. *Critical Reviews in Plant Sciences.* PP: 149-179.

Mc cube. (1988). Gene transfer and genetic transformation. *Transgenic plants.* PP: 125-135.

Negrutiu, I., Shillito, R., Potrycus, I., Biasini, G., and Sala, F. (1987). Hybrid genes in the analysis of transformation condition. *Plant Molecular Biology.* PP: 237-24.

Petit, A., Chantal David., Gary, A. Dahl., Jerry g Ellis. (1970). Further Extension of the opine concept plasmids in *Agrobacterium Rhizo* genes co-operate for opine degradation. *Molecular and General Genetics.* PP: 204-214.

Schiweck., Cartarius., Lichtenthaler. (2004). Characterization of Pantoea dispersa UQ68J: producer of a highly efficient sucrose isomerase for isomaltulose biosynthesis. *Applied Microbiology* .PP: 93-103.

Sharma, K.K., Ortiz, R. (2000). Program for the application of genetic transformation for crop improvement in the semi-arid tropics. *Invitro Cellular and Develoment Biology.* PP: 83-92.

Smith, and Townsend. (1907). Agrobacterium: a natural tool for plant Tumefaciens. *Electronic Journal of Biotechnology (EJB).*PP: 0717-3458.

Stachel, S.E., Zambryski, P., Mesiens, E., and Van montagu, M. (1986). Identification of the single molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium Tumefaciens. NASA Astrophysics Data System.* PP: 624-629.

Stodola., Weidenhagen., Lorenz., Siddique, and Furgala. (2004). Characterization of Pantoea dispersa UQ68J: producer of a highly efficient sucrose isomerase for isomaltulose biosynthesis. *Applied Microbiology.* PP: 93-103.

Takazoe., Schiweck. (1989). Characterization of Pantoea dispersa UQ68J: producer of a highly efficient sucrose isomerase for isomaltulose biosynthesis. *Applied Microbiology.* PP: 93-103.

Willmitzer. L., Dhaest, P., Schreler, P.H., Schmalenbach, W., Van montagut, M., schell, J. (1983). Size location and polarity of T-DNA encoded transcripts in octopine crown gall disease, common transcripts for octopine and nopaline tumors. *Cell Press.* PP:1045-1056.

W.U.L, Birch R.G. (2007). Double sugar content in sugarcane plants modified to produce a sucrose isomer. *Plant Biotechnology*. PP: 107-117.

Yadav, N.S., Vander Leyden, J.O.S., Donald, R., Bennet., Wayne, M., Barnes and Mary-Dell Chilton. (1982). Short direct repeats flank the T-DNA on a nopaline Ti plasmid. *Cross Mark.* PP: 6322-6326.

Zhao,Z., Tishu,C., Laura,T., Mike ,M., Ning, W, and Hong, P. (2000). Agrobacterium - mediated transformation. *Plant Molecular Biolog.* PP 789-798.

Zhao., Able. (1998). Biolistic mediated Sorghum bicolar transformation Via mannose based selection systems. *Asian Network for Scientific Information.* PP: 1811-9700 **.**

APPENDIX

Chapter 8

APPENDIX

Requirements for tissue culture and Bacterial culture

Appendix:1 Murashige and Skoog medium.

MS Major (400 ml)

NH_4NO_3	66.0g
KNO_3	38.0g
KH_2PO_4	06.8g
$CaCl_2$	17.6g
$MgSO_4.7H_2O$	14.8g

MS-Minorsalts (mg/ml)

H_3PO_3	6.20
KI	0.83
$MnSO_4.H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$Na_2EDTA.\ 2H_2O$	37.30
$FeSO_4.7H_2O$	27.80

Fe- EDTA(1 L)

EDTA	3.73mg
Ferroussulphate	2.78mg

MyoInositol

NH_4NO_3	165gm/L
$MgSO_4$	37gm/L
KH_2PO_4	17gm/L
$CaCl_2$	44gm/L

Appendix 2: Media used for the regeneration and genetic transformation of Sorghum.

Medium	**Composition**
Agro-infection Medium	MS basal
Co- Cultivation medium on to which seeds are to be transferred after 72 h	MS basal +250mg/L cefatoxime + hygromycin.
Shoot Elongation Medium	MS basal +250mg/L cefatoxime +hygromycin
Root Induction Medium	MS basal +250 mg/L cefatoxime + hydromycin.

Table no. 3

Appendix 3: Composition of YEB medium (1 litre)

Bacto Peptone	5 gm.
Yeast extract	1 gm.
Beef extract	5 gm.
Sucrose	5 gm.
Magnesium sulphate Heptahydrate	0.5 gm.

Appendix 4: Composition of LB medium (1 litre)

Bacto Peptone	10 gm.
Yeast extract	5 gm.

Sodium chloride	10 gm.

Table no. 4

Required concentration of all these constitutions was added to the distilled water and dissolved (10% less than the final volume). Then mixed till they dissolve and final volume was made using distilled water and pH is checked as 7 then it is autoclaved and can be used.

Appendix 5: CTAB Extraction Buffer for DNA Isolation

S.No	Name of the reagent	Volume for 100ml
1.	Distilled water	46 ml
2.	1M Tris (pH 8.0)	20 ml
3.	5M NaCl	28 ml
4.	0.5 M EDTA	4 ml
5.	β – mercaptoethanol	2 ml
6.	CTAB	2 ml

Table no. 5