Development of transgenics for fungal resistance and discovery of chemically induced mutations in Pearl Millet (*Pennisetum glaucum* L.) population by TILLING

Thesis Submitted to
Osmania University, Hyderabad

For the Award of the Degree of
Doctor of Philosophy in Genetics

By
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Under the Supervision of
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Dedicated …

To My Father

CERTIFICATE

This is to certify that the thesis entitled "Development of trangenics for fungal resistance and discovery of chemically induced mutations in Pearl Millet (*Pennisetum glaucum L.)* population by TILLING " in partial fulfillment of the requirement for the award of degree of Doctor of Philosophy in Genetics, Osmania University, Hyderabad, embodies the results of bonified research work carried out by Ms. N. Jalaja, under my supervision and guidance and that no part of the thesis has so far been submitted anywhere for any other degree or diploma.

The assistance and help taken during the course of the investigation and the source of literature and material has been duly acknowledged by her.

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(Research Co-Supervisor)

DECLARATION

I hereby declare that the research work entitled "Development of transgenics for fungal resistance and discovery of chemically induced mutations in Pearl Millet (*Pennisetum glaucum L.)* population by TILLING" has been carried out by me in the Applied Genomics Laboratory, GT-BT, ICRISAT, Patancheru, Hyderabad, and at the Department of Genetics, Osmania University, Hyderabad under the supervision of Dr. Rajeev K Varshney, Principal Scientist, ICRISAT and co-supervision of Prof. P. B. Kavi Kishor, Osmania University respectively. The work done is original and no part of the thesis has been submitted for any other degree or diploma of any other university.

Date:
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OUTLINE

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%	:	Per cent
°C	:	Degree Celsius
/	:	Per
µg	:	Microgram
µl	:	Microlitre
µM	:	Micromolar
mM	:	Millimolar
AS	:	Acetosyringone
APS	:	Ammonium C persulphate
BAP	:	6-Benzylaminopurine
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pair
BSA	:	Bovine serum albumin
4CL	:	4-Coumarate: coenzyme A ligase
CAD	:	Cinnamyl alcohol dehydrogenase
CAld5H	:	Coniferaldehyde 5-hydroxylase
CCoAOMT	:	Caffeoyl coenzyme A O-methyltransferase
CCR	:	Cinnamoyl-CoA reductase
cDNA	:	Complementary DNA
CoA	:	Coenzyme A
COMT	:	Caffeic acid *O*-methyltransferase
cm	:	Centimeter (s)
CTAB	:	Cetyl Trimethyl Ammonium Bromide
CODDLE	:	Codons to Optimize Discovery of Deleterious Lesions
CLUSTAL	:	Cluster Alignment
dH_20	:	Distilled water
DNA	:	DeoxyriboNucleic Acid
dNTP	:	Deoxyribose Nucleotide Tri-Phosphate
ds	:	Double stranded
DREB2A	:	Dehydration responsive element binding protein
E. coli	:	Escherichia coli
EDTA	:	Ethylene Diamine Tetra Acetic acid
EtOH	:	Ethanol
EMS	:	Ethyl methane sulphonate
EDR2	:	Enhanced disease resistance

F	:	Forward
F5H	:	Ferulate 5-hydroxylase
G unit	:	Guaiacyl unit of lignin
g	:	Gram (s)
GR	:	Glutathione reductase
H unit	:	p-Hydroxyphenyl unit of lignin
h	:	Hour (s)
$HgCl_2$	:	Mercuric chloride
HPLC	:	High Performance Liquid Chromatography
IAA	:	Iso-amyl Alcohol
IBA	:	Indole-3-butyric acid
IRDye	:	Infrared dye labeling
ICRISAT	:	International Crop Research Institute for Semi-arid Tropics
2iP	:	Isopentenyladenine
kb	:	Kilo-basepair
kg	:	Kilogram
L	:	Litre (s)
LB	:	Luria Bertani
LIMS	:	Laboratory Information Management Systems
M	:	Molar
m	:	Metre
max	:	Maximum
mg	:	Milligram
min	:	Minute (s)
ml	:	Millilitre (s)
mM	:	Millimolar
mm	:	Millimeter (s)
mol.wt.	:	Molecular weight
MOPS	:	4-Morpholino propanesulfonic acid
mRNA	:	Messenger RNA
NAA	:	α-Naphthaleneacetic acid
NaCl	:	Sodium chloride
NADPH	:	Nicotinamide adenine dinucleotide phosphate (reduced)
NCBI	:	National Centre for Biotechnology Information
No	:	Number (s)
nt	:	Nucleotide (s)
OD	:	Optical Density

ORF	:	Open Reading Frame
PCR	:	Polymerase Chain Reaction
PTGS	:	Post-transcriptional gene silencing
pH	:	Hydrogen ion concentration
R	:	Reverse
RNase	:	Ribonuclease
rpm	:	Revolutions per minute
RT-PCR	:	Reverse transcription polymerase chain reaction
s	:	Seconds
S unit	:	Syringyl unit of lignin
SDS	:	Sodium Dodecyl Sulphate
SSC	:	Sodium chloride / Sodium citrate
SNP	:	Single Nucleotide Polymorphism
TBE	:	Tris Borate EDTA
TE	:	Tris-HCl /EDTA
TDZ	:	Thiadiazuron
Tm	:	Temperature of melting
TEMED	:	N,N,N,N-tetramethylethylenediamine
TILLING	:	Target induced Local Lesions in Genomes
U	:	Unit(s)
UV	:	Ultra violet
V	:	Volt (s)
W	:	Watt (s)
YEM	:	Yeast extract Mannitol
YEP	:	Yeast extract Peptone
α	:	Alpha
β	:	Beta
δ	:	Delta

One letter codes for nucleotides

A	:	Adenosine
C	:	Cytosine
G	:	Guanosine
T	:	Thymidine
U	:	Uracil

CHAPTER I

INTRODUCTION

1.1 Importance of Pearl millet

Pennisetum glaucum (L.) R.Brown (*Pennisetum typhoides* (Burm.) Stapfet Hubb.) (Pearl millet) is the most important member of the genus *Pennisetum* of the tribe Paniceae in the family Poaceae. The name *Panicum glaucum* (L.) R.Brown, was adopted by Hitchcock and Chase (1951) in their Manual of Grasses of the United States, and hence accepted by American workers. Pearl millet is a dual-purpose crop used for grain and fodder and is grown primarily in Asia and Africa, where it occupies some 27 million ha (ICRISAT 1996). It is capable of growing on some of the poorest soils in dry, hot regions of Africa and Asia, where, as a poor man's source of dietary energy, it sustains a large proportion of the populace. It is also grown in other countries where, under relatively more favorable conditions, it provides grain for bullocks, dairy cows, and poultry.

Pearl millet is the most important member of this genus. With 2n = 14 large somatic chromosomes, it lends itself to investigation from the stand points of classical and molecular cytogenetics, gene location by aneuploid analyses, and studies on haploid and chromosome pairing. Its short life cycle; protogynous flowers, open pollinated breeding system, and ability to set a large number of seeds per ear make pearl millet highly suitable for intra and inter specific hybridization. This breeding system facilitates the flow of genes between cultivated annual species and related wild species. Pearl millet's has large chromosomes, larger than in most other species in the tribe Paniceae, and a distinctive pair of nucleolar organizers make it possible to study intergenomic and intragenomic chromosome pairing in interspecific hybrids (Jauhar, 1968). Its outbreeding nature makes pearl millet an ideal crop for heterosis breeding. It is generally agreed that pearl millet is of African origin, although the specific region where it originated is controversial. Harlan (1971) suggested the center of origin in a belt stretching from western Sudan to Senegal. Based on the present-day distribution, Brunken et al., (1977) considered the Sahel zone of West Africa to be pearl millet's original home, the view favored by Clegg et al., (1984) based on chloroplast DNA studies. Based on the available evidence, Appa Rao and de Wet (1999) concluded that pearl millet originated in western Africa some 4000 years ago.

1.2 *In vitro* regeneration

In vitro response of millets shows that strong genotype dependences and production of embryos are limited to selected genotypes. Moreover, majority of cereals including pearl millet lose their morphogenetic potential with increasing number of subclutures (Kavi Kishor et al., 1992; Pinus et al., 1993; Lambe et al., 1999). Plant regeneration in pearl millet was reported from a range of tissues such as immature zygotic embryos (Vasil and Vasil, 1981; Oldach et al., 2001; Goldman et al., 2003), mature embryos (Taylor and Vasil, 1996), immature inflorescences (Vasil and Vasil, 1981) and shoot apices (Devi et al., 2001). Even though major improvements were made in terms of regeneration frequency, genotype dependency and the lack of ideal explants are still the major bottlenecks for routine high frequency regeneration of fertile plants in pearl millet. It is therefore necessary to optimize conditions for regeneration in selected pearl millet breeding lines/genotypes prior to attempting genetic transformation. In general, auxins are the principal growth regulators for callus induction in monocots. Mainly 2,4-

dicholophenoxyacetic acid (2,4-D) alone was sufficient to produce good totipotent callus (Green 1978; Mikami and Kinoshita, 1988). Many workers used ethylene inhibitors such as silver nitrate, cefotaxime, cobalt chloride and nickel chloride (Pinus et al., 1993) that improved the frequency of regeneration. However, high frequency shoot regeneration is still a problem in majority of the pearl millet lines.

1.3 Transgenic plants for downy mildew resistance

Downy mildew caused by *Sclerospora graminicola* (Sacc.) J. Schroet is the most widespread and destructive disease of pearl millet causing severe economic losses. Other major diseases affecting pearl millet are smut (*Moeszimyces penicillatiae*), ergot (*Claviceps fusiformis*) and rust (*Puccinia substriata*). There is 50-60% of these pathogenic incidences in India depending upon the season and state and yield losses due to downy mildew disease in pearl millet are very high. The pathogen adapts its virulence and continues to threaten popular hybrids all over India. Symptoms of this disease often vary as a result of systemic infection. Leaf symptoms begin as chlorosis at the base and successively higher leaves show progressively greater chlorosis. Severely infected plants are generally stunted and do not produce panicles. Green ear symptoms result from transformation of floral parts into leafy structures. Many attempts to get downy mildew resistant plants using conventional plant breeding methods did not succeed till date. Therefore, there is an urgent need to evolve a line or variety that is tolerant to this fungus using genetic engineering techniques. Some of the pathogenesis related (PR) proteins show antifungal activity. For example, chitinase is one among them. In theory, constitutive expression of PR proteins, either singly or combined might confer decreased susceptibility to a specific group of pathogens. Pearl millet is generally grown in the form of hybrids only. Attempts to obtain downy mildew resistant plants by regular breeding methods did not succeed. Therefore, there is an urgent need to evolve pearl millet plants that are tolerant to the fungus *Sclerospora* and other fungi using genetic engineering techniques. As the world population continues to increase, food supplies must also grow to meet nutritional requirements. One means of insuring stability of food maintenance is to limit yield loss caused by plant pathogens mainly fungus, bacteria and virus. Since pearl millet is one of the most important cereals in India, it is of great interest to establish a system for production and generation of transgenic plants. Attempts to produce hybrids for downy mildew resistance have failed so far in imparting resistance. Since the fungus can reproduce both sexually and asexually, it may be difficult to produce a hybrid that is tolerant to *Sclerospora*. On the other hand, multiple genes if transferred can effectively control this fungus or multiple fungi. Hence, virulence is common in this fungus. Because of severe infections, plants are generally stunted and do not produce any panicles. This will result in the production of leafy structures instead of reproductive organs. Plants defend themselves from pathogenic fungi by a variety of means, including the production of several proteins with antifungal properties. Plant intrinsic responses that can be engineered to attain a wider, more durable resistance include the Hypersensitive Response (HR) and Systemic Acquired Resistance (SAR). Although these phenomena are complex, plant genes encoding cell wall degrading enzymes, especially chitinases have been used to alter plant resistance to fungal pathogens. But no single gene can give an adequate level of resistance and very few reports exist for resistance to multiple pathogens. It is expected that the use of chitinase and also osmotin as transgenes should produce a high level of resistance in crop plants against a variety of fungal pathogens.

1.4 Genetic transformation

Alternately has conducted transformation in pearl millet by *Agrobacterium* and biolistic approaches using hand gene gun and stated that particle gun method of gene transfer was scored over *Agrobacterium* mediated transformation. Biolistic bombardment using particle inflow gun device (Finer et al., 1992) has been a very useful technique to introduce foreign DNA into plant cells of monocotyledons and dicotyledonous plants. Particle bombardment is now being used as popular and inexpensive device for direct gene delivery into cell, tissue and organ since it requires minimum pre and post bombardment manipulations (Sugimura et al., 1999; Somika Bhatnagar et al., 2002). Stable genetic transformation by particle inflow gun has also been reported in some plants like, marigold (Vanegas et al., 2006) and *Dendrobium* (Suwanaketchanatit et al., 2007).

1.4.1 Functions of osmotin and chitinase genes

The transcript levels for many pathogenesis-related (PR) proteins and the PR3 chitinase gene have been shown as associated in plant defense (Li et al., 2001; Pritsch et al., 2000, 2001). The availability of genes encoding PR-proteins and the demonstration that PR-proteins exhibit strong *in vitro* antifungal activity (Mauch et al., 1988) has led to their deployment for enhancing disease resistance in crop plants. Several groups have reported that the introduction of a single transgene encoding different antimicrobial proteins including PR-proteins resulted in enhanced resistance (as measured in greenhouse trials) to a wide range of disease resistance including powdery mildew (Bliffeld et al., 1999; Schweizer et al., 1999; Bieri et al., 2000; Oldach et al., 2001), *Tilletia tritici* (Clausen et al., 2000), barley stripe mosaic virus (Zhang et al.,2001), scab (Chen et al., 1999), and other fungal pathogens (Leckband and Lorz, 1998). The increase in resistance to the pathogen varied widely and in most cases resistance was only partial. Following the initial reports of significant improvement in disease resistance in transgenic plants expressing PR proteins (Broglie et al., 1991; Zhu et al., 1994; Jach et al., 1995; Jongedijk et al., 1995; Lin et al., 1995) there have been numerous studies that have exploited a similar strategy (Datta et al., 1999). Osmotin is a member of a family of ubiquitous plant proteins, referred to as plant pathogenesis-related proteins of family 5 (PR-5), that is implicated in defense against fungi (Veronese et al., 2003). Osmotin gene and protein expression is induced by biotic stresses, and overexpression of osmotin delays development of disease symptoms in transgenic plants (LaRosa et al., 1992; Li et al., 1999). The specific interactions of osmotin with the plasma membrane are responsible for cell death signaling. However, because the cell wall governs access of osmotin to the plasma membrane, differences in cell wall composition largely account for the differential osmotin sensitivity of various strains, and specific cell wall components play a significant role in modulating osmotin toxicity (Ibeas et al., 2000, 2001). Osmotin, like other plant defense antifungal proteins, has specific but broad-spectrum antifungal activity. One of the most osmotin-sensitive phytopathogenic fungi is *Fusarium oxysporum*. *F. oxysporum* is an ascomycete fungus, like *S. cerevisiae*, and has been touted as an appropriate multihost model for studying fungal virulence.

1.5 New approaches to molecular resistance breeding

Genetically modified (GM) crops are deployed globally to control various insect pests and virus diseases. The development of GM crops to resist fungal and bacterial diseases has been a failure. Interest has now turned to the identification and exploitation of the natural diversity at R loci. Gene sequence diversity searches, achieved through techniques such as polymerase chain reaction (PCR), targeting induced local lesions in genomes (TILLING) may be targeted to the entire R protein or to specific R domains known to be required for pathogen recognition. Diversity searches will also allow us to define novel R sources so that effective R gene pyramiding can be implemented. R gene sequences can be used to create precise within the gene (WTG) molecular markers, thereby obviating the need for pathogen testing during introgression breeding. Searches for resistance gene analogues (RGAs) in the syntenic regions between closely related plant species are now a realistic option. GM approaches are also becoming more sophisticated and have a conceptual basis. Some of the focus has shifted to exploiting master-switch defense signaling proteins in recent times. Such proteins appear to function at taxonomically greater distances than R protein, can activate multiple component defense to provide broader spectrum pathogen control, and ideally would be key components of non-host defense.

1.6 Genes resistant to biotic and abiotic stresses

The expression as well as published reports (Cooper et al., 2003), say that a panel of candidate genes are potentially involved in stress tolerance. Candidate gene is used as a target for targeting induced local lesions in genomes (TILLING) (Till et al., 2003). These genes are selected based on several criteria: (1) high-quality sequence information (2) implicated function based on sequence annotation (3) evidence that they are within QTL regions based on mapping studies.

1.6.1 *Enhanced disease resistance (EDR2)* gene

Few mutants have been described that do not develop spontaneous lesions but display HR-like lesions only in response to a stimulus such as pathogen attack. *Enhanced disease resistant 1* (*edr1*)-*edr3* are examples of such mutants. *edr1* and *edr2*, but not *edr3*, also show elevated defense responses following powdery mildew attack. These phenotypes were suppressed in mutants with defects in the salicylic acid (SA) signal transduction pathway but not by those with defects in the ethylene/jasmonate pathway, suggesting that these mutants are hypersensitive to or have a lower threshold for responding to stress and activating the SA pathway. *EDR1* encodes a kinase, *EDR2* a novel protein, and *EDR3* a dynamin-like protein. The *EDR1* and *EDR2* mutants have a second phenotype that is SA-independent; they are hypersensitive to ethylene-induced senescence, implicating these two genes in the regulation of senescence as well as defense signaling. The diverse nature of processes interrupted in these mutants suggests that much remains to be uncovered about the mechanisms controlling cell death in plants. We initiated a screen for mutants that developed an exaggerated cell death response due to downy mildew, caused by *Sclerospora graminicola* in pearl millet as a means of identifying components of the HR programmed cell death. Lesion mimic mutants with spontaneous lesions were discarded from this screen to minimize the likelihood of recovering mutants with a metabolic dysfunction or that were compromised in the mechanisms protecting plants from the oxidative stress that arises during photosynthesis.

1.6.2 *Dehydration responsive element binding protein (DREB2A)* gene

Plants in the field are not subjected to only a single stress at a time, but they face numerous stresses collectively, whether it is biotic or abiotic. Some recent reports have highlighted the connection between disease resistance and drought tolerance. Inoculation of *Arabidopsis* plants with growth promoting rhizobacteria enhanced the protection against both *Erwinia carotovora* and dehydration stress (Timmusk and Wagner, 1999). Also distinct abiotic stresses induced the expression of antifungal protein cystatin in *Castanea sativa* (Pernas et al., 2000). The ABA-independent dehydration responsive signaling pathways marked by *DREB2A* were found to cross talk with adr1, activated signaling pathways (Chini et al., 2004). Constitutive or conditional enhanced expression of ADR1 conferred significant tolerance to drought but not for thermal and salt stress. The *DREB2A* expression was SA-dependent, since ROIs are also reported to signal *DREB2A* expression (Desikan et al., 2001). Therefore, *DREB2A* expression might have resulted from SA-amplified ROI synthesis, which suggests redox control of *DREB2A* expression. Microarray analyses of plants containing a conditional *adr1* allele demonstrated that a significant number of drought responsive genes were up-regulated (Chini et al., 2004). Hence, there may be significant overlap betweenbiotic and abiotic stress signaling. This summarizes that *DREBs* are important transcription factors regulating stress responsive gene expression through *DRE/CRT cis*-elements and its DNA binding domain. They play a crucial role in providing tolerance to multiple stresses and display overlapping responses to different stress conditions. *DREBs* control the expression of stress-responsive genes via ABA-independent pathways in both abiotic and biotic stresses. The highly conserved domains in *DREB* proteins are important for their specific biological functions and identifying such critical domains will help in achieving efficient crop improvement strategies by genetic engineering.

1.7 Targeting Induced Local Lesions in Genomes (TILLING)

Targeting Induced Local Lesions in Genomes is a method in molecular biology that allows directed identification of mutations in a specific gene. TILLING has been demonstrated to be effective in maize, wheat, *Lotus* and barley. Furthermore, several publicly funded TILLING projects are under development in crops including wheat and *Triticum monococcum*, poplar and brassicas, pea, soybean and *Medicago*. TILLING has become an accepted and commonly used technique for most chemical mutagenesis-based reverse genetics in the plant research community and its potential for application in both basic and applied research has been widely recognized. The breakthrough in exploiting point mutations for reverse genetics came in 2000, when Claire McCallum and colleagues in Seattle developed a general strategy that they christened Targeted Induced Local Lesions in Genomes or TILLING. EMS mutagenesis with a sensitive method for detecting induced point mutations in pooled DNA samples. Since then, the emergence of TILLING has been impressive. A patent application was filed and a company (Anawah, Inc., Seattle, WA, USA) spun out to exploit TILLING for commercial crop improvement. The function of pearl millet genes is the challenge for researchers, particularly to those involved in millet species. Unfortunately, many of the reverse genetic tools, such as T-DNA tagging and transposon-tagging are still not available in pearl millet. RNAi has emerged as an effective gene knockout/knockdown tool for many but, has yet to be applied to pearl millet.

Development of RNAi technology requires genetic transformation and very little work on transformation protocols in pearl millet due to the regeneration response of millet shows strong genotype dependence. Given these limitations, the ability to use traditional mutagenesis techniques coupled to efficient targeting of genes would be advantageous. Fast neutron mutagenesis can generate small to medium size deletions in genomes. In contrast to transgenic methods, chemical mutagenesis can be applied to most species including pearl millet. Chemical mutagenesis does not require gene transfer and is therefore not subject to biosafety and extensive regulatory concerns (Henikoff, 2004).

1.7.1 Creating structured mutant populations

Chemical mutagens have been used for forward genetic screens in a variety of organisms (Guenet, 2004). Ethyl methane sulphonate (EMS) induces single nucleotide changes by alkylation of specific nucleotides (Sega, 1984; Vogel, 1995), resulting in mutations that are high in density and essentially randomly distributed. Therefore, a relatively small population of individuals can provide an allelic series that includes a variety of missense changes with differing effects on protein function, and nonsense or splice site changes that cause truncation of the gene product. TILLING is a general reverse genetic technique that uses traditional chemical mutagenesis methods to create libraries of mutagenized individuals that are later subjected to high throughput screens for the discovery of mutations (Comai, 2006; Henikoff, 2003). Indeed, TILLING results have been reported for a variety of plants and animals (McCallum et al., 2000). The application of TILLING to pearl millet, however, has been hampered by the difficulty in obtaining a population with a sufficiently high mutation density. For reverse genetics, structured populations are essential to track any discovered mutations back to the families from which they originated the approach adopted in inbreeding *Arabidopsis*, *Lotus*, barley and wheat. *Arabidopsis* had been screened for mutations, detecting 5600 mutations that have been distributed back to the community as M_2 seed and is the current levy to academic laboratories for TILLING of 1 Kb fragments, which is a cost-effective approach and the basic platform.

1.7.2 Mutation detection and verification

In the original TILLING method, the authors used heteroduplex analysis by endonuclease to detect mutations in pooled DNA samples. The development of a DNA mismatch cleavage assay using an endonuclease SNiPerase that cleaves heteroduplex dsDNA at single base mismatches is a significant discovery. The SniPerase is distributed by Transgenomic kit (http://www. Transgenomic.com). In the present study, TILLING combines SniPerase cleavage with gel electrophoresis on LI-COR, fluorescence-based fragment analysis systems. The system is rapid, high throughput and relatively tolerant of PCR amplicon quality. In addition, software has been developed that facilitates the rapid analysis of the gel images. By adding different fluorescent dye labels to each of the PCR primers, SniPerase cleavage generates different sized and coloured fragments that together total the size of the uncleaved amplicon. This key piece of information allows potential false positives to be excluded from subsequent analyses and provides accurate information regarding the location of the mutation. The equal quantities of the DNA of eight individuals are pooled and mutations detected in four steps: PCR amplification, heteroduplex formation, SniPerase cleavage and detection of the cleaved products by fragment analysis. In addition to gel electrophoresis, fragment

analysis by capillary-based DNA sequencers has also been assessed. After de-convoluting pooled DNAs and re-sequencing alleles from individual plants, sequence comparison with the parental allele will confirm an induced mutation, define its nature and determine whether it is consistent with the known mode of action of the mutagen used. Mutant alleles are detected as bands running ahead from the main size band.

1.7.3 TILLING gel on LICOR analyzer

Although our own experience has been exclusively with LICOR 4300 series slab gel analyzer and all of our protocols are for these instruments, other slab and capillary instruments should also be adaptable for TILLING (Perry et al., 2003; Augustin et al., 2005). The loading capillary instruments and their high throughput make them potentially highly desirable for TILLING. The advantage of physically separating tracks with capillaries which makes these instruments superior to slab gels may be a disadvantage for TILLING, which currently relies on the background patterns present in all lanes to identify novel bands (Colbert et al., 2001).

1.8 SNP detection

EcoTILLING uses the single-base mismatch discovery method developed for TILLING, but instead of being pooled from a standard accession. Because naturally occurring polymorphisms are more frequent than those produced by a single round of chemical mutagenesis, TILLING generates many more cleavage fragments: typically a hundred or more per gel versus 1–5 for the mutation. Several implementation details facilitate the analysis of TILLING gel images. This simplification is feasible because TILLING applications do not require highly accurate fragment length calculation and because TILLING images contain a consistent, primer-specific pattern of background bands in each lane. The exact nucleotide change is then determined using standard DNA sequencing methods. With eight-fold pooling, 200 samples can be screened per day for mutations in a single 1.5 kb gene target using a single LI-COR analyzer. Higher throughput and economy of scale can be achieved by using multiple thermal cyclers and analyzers. Screening for mutations begins with PCR amplification of a target fragment of up to 1.0 kb using gene-specific infrared dye-labeled primers. The forward primer is 5'-end labeled with a fluorescent dye that is detected at B700 nm (IRDye 700) and the reverse primer is labeled with the IRDye 800, which is detected at B800 nm. In addition, TILLING can be performed using alternative readout platforms including slab gel and capillary systems. Although many groups use an enzymatic mismatch cleavage approach similar to that described in this protocol, other mutation discovery methods can potentially be substituted including denaturing HPLC and resequencing. When choosing a single nucleotide polymorphism (SNP) discovery platform, it is important to consider criteria such as equipment cost, reagent cost, maintenance cost and automation and assay sensitivity.

CHAPTER II

REVIEW OF LITERATURE

2.1 Distribution and uses of Pearl millet

Millets are part of the diet of the people of China, Japan, Africa and India. In Western countries they are used mainly as birdseed. Millets embrace 10 genera and at least 14 species. They are important because they are grown in poor soils with limited inputs and they constitute a major source of food for resource poor farmers of the areas of their cultivation. The food demand for 2025 (Borlaug, 2002) will require the yield of millets to rise from 2.5 to 4.5 million tones. This increase will largely come from improved varieties, transgenetically modified for resistance to abiotic and biotic stress, using a tertiary gene pool. It is the fifth most important cereal crop and most important millet occupying 55% of global millet production. It is grown over 40 countries, predominantly in Africa and Asia. It is staple diet for the vast majority of poor farmers and also forms an important fodder crop for livestock population in arid and semiarid regions of India. Pearl millet is the sixth most important cereal crop in the world, ranking after wheat, rice, maize, barley, and sorghum. It is a valuable grain and fodder crop and is cultivated in many parts of the world, although in the U.S. it is grown primarily as a forage crop on less than 1 million ha. In tropical and warm temperature regions of Australia and some other countries, it is grown as a forage crop (Jauhar, 1981a). It is grown in other countries where, under relatively more favorable conditions, it provides grain for bullocks, dairy cows, and poultry. In Brazil, it occupies about 2 million ha and is mainly grown as a mulch crop in the soybean production system. The need for collection and conservation of pearl millet germplasm for its improvement for present and future needs cannot be over emphasized. Appa Rao (1999) described the status of germplasm collections and genetic resources for pearl millet, particularly those at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in India. The ICRISAT collection includes the cultivated as well as weedy forms of pearl millet that belong to its primary gene pool.

Pearl millet is an ideal organism for basic and applied research. In their extensive reviews, Jauhar (1981a) and Jauhar and Hanna (1998) compiled the available literature on cytogenetics and breeding of pearl millet and related species. Pearl millet has received several different taxonomic treatments and hence different Latin names. It was treated as a constituent of at least six different genera, viz., *Panicum, Holcus, Alopecuros, Cenchrus, Penicillaria,* and *Pennisetum* (Jauhar, 1981c). The name *Pennisetum typhoides* (Burm.) Stapf et Hubb., accepted by Bor (1960), was widely used by workers outside of the U.S. and adopted by Hitchcock and Chase (1951) in their *Manual of Grasses of the United States,* and hence accepted by American workers.

2.2 *In vitro* regeneration in millets

In vitro culture of multicellular explants has been carried out in all major cereal crops during the past 10 years, and plant regeneration has been obtained by either organogenesis or somatic embryogenesis (Vasil, 1987; Bhaskaran and Smith, 1990). These studies have contributed to the development of genetic engineering technology for the improvement of cereal crops by *Agrobacterium* or biolistic transformation (Vasil, 1994). However, in most species and with both approaches, the *in vitro* culture step remains the limiting factor for two major reasons. First, *in vitro* culture of cereals shows strong genotype dependence and production of the appropriate culture type is generally

limited to few cultivars or cell lines (Bhaskaran and Smith, 1990; Christou, 1993). Second, the majority of cereal cultures lose their morphogenic capacity after several sub-cultures (Kishor et al., 1992; Pius et al., 1993). The culture response is also influenced by media composition, carbon source, genotype, explant source, growth conditions of the donor plant, other additives in the medium and the physical conditions of growth of the cultures (Morrish et al., 1987; Vasil, 1987).

In Pearl millet (*Pennisetum glaucum* or *P. americanum*), procedures for plant regeneration by somatic embryogenesis have been described from a range of tissues such as immature embryos (Vasil and Vasil, 1981), immature inflorescences (Vasil and Vasil, 1981; Pius et al., 1993) or shoot apices (Botti and Vasil, 1983). However, the efficiency of embryogenic callus induction or the regeneration ability was rarely quantified and little is known about the behaviour of different explants within a given genotype. In these previous reports, a two-step procedure was generally used for plant regeneration, including induction of embryogenic calli on a 2,4-D-containing medium followed by onset of embryos on a medium without growth regulator or supplemented with cytokinin or auxin. Callus of pearl millet (Lambe et al., 1995) could not be regenerated due to the long period of culture needed for the selection of transgenic callus.

Several attempts have been made to develop an efficient, reproducible and genotype independent protocol for pearl millet transformation, to compare the embryogenic behaviour of different explants from ten pearl millet genotypes and to optimise the rate and efficiency of regeneration via somatic embryogenesis during long-term callus culture. Different explants have been used for raising regenerable cultures in millets. Immature inflorescence was used for initiating cultures (Table.1). The cultured explants form callus, and then plant regeneration occurs through either somatic embryogenesis or organogenesis. Microtillering has also been noted in some cases.

Table 1. *In vitro* plant regeneration in millets

Species	Growth regulators used for callus induction/plant regeneration	References
Panicum bisulcatum	2,4-D	Fladung and Hasselbach 1986; Akashi and Adachi 1991
P. maxicum	2,4-D, NAA	Lu and Vasil 1981,1982; Kothari et al., 1994
P. miliaceum	2,4-D, 2,4,5-T, BAP	Nabors et al., 1983; Jain et al., 2001
P. milioides	2,4-D	Fladung and Hasselbach 1986
Paspalum dilatatum	2,4-D	Akashi and Adachi 1992

P. n otatum	2,4-D, NAA	Marousky and West 1987, 1990; Bovo and Mroginsky 1989; Akashi et al., 1993; Chen et al., 2001
P. scrobiculatum	2,4-D, Kn, NAA, BAP	Rangan 1976; Nayak and Sen 1989; Kavi Kishor et al., 1992; Vikant and Rashi 2001; Arockiasamy et al., 2001; Vikrant and Rashid 2002a, b; Kaur and Kothari 2003, 2004; Vikrant and Rashid, 2003
P. simplex	2,4-D, Kn, NAA, BAP	Molinari et al. 2003
P. vaginatum	2,4-D	Cardona and Duncan 1997
Pennisetum americanum	2,4-D, IAA	Vasil and Vasil 1981a, b; Botti andVasil1983, 1984; Taylor and Vasil1995,1996
P. g laucum	2,4-D, pCPA, Kn, BAP,NAA	Mythili et al., 1997, 2001; Devi et al., 2000; Devi and Sticklen 2001; Oldach et al., 2001; srivastav and Kothari 2002
P. typhoides	2,4-D, 2,4,5-T, IAA	Nabors et al. 1983
E. indica	2,4-D, Kn, GA_3	Kumar et al., 2001; Yemets et al., 2003
Eleusine coracana	2,4-D, NAA, IBA, BAP, Kn,Pic,GA_3	Thiru and Mohan Ram, 1980; Mohanty et al., 1985; Wakizuka and Yamaguchi 1987; Eapen and George 1989; Sivadas etal., 1990; Vishnoi and Kothari 1995; Poddar et al., 1997; Kumar et al., 2001; Kothari et al., 2004

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; NAA, α-naphthalene acetic acid; BAP- 6-benzylaminopurine; Kn- Kinetin; IAA, indole-3-acetic acid; pCPA- *para* chlorophenoxyacetic acid; Pic-4- amino-3,5-6-trichloropicolinic acid; TDZ- thidiazuron; IBA- Indole-3-butyric acid; GA_3- gibberellic acid.

2.3 Transformation of millets

Improvement of millets using biotechnology has been overlooked due to economic or regional considerations. Development of resistance against biotic and abiotic stresses and improvement of quality for consumer must receive top priority.

2.3.1 Explants and transgene delivery method

Introduction of DNA was feasible by electroporation or chemical methods (Hauptmann et al., 1987). After the development of the particle gun, it became possible to introduce DNA into intact cells and tissues and this became the preferred mode of gene transfer. Embryogenic calli were bombarded with DNA coated particles to obtain transgenic plants. Particle delivery devices such as PDS 1000/He (Du Pont or Biorad, Munich, Germany) or the particle inflow gun (PIG) (Finer et al., 1992) were used to introduce gold/tungsten particles coated with desired vectors into the cells. Osmotic treatment of explant during the bombardment was reported to be helpful for the transformation (Girgi et al., 1992; Goldman et al., 2003).

2.3.2 Vectors

Promoter, enhancer, introns and polyadenylation regions can affect the expression of transgenes in transgenic plants (Birch, 1997). Lambe et al., (1995, 2000) tested several vectors having *gus, hph, bar* or *nptII* regions in various combinations of CaMV 35S and terminator nos in pCAMBIA. They reported highest transient expression of *gus* gene by using the plasmid having *gus* gene with recombinant *Emu* promotor with *Adh1* intron and *nos* terminator in their experiments on pearl millet transformation.

2.3.3 Selection and plant regeneration

In an early report, Hauptmann et al. (1988) used hygromycin, phosphinothricin, kanamycin and methotrexate to test their efficacy for protoplast cultures of *T. monococcum*, *Panicum maximum*, *Saccharum officinarum* and a tri-specific hybrid of *Pennisetum* species, transformed with resistance genes. Gene integration was confirmed by PCR and Southern analysis. All tested cell lines showed resistance to high levels of kanamycin. However, no plants were regenerated. Lambe et al., (1995) reported maintenance of hygromycin resistant transformed callus of Pearl millet for two years without loss of resistance phenotype, but plants were not regenerated. In a later study on Pearl millet, Lambé et al., (2000) reported regeneration of hygromycin resistant plants obtained from callus.

In an important publication on pearl millet, Girgi et al., (2002) reported transgenic plants using *bar* and *gus* genes. Four breeding lines of pearl millet were bombarded with *gus* reporter and *bar* marker genes. The Ti progeny of

regenerated plants showed co-segregation of both marker genes indicating that both genes integrated at the same locus in the pearl millet genotype. Goldman et al., (2003) selected the transgenic tissues bombarded with *bar* gene on a medium containing 15 mg /l phosphinothricin but later on, the embryogenic cultures were transferred to a phosphinothricin free medium. The germinated somatic embryos were then exposed to 3–10 mg /1 phosphinothricin. In a more recent report, O'Kennedy et al., (2004) reported production of fertile transgenic pearl millet plants expressing a phosphomannose isomerase gene under the maize ubiquitin promoter.

2.3.4 Integration and expression of the transgene

Genetic transformation is being used as a method of choice for introducing beneficial exotic genes into crop varieties for improving different agronomic characters. Particle-inflow-gun (PIG) method has been successfully used for producing transgenics in diverse crop plants (O'Kennedy et al., 1998, 2001, 2004; Abedina et al., 2000; Zhang and Puonti-Kaerlas, 2000; Girgi et al., 2002; Madhavi et al., 2005). However, to date, limited attempts have been made to develop protocols for genetic transformation of pearl millet. Taylor and Vasil (1991) and Taylor et al. (1993), using microprojectile bombardment method, incorporated *gus A* gene into the scutellum of immature embryos of pearl millet and reported transient GUS expression. Lambe et al., (1995, 2000) transferred *gus A*, *hpt*, *nptII*, and *bar* genes into pearl millet through microprojectile method but could not obtain any transgenic plants. Girgi et al., (2002) transferred *gus A* and *bar* genes into the scutellar tissue of immature embryos and recovered limited number of transformants using particle delivery system (PDS) and PIG methods. Transgenic pearl millet plants harboring *bar* and *gfp* genes (Goldman et al., 2003), and phosphomannose isomerase gene (O'Kennedy et al., 2004) were also developed using PDS and PIG methods, respectively. Efforts made, thus far, to improve pearl millet through genetic engineering methods proved unsuccessful in developing stable resistance against the downy mildew disease. Genetic enhancement of pearl millet by incorporating novel antifungal genes, using molecular approaches, is highly desirable to achieve durable resistance against this major pathogen. Using the established protocol for plant regeneration from the shoot-tip cultures of pearl millet, a reproducible method for genetic transformation has been developed employing *gus A* gene with PIG method. Further, a chemically synthesized antifungal *pin* gene (Madhavi Latha et al., 2005) has been used for producing transgenic pearl millet resistant to downy mildew.

2.4 PR related chitinase and osmotin functions

The results for fungal disease resistance have been mixed with some notable failures (Neuhaus et al., 1991). The current consensus is that combinations of PR-proteins are required to achieve effective disease control. Transgenic wheat lines that stably and constitutively express one or both of two PR-protein genes have been reported (for a chitinase and a β-1,3-glucanase). While genetic resistance is the most effective disease control, suitable resistance genes are not always available. Resistance to fungal disease might be achieved by overexpressing PR-proteins that could degrade structural components of pathogenic filamentous fungi. A large group of pathogenesis related (PR) proteins has been shown to be rapidly and massively induced both locally around infection sites and systemically. The PR proteins encompass several different classes of structurally and functionally unrelated proteins that have been grouped into 11 protein families according to sequence similarities, although additional pathogen-induced

proteins with potential antipathogenic action are described. Members of several of these families were demonstrated to have a damaging action on the structures of the parasite, thus exhibiting antifungal activity in *in vitro* bioassays and supporting a possible role for these proteins in plant defense. These include PR-1 and PR-5 (thaumatin- like proteins and osmotins), which are thought to create transmembrane pores and therefore have been named permatins; PR-2 (1,3-glucanases) and PR-3, 4, 8, and 11 (chitinases), which attack 1,3-glucans and chitin, respectively, components of the cell walls in most higher fungi. Several reports demonstrated that transgenic plants over expressing some PR genes show enhanced resistance to fungal pathogens. Although some of these have been shown to have specific enzymatic activity i.e. chitinase (PR-3) and glucanases (PR-2), many are known to have catalytic function and their mechanisms of action are not understood clearly. Osmotin is a member of the PR-5 family that was originally identified as the predominant protein that accumulated in tobacco cells as a function of osmotic adaptation. Subsequently osmotin and other osmotin-like proteins were shown to have antifungal activity *in vitro* against a broad range of fungi, including several plant pathogens. Many of the PR proteins, including osmotin, exhibit clear specificity and resistance to fungal cells.

2.5 Downy mildew disease

Pathogen of plants is a significant and growing threat to crop production worldwide. The goal of producing crops with increased and durable resistance to spectrum diseases is therefore a major focus in plant research. Increasing knowledge of plant defense has led to more sophisticated transgenic approaches to enhancing resistance. The number of candidate genes put forward by transcriptomics, proteomics and protein interaction studies gives us a large choice of genes to be used. Potentially, these genes can be manipulated by over expression, induced expression and tissue specific expression. Downy mildew caused by *Sclerosopora graminicola* (sacc.). Schroet is the most widespread and destructive disease of pearl millet causing severe economic losses. This pathogen not only reproduces asexually but also sexually. Hence, virulence is common in this fungus. Other major diseases affecting pearl millet are smut (*Mesozimyces penicillariae*), ergot (*Claviceps fusiformis*) and rust (*Puccinia substriata*). There is 50-60% of this pathogenic incidence in India depending upon the season and state and yield losses due to downy mildew disease in pearl millet are very high (50%). The pathogen adapts its virulence and continues to threaten popular hybrids all over India. Symptoms of this disease often vary as results of systemic infection. Leaf symptoms begin as chlorosis at the base and successively higher leaves show progressively greater chlorosis. Severely infected plants are generally stunted and do not produce panicles, green ear symptoms result from transformation of floral parts into leafy structures. Many attempts to get downy mildew resistant plants using conventional plant breeding methods did not succeed till date. Therefore, there is an urgent need to evolve a line or variety that is tolerant to this fungus using genetic engineering techniques.

As the world population continues to increase, food supplies must also grow to meet nutritional requirements. One means of insuring stability of food maintenance is to limit yield loss caused by plant pathogens mainly fungus, bacteria and virus. Since pearl millet is one of the most important cereals in India, it is of great interest to establish a system for production and generation of transgenic plants. Attempts to produce hybrids for downy mildew resistance have failed so far in imparting resistance. Since the fungus can reproduce both sexually and asexually, it may be

difficult to produce a hybrid that is tolerant to *Sclerospora*. On the other hand, multiple genes if transferred can effectively control this fungus or multiple fungi. Naturally occurring variation and induced variation caused by artificial mutation or genetic engineering technique, shown by single nucleotide polymorphism (SNPs), insertions and deletions (Indels), can be indentified and discovered using small-scale to high throughput systems. Currently, mutations are mainly discovered by DNA sequencing, where after PCR, primers must be developed for the screening of any polymorphism. Several alternatives to sequencing, which is costly for large-scale screening have been proposed and were recently reviewed by Yeung et al., (2005).

2.6 Abiotic Stress

Drought and salinity are two major environmental factors determining plant productivity and plant distribution. Drought and salinity affect more than 10% and they are rapidly increasing on a global scale declining average yields for most major crop plants by more than 50% (Bray et al., 2000). Plants can perceive abiotic stresses and elicit appropriate responses with altered metabolism, growth and development. The regulatory circuits include stress sensors, signalling pathways comprising a network of protein-protein interactions, transcription factors and promoters, and finally the output proteins or metabolites. Classical breeding approaches revealed that stress tolerance traits are mainly quantitative trait loci (QTLs) which make genetic selection of traits difficult. Stress tolerant crops have been obtained mainly by introducing traits from stress-adapted wild relatives. Differential stress tolerance could be attributed to differences in plant reactivity in terms of stress perception, signal transduction and appropriate gene expression programs, or other novel metabolic pathways that are restricted to tolerant plants. The hypothesis that the genetic program for tolerance is at least to some extent also present in non- tolerant plants is supported by the observation that gradual acclimation of sensitive plants leads to acquisition of tolerance to some degree. These plants may need gradual adaptation for proper expression of genes responsible for acquisition of tolerance (Zhu, 2001).

2.7 Candidate genes

2.7.1 Searching candidate drought responsive genes for TILLING

Drought tolerance is a complex phenomenon controlled by multiple genes. Genes found to control drought tolerance in rice, for example, may predict those genes that play an important role in pearl millet. Comparative mapping of rice, foxtail millet, sorghum and pearl millet has demonstrated the genomic relationship among the cereal crops. Although at present only 2853 ESTs are available for pearl millet in public domain (as on May 2007), various genome or EST sequencing projects in major cereal species like rice, maize, sorghum, wheat and barley have provided a wealth of sequence information. These gene/EST sequence data can be mined for selecting the promising drought responsive genes. In parallel, literature search has been initiated to identify the other interesting candidate genes that can be used for TILLING in pearl millet.

2.7.2 *DREB2A* Gene Function

Abiotic stress-mediated gene expression is regulated via different transcription factors of which drought-responsive element-binding (DREB) proteins play an important role. There are two types of DREBs. Presently, the function of *DREB1* type protein is well studied; however, much less information is available for *DREB2*. A cDNA with an open reading frame of 332 amino acids, encoding the transcription activation factor *DREB2A* was isolated from *Pennisetum glaucum*, a stress tolerant food grain crop. Phylogenetic tree revealed that *PgDREB2A* is more close to DREBs isolated from monocots, though it forms an independent branch. The *PgDREB2A* transcript was up-regulated in response to drought within 1 h of the treatment, whereas the induction was delayed in response to cold and salinity stress. The DREBs belongs to ethylene-responsive element binding proteins (EREBPs)/AP2 (APETLA2) transcription factor family that is unique to plants. The DREBs bind to a 9-bp conserved sequence TACCGACAT, termed the DRE. They are essential for the regulation of dehydration-responsive gene expression in an ABA-independent stress signal transduction pathway (Liu et al., 1998).

Although transformation with individual genes has been shown to confer some degree of tolerance in transgenic plants, it is felt that regulated expression of more genes via over-expression of transcription factors can lead to sustained tolerance. It is important to enhance regulatory ability of an important transcription factor that can activate the expression of many target genes controlling correlated characters. In fact in many studies, overexpression of stress inducible DREB transcription factor was found to activate the expression of many target genes having DRE elements in their promoters and the resulting transgenic plants improved stress tolerance. The level of stress tolerance and growth retardation in the *35S:OsDREB1A* transgenic *Arabidopsis* was relatively lower than that in the *35S:AtDREB1A* transgenic *Arabidopsis*. This might be due to the difference in the number of target stress genes induced. The DREBs follow an ABA-independent signal transduction pathway. However, in *aba* (ABA-deficient) or *abi* (ABA-insensitive) *Arabidopsis* mutants a number of other genes were induced by drought, salt and cold. This suggests that some of the genes do not require ABA for their expression under drought, salt and cold conditions (Thomashow, 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). The *CBF4* transcription factor is ABA responsive and involves CRT/DRE elements in ABA-dependent pathway. Structural studies have shown that the DREB family of transcription factors has unique conserved regions in them that allow them to interact with a series of downstream genes in an ABA-independent fashion. The involvement of DRE in ABA-dependent regulation of stress response suggests a further interaction or a cross talk between the ABA dependent and ABA-independent signal transduction pathways. This interaction highlights co-ordination between the stress signals and ABA in the regulation of various stress induced genes.

2.7.3 Searching candidate fungal responsive genes for TILLING

At the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad several candidate genes are being evaluated for their biological efficacy against the sorghum shoot fly *(Atherigona soccata*), spotted stem borer *(Chilo partellus*), tobacco caterpillar *(Spodoptera litur*a) and cotton bollworm or legume pod borer *(Helicoverpa armigera),* which are major crop pests in the semi-arid tropic*s.* The need for genetic transformation of crops to improve crop production in the developing world has been discussed by Ortiz (1998), Sharma and Ortiz

(2000) and Ortiz et al., (2000). Information on candidate genes that can be used for genetic transformation of crops has been reviewed to evaluate their potential for genetic improvement of crops for pest management.

2.7.4 Enhanced Disease Resistance *(EDR2)* gene function

The *EDR2* mutants exhibit properties consistent with the assumption that *EDR2* acts as a negative regulator of cell death. The chlorosis and necrosis phenotypes do not develop spontaneously and do not develop in response to various abiotic stresses, such as wounding, heat stress, light stress, or drought stress. The chlorosis and necrosis were elicited only following inoculation with the pathogens *G. cichoracearum*, *B. g. hordei* or *H. parasitica*. The *EDR2* plays a role specifically in the cell death associated with plant-pathogen interactions and do not have a general role in cell death. These features distinguish the *EDR2* mutants from typical lesion mimic mutants such as the *acd* and *lsd* classes. The occurrence of chlorotic and necrotic tissue was restricted to inoculation sites and did not spread suggesting that *EDR2* restricts the initiation of cell death rather than its spread. Because both *G. cichoracearum* and *H. parasitica* are biotrophic pathogens, the chlorosis and necrosis that develop may be sufficient to account for the restricted growth of these pathogens in the *EDR2* mutants. The Salicuclic acid (SA) pathway, but not the ethylene/jasmonate pathway, appears to be somewhat deregulated in that PR1 transcript levels are elevated in *EDR2* mutants relative to wild type following elicitation by pathogen attack. Plants deficient in SA accumulation or signaling suppress both the development of chlorosis and necrosis as well as the disease resistance phenotypes of *EDR2* mutants. Thus, it is also possible that SA-dependent defenses unrelated to cell death contribute to the disease resistance phenotype of *EDR2* mutants. The SA signal transduction pathway is required for the HR elicited by incompatible plant-pathogen interactions. Thus, it is difficult to know whether *EDR2* acts upstream of SA to limit SA activation of cell death or downstream of SA. That *EDR2* may bind lipid-like molecules via both its PH and START domains, *EDR2* may have a direct or indirect inhibitory effect on *PAD4 or EDS1* via a lipid-like intermediate. Candidates for this lipid-like intermediate could be a sphingolipid, phosphatidic acid or oleic acid. Because loss-of-function mutations in the *EDR2* gene confer enhanced disease resistance to powdery mildew, *EDR2* probably functions as a negative regulator of powdery mildew resistance. Combined, these observations strongly suggest that *EDR1* and *EDR2* may function in the same pathway to regulate senescence and cell death.

2.8 Molecular Markers

DNA markers reveal genetic differences that can be visualized by using a combination of gel electrophoresis and staining with chemicals (ethidium bromide or silver) or detection with radioactive or colorimetric probes. Recently Mohan et al., (1997), kumar (1999) and Gupta and Varshney (2000) extensively reviewed the details for these markers system. Several resources are available for genetic/genomic research in plants are the following: (i) wild species and mutant collections; (ii) marker collections; (iii) F2 synteny mapping population and permanent recombinant inbred (RI) mapping populations; (iv) BAC libraries and an advanced physical map; (v) TILLING populations; and (vi) microarrays, gene silenced lines, and VIGS libraries (for transient silencing)

2.8.1 Strutural Genomics

Beginning in the 1980s, different types of molecular markers have been developed. Now in crop species, the richest in the number and type of these genetic markers. The PCR-based markers were developed both as random markers, such as random amplified polymorphic DNA (RAPD), including restriction fragment length polymorphisms (RFLPs), simple sequence repeats (SSRs), cleaved amplified polymorphic sequence (CAPS), amplified fragment length polymorphisms (AFLPs), and single nucleotide polymorphism (SNP) and many of them have been mapped onto the high-density tomato genetic map. These sequences are available from the SGN Database, thus allowing specific primers for PCR reaction to be designed.

2.8.2 Insertional mutagenesis

Both classical and insertional mutageneses have been used in tomato. Indeed, together with barley, *Arabidopsis*, sorghum, wheat and maize, tomato was the focus of early, extensive mutagenesis programs. In a paper published in 1964, Hans Stubbe reviewed over 250 tomato mutants arising from the seminal work of the Gatersleben group. Insertional mutagenesis systems exploiting exogenous transposon systems have also been described. Highly efficient protocols for transformation of Micro-Tom have been described, which may serve as a tool for extensive T-DNA mutagenesis programs also.

2.9 Targeting induced local lesions IN genomes

In addition to the above, a more recent strategy called targeting induced local lesions IN genomes (TILLING) was described by McCallum et al., 2000 for targeting local mutations in the genome. This is a PCR-based strategy that provides an allelic series of induced point mutations in genes of interest. As such, it can be applied to most organisms, even to those for which an efficient transformation system is not available. TILLING has been used for high-throughput isolation of mutants in *Arabidopsis* as well as in several crop plants.

2.10 Muatagenesis

TILLING is a chemical mutagenesis with high throughput genome-wide screening for point mutations in genes of interest that effectively complements classical forward mutation screening based on phenotype. Chemical mutagens like ethyl methanesulphonate (EMS) induce high-density single nucleotide changes that are randomly distributed in the genome. These DNA damaging agents induce allelic variation that includes missense mutations in coding sequences (leading to altered protein structure and function), mutations of non-coding sequences that may affect gene regulation, premature stop codon mutations and splice site changes that may result in knockout/knockdown of a protein. TILLING introduces new aspects into plant breeding, such as the possibility of surveying specifically for allelic series of economically interesting genes. This is a new reverse genetic strategy that combines the high density of point mutations provided by traditional chemical mutagenesis with rapid mutational screening to discover induced lesions (McCallum et al., 2000). The general applicability of TILLING makes it appropriate for genetic modification of crops, and there may be agricultural interest in producing phenotypic variants without introducing foreign DNA of any type into a plant's genome. Reverse genetics generally refers to the generation or targeted

discovery of a mutation in a gene that is known by its sequence. TILLING minimizes the effort required to find mutations, ascertaining the resulting phenotype. Chemical mutagenesis introduces background mutations that can make phenotypic analysis uncertain, and multiple generations of outcrossing may be desirable. A phenotype attributable to the two non-complementing mutations will be found in every heteroallelic individual.

The primary importance is the method that is used to generate chemically induced mutations. To ensure that useful mutations are identified, a sensible balance must be struck between the amounts of mutagenesis and the number of individuals analysed. There are well-established chemical mutagenesis methods for many organisms and many of these are likely to be directly applicable to a TILLING project.

Mutagenesis has remained popular over the past 70 years because it is simple, cheap to perform, applicable to all plant species and usable at a small or large scale. By varying mutagen dose, the frequency of induced mutations can be regulated and saturation can be readily achieved. Historically, mutation screens have assembled rich collections of phenotypic variants. The approach, which couples mutagenesis with sequence-specific mutation detection, promises to provide a general and effective platform for 'reverse genetics' in a wide range of plants. Today, several variants of this basic strategy have been developed and acronyms such as TILLING, Deleteagene and DEALING (Detecting Adduct Lesions IN Genomes) have been coined. Reverse genetics is a generic term for approaches that attempt to determine the biological function of a gene or protein by analysing individuals in which that gene has been deliberately perturbed. In plants, reverse genetics has relied heavily on insertional mutagenesis using mobile genetic elements such as T-DNA and endogenous or introduced transposons. In *Arabidopsis*, rice and maize, large populations have been developed that contain insertions dispersed throughout the genome.

2.11 TILLING will give mutants

S. Henikoff and colleagues have assembled a process that is a good general solution to the problem of identifying mutations in genes that are known only by their sequence. In addition to being a general method for the identification of mutations in an arbitrary gene, TILLING also provides an ALLELIC SERIES of mutations. Many amino-acid substitutions, splicing mutations and stop mutations can be identified by TILLING. This has several advantages over other methods. With an allelic series, which is not biased by phenotypic selection, partial loss-of function and novel-function alleles can often provide a more informative insight into the true function of a gene product. In addition, by using different chemical mutagens, it is possible to identify different constellations of mutations. This has the added advantage of increasing the diversity of an allelic series, raising the possibility of identifying specific amino-acid changes that are made interesting by results from other biochemical, structural or genetic studies.

2.12 TILLING in Plants

TILLING has been embraced by the *Arabidopsis* research community and has been made widely accessible through the *Arabidopsis* TILLING Project. The success with *Arabidopsis* has inspired researchers who work with other model organisms to use the method, which has the potential to be applied to many other genetic systems, especially

animal species for which there are no other, or limited, reverse genetic methods. Although the *Arabidopsis* research community has the distinct advantage that seeds are relatively easy to store and distribute, there are good reasons to believe that community-wide efforts to support TILLING for other organisms should be undertaken. In 2003, Greene et al. reported that the *Arabidopsis* TILLING Project (ATP), which was set up and introduced as a public service for the *Arabidopsis* community, had detected 1,890 mutations in 192 target gene fragments. Heterozygote mutationswere detected at twice the rate of homozygote mutations. The mutational density for treatment of *Arabidopsis* with EMS was approximately 1 mutation per 300 kb of DNA screened with these mutations distributed throughout the genome. The numerous mutations in *Arabidopsis thaliana* that have been identified *via* TILLING have provided an allelic series of phenotypes and genotypes to elucidate gene and protein function throughout the genome for *Arabidopsis* researchers. Another model plant, *Lotus japonicus*, has also been the focus of elucidating gene function through TILLING. EMS null allele mutants were examined and shown to have reduced sucrose synthase activity compared to the wild type; however, mutants still retained the ability for nitrogen fixation. In a separate study of pea (*Pisum sativum*), which also fixes nitrogen and is a member of the legume family, TILLING was applied to identify an allelic series of mutations in five genes with a total of 60 mutants identified. Some of the mutations discovered in the LE gene, which encodes 3 β-hydroxylase, were further characterized and determined to affect internode length. Mutants were backcrossed to the wild type and the segregation of the mutations and their respective phenotypes were examined. In 2004, maize, which is an important staple crop with a large genome, was shown to be conducive to the TILLING method.

In contrast to transgenic methods, chemical mutagenesis can be applied to most species including sorghum. Chemical mutagenesis does not require gene transfer and is therefore not subject to biosafety and extensive regulatory concerns. Chemical mutagenesis has been used in sorghum breeding previously; unfortunately, these mutagenized populations were not annotated and preserved. Traditional mutagenesis is used extensively in floricultural plants and fruit trees. In such material, irradiation of rooted stem cuttings, detached leaves or dormant plants is common, although *in vitro* cultured or micropropagated plants, regenerable callus cultures, stolons, axillary or adventitious buds have also been used. The major hurdle is overcoming the need for sex to purify mutations biologically.

2.13 Genotyping method

Another factor to consider is the choice of genotyping method. Although Cel-I is clearly an effective way to detect heterozygous mutations, even in pools of individuals, other methods might be more applicable for an existing set-up or might provide more information. Perhaps the most complete information is given by resequencing the target exons of each individual in the array. This was done on an array of F1 progeny of *N*-ethyl-*N*-nitrosourea (ENU) mutagenized including 9 amino-acid substitutions and 1 premature stop codon mutation. This method is designed to detect premature stop codons in the target gene. This method has the advantage of allowing the efficient detection of nonsense mutations and the detection of mutations that are manifested in expressed mRNA, but it cannot detect the more subtle alleles that can be identified by the Cel-I or re-sequencing strategies.

2.14 CODDLE

CODDLE was developed by Nicholas Taylor and Elizabeth Greene (2003) as a general tool that can also be used for polymorphism analysis and for conveniently designing primers for any organism and any mutagen. Few researchers used protein sequence conservation as the basis for evaluating whether a missense mutation is likely to have an effect on the encoded protein. The program CODDLE (for codons optimized to discover deleterious lesions) takes genome sequence data as input, determines which exons are most likely to yield nonsense and missense mutations given a particular type of mutagen, then runs another program Primer3 to generate PCR primers to amplify selected exons. After mutations have been identified and sequenced, the program PARSESNP (for project aligned related sequences and evaluate SNPs) shows the locations of induced polymorphisms, including restriction enzyme polymorphisms.With a homology model of related sequences for the target protein, PARSESNP produces predictions of the severity of missense changes.

Several computer programs have been developed or adapted to facilitate the TILLING process. As described above, CODDLE provides the front end for TILLING (Till et al., 2003). It has multiple entry options for submitting a genomic sequence and for obtaining an exon-intron model for the gene of interest using public sequence databanks. CODDLE also aligns conserved protein regions from the Blocks database (http://blocks.fhcrc.org) with the gene model. CODDLE uses the Primer3 algorithm and reports suitable primers for amplification of the chosen fragment. CODDLE enters the information automatically into the order form, then a submitted order is checked by BLAST searching, the user is billed, and the primers are ordered to initiate the process. For the recovery of mutations after identification, it is useful to have a routine method to genotype individual carriers. Neff and colleagues have developed a useful method that is known as dCAPS (for derived cleaved amplified polymorphic sequence), which uses a mismatched PCR primer to generate restriction enzyme polymorphisms in the amplified product, allowing mutant and wild type alleles to be distinguished. A web based program is used to design primers and identify restriction sites.

2.15 IR labeling primers

TILLING and EcoTILLING are based on a common set of methods. Target fragments of 1.5 kb are amplified by PCR with gene specific oligonucleotide primers that are 50-end-labeled with fluorescent IRDye 700 or IRDye 800 dyes. After amplification, samples are denatured and then annealed to form heteroduplexes between strands of DNA harboring nucleotide polymorphisms. Heteroduplexes are digested using a singlestrand specific nuclease and then size-fractionated by denaturing PAGE. To increase the efficiency of discovery of rare alleles, we applied an 8-fold pooling in the discovery.

2.15.1 IRDye 800:

IRDye 800 is a heptamethine cyanine dye absorbing and fluorescing in the near infrared region of the spectrum. The absorption maximum at 787 nm is well matched to the 785 nm laser in the detection microscope. The extremely high absorptivity and good quantum efficiency of the dye provide excellent sensitivity. Bands containing less than 10 attomoles of IRDye 800 label have been detected.

2.15.2 IRDye 700:

IRDye 700 is a pentamethine carbocyanine dye fluorescing in the near infrared region of the spectrum. The absorbtion maximum (681 nm) is just outside the visible region and matches the 685 nm laser of the detection microscope. While the absorptivity of IRDye 700 is slightly less than that of IRDye 800, higher fluorescence efficiency compensates for the absorption difference. Bands containing less than 10 attomoles of IRDye 700 label have been detected.

2.16 Limitations of TILLING

TILLING project depends on the development of a densely mutagenized population and the preparation of DNA of suitable quality for PCR. Because protocols for chemical mutagenesis in different plant and animal species can vary dramatically, no single protocol can be considered generally. Although many species have been successfully mutagenized, others have been less tractable for reasons that are not clear. In cases where mutagenesis is unfeasible, natural nucleotide diversity can nevertheless be discovered by EcoTILLING. In theory, mutation discovery may be limited in highly heterozygous species where the large number of bands from natural polymorphisms could potentially inhibit the detection of rarely induced mutations. When encountering problems to obtain consistent PCR amplification, alternative extraction methods may be developed or additives may be added to the PCR mixture.

2.17 Advantages and Disadvantages of TILLING and EcoTILLING

As discussed earlier, using chemical mutagenesis and TILLING to pinpoint these mutations has been highly effective in the elucidation of gene function in plants and animals without the production of transgenic material. TILLING has been demonstrated to be sensitive enough to detect induced mutations and naturally occurring SNPs, as well as the detection of heterozygotes. EcoTILLING, which has been less frequently employed in the current literature, can also be a valuable tool for mining for SNPs in germplasm, assessing heterozygosity, uncovering variants for disease resistance, or ascertaining the function of a gene or regulatory element by detecting natural variants. EcoTILLING can be a good technique to employ especially when working with a well established population with thoroughly characterized morphological data. One of the main advantages of TILLING is the amount of time and money this method can potentially save by not requiring resequencing of all individuals in a population to mine for frequent or rare SNPs. As a general rule for a diploid organism, TILLING is performed by pooling eight individuals of a population at a time and assessing differences by endonuclease digestion of mismatches in a heteroduplex. The majority of the samples screened in TILLING have the same haplotype with very few samples in the population having an induced mutation in the gene of interest due to a relatively low frequency of induced mutants by utilizing chemical mutagenesis. TILLING is sensitive enough to detect homozygous mutations as well as heterozygous mutations in an 8 fold pool. This method allows one to weed out the identical individuals and only focus resources on sequencing individuals with rare chemically induced DNA polymorphisms.

TILLING is a non-transgenic, high throughput reverse genetic approach. This technique unlike other SNP detection methods provides the approximate location within a few 100,000 base pairs of the induced mutation, which allows

targeted sequencing in the area of the induced mutation supposed to be sequencing the entire fragment. Therefore, through mutagenesis one can obtain partial loss or complete loss of function and new novel functions, which can provide valuable insight into the true role of a gene in a species of interest. Another advantage of TILLING is that the likelihood of recovering a deleterious mutation can be calculated in advance. A calculation is possible, since EMS produces primarily C/G to T/A transitions (Ashburner, 1990). For example, 20 of 23 LEAFY EMS-generated mutations are from C to T, resulting in C/G to T/A transitions (http://www.salk.edu/LABS/pbio-w/ lfyseq.html). The probability of discovering deleterious alleles can be maximized by judicious choice of the region to be TILLed. By choosing coding regions that are evolutionarily conserved, it becomes more likely that missense mutations with detrimental effects on gene function will be obtained. Splice junction mutations are also potentially deleterious.

Insertional mutagenesis is an immensely powerful tool but the drawbacks are: (i) insertion sites are non-random, with certain genes less susceptible to insertion than others; (ii) induced mutations generally produce a complete loss of function allele that might not provide much insight into the normal function of the gene; (iii) the approach has been restricted to a few species; (iv) because many insertional-mutagenesis strategies rely on genetic manipulation to develop the resource, the incorporation of promising experimental lines directly into breeding programmes is highly restricted.

2.18 Application Overview in TILLING

Rapid acquisition of genomic sequence data has elevated a new discipline, functional genomics, which focuses on determination of gene function. Reverse genetics methodologies are an important part of functional genomics. Traditional reverse genetic methods, such as the use of transposons to "knock out" a specific gene, can accurately determine phenotype but require time consuming transgenic or sophisticated tissue culture methodologies. Such "knockout" methods are limiting because the entire gene is knocked out the effects of partial loss of function of an active gene cannot be observed. To overcome the limitations of knocking out an entire gene and to expand knowledge of active gene mutations, researchers from Fred Hutchinson Cancer Research Center developed a process for Targeting Induced Local Lesions In Genomes, or TILLING. Elegantly simple, highly efficient, TILLING uses chemical mutagenesis to yield a traditional allelic series of point mutations for virtually all genes. The TILLING process is of particular value for essential genes where sublethal alleles are required for phenotypic analysis. The value of TILLING for genetic research is enhanced by its proven viability for a rapidly growing range of organisms.

CHAPTER III

MATERIALS AND METHOD

3.1 In vitro plant regeneration studies in different varieties of Pennisetum gluacum (Pearl millet)

For callus initiation and subsequent plant regeneration, seeds of different genotypes of pearl millet namely PT 732 B-P2, LGD-1-B-10, ICMB 99022, 863B-P2, 841-P3, 81B-P6, P1449-2-P1, Tift-23D2B1-P1-P5, that are susceptible and tolerant to fungal pathogens especially to downy mildew were collected from ICRISAT, Patancheru, Hyderabad and the germplasm was maintained in the Experimental Farm, Department of Genetics, Osmania University for further experimental studies.

3.2 Preparation of medium

All *in vitro* experiments were conducted using Murashige and Skoog's (1962) medium (MS) fortified with different plant growth regulators. Test tubes made of Borosil glass (150 mm × 25 mm), plugged with non-absorbent cotton and autoclavable plastic caps were used for callus initiation and 250 ml capacity bottles (11 cm height, 6 cm in diameter) containing a plastic cap were used for elongation of shoots. Most of the inorganic salts and chemicals were obtained from Hi Media, Merck and Qualigens. Plant growth regulators were of high purity and purchased from Sigma Inc., St. Louis, MO, USA. All other chemicals were purchased from the Fermentas Inc., Hanover, MD, USA and from Qiagen Inc., CA, USA. MS media with different plant growth regulator combinations were tried for callus induction, shoot regeneration and rooting. The composition of MS medium employed in the present study is shown in Table 1. All the media stock solutions were prepared according to the composition of the nutrient medium using glass-distilled water. The stock solutions were stored in the refrigerator until use. Whenever hydrates of salts were used, appropriate corrections were made. All supplements to the basal medium were added prior to the final adjustment of volume. Media pH was adjusted to 5.7 with 0.1N sodium hydroxide or 0.1N hydrochloric acid (as found necessary) before autoclaving. The media were solidified with 1% Hi Media agar. After adding agar, the medium was heated gently with constant stirring, till the agar is dissolved. Later 50 ml of medium was transferred to each of the culture vessels. The mouth of the culture vessels was closed immediately with a plastic cap. Sterilization of media, test tubes and bottles was performed by autoclaving the contents at 121 ºC and 15 psi (1.05 Kg cm^{-2}) pressure for 15-20 minutes. Heat labile compounds were subjected to filter sterilization using Millipore filters (0.22 μM) under pressure.

Table 2. Composition of Murashige and Skoog's (1962) medium

Nutrients	Formula	Quantity mg/l

I	**Macro nutrients**		
	1. Ammonium nitrate	NH_4NO_3	1650
	2. Potassium nitrate	KNO_3	1900
	3. Magnesium sulphate	$MgSO_4.7H_20$	370
	4. Potassium dihydrogen ortho phosphate	KH_2PO_4	170
	5. Calcium chloride	$CaCl_2.2H_2O$	440
II	**Micro nutrients**		
	1. Boric acid	H_3BO_3	6.20
	2. Sodium molybdate	$NaMoO_4.2H_2O$	250 µg
	3. Cobalt chloride	$CoCl_2.6H_2O$	25 µg
	4. Manganese sulphate	$MnSO_4.4H_2O$	22.30
	5. Zinc sulphate	$ZnSo_4.7H_2O$	8.60
	6. Copper sulphate	$CuCO_4.5H_2O$	25 µg
	7. Potassium Iodide	KI	0.83
III	**Fe Na_2EDTA**		
	1. Ethylene diamine tetra acetic acid disodium salt	$C_{10}H_{14}N_2Na_2O_8.2H_2O$	373
	2. Ferrous sulphate	$FeSO_4.7H_2O$	278
IV	**Vitamins**		
	1. Thiamine hydrochloride	$C_{12}H_{17}N_4$ OSCl.HCl	0.1
	2. Phyridoxin hydrochloride	$C_8H_{11}NO_3$ HClS	0.5
	3. Nicotinic acid	$C_6H_5NO_2$	0.5
	4. Meso-inositol		100
V	**Others**		
	1. Glycine	NH_2 CH_2COOH	2.0 mg/l
	2. Sucrose	$C_{12}H_{22}O_{11}$	20 g/l

3.3 Aseptic conditions: Surface sterilization of plant material

All operations including inoculations and transfers were conducted in a sterile ultraviolet light (UV) treated laminar airflow transfer hood. The surface of the hood was rubbed down first with 5% soap solution, rinsed with water, dried and finally sterilized with 70% alcohol prior to each use. The interior of the chamber was saturated with an aerosol of 2% thymol and 2% glycerine in 90% ethyl alcohol. All the surgical instruments were sterilized by autoclaving before use. Spatula, inoculating loop, anatomical scissors, forceps, needles and scalpels were further sterilized by flaming with absolute alcohol inside the laminar flow cabinet. To maintain maximum sterility inside the laminar flow hood, it was swabbed with 70% ethanol and the UV lights were switched on for 10–15 minutes before every operation. All the test tube cultures were incubated under continuous white fluorescent light (30 µ Em^{-2} S^{-1}) at an ambient temperature of 24 ± 2 ºC.

3.4 Callus induction

3.4.1 Callus initiation from different genotypes and explants (immature embryos, mature embryos, immature inflorescence and shoot tips)

Different explants such as shoot tips, mature embryos, immature embryos and immature inflorescences were inoculated onto Murashige and Skoog's medium containing different concentrations of thidiazuron, dicamba, picloram, 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxypropionic acid (2,4,5-TP), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), 6-benzylaminopurine (BAP), kinetin, zeatin and 2-isopentenyladenine (2- iP). The inflorescences were cut aseptically into explants and inoculated onto Murashige and Skoog's medium (MS) containing different concentrations of 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, 2,4,5-trichlorophenoxypropionic acid thiodiazuron (TDZ), dicamba, picloram and napthaleneacetic acid. Similarly, shoot tips from different genotypes measuring 5-10 mm were cut and inoculated onto the above media for callus initiation.

Callus was also initiated from mature embryos and shoot tips of different varieties of *Pennisetum* in Murashige and Skoog's basal medium containing 2 mg/l 2,4-D and 6 mg/l 2,4,5-trichlorophenoxyacetic acid. Calli obtained were subcultured onto the same medium with an interval of two-weeks. In all these varieties, 10% callus was found embryogenic which was subsequently transferred to MS medium containing different concentrations of BAP and kinetin.

Seeds of all the inbreed lines were sown in the field and the immature inflorescences were collected from the boot leaves after 35-45 days old plant (from the date of sowing in the field). The boots were collected and 3-7 cm long young inflorescences were separated. Then they were surface sterilized with 0.1% mercuric chloride for 5 min and the boots were washed again 3 times with sterile distilled water to remove traces of $HgCl_2$ by changing the water each time at least for 30 min. The florets were separated from the panicles and inoculated onto MS basal medium containing 2,4-dichlorophenoxyacetic acid (2 mg/l). Cultures were maintained at 25+2 ^{0}C under dark. Callus was subcultured after every 20 days and was maintained on MS medium supplemented with 2 mg/l 2,4-D. This callus was also transferred onto 2 mg/l 2,4-D +10 mM NaCl for three subcultures and onto 0.01 mg/l TDZ for one subculture. Likewise, shoot tips from different genotypes were also used for callus initiation on MS medium containing different concentrations of plant growth regulators.

3.5 Shoot regeneration from immature inflorescence explants

Embryogenic callus and somatic embryos obtained on the TDZ media were later transferred onto regeneration media (MS medium plus different growth regulators) supplemented with 6-benzylaminopurine or kinetin in combination with indole-3-acetic acid (IAA) and naphthaleneacetic acid (NAA). Cultures were maintained at 25+2 ^{0}C under 16 h light and 8 h dark cycles at a light intensity of 30 µ Em^{-2} S^{-1} provided by white fluorescent bulbs . The influence of different concentrations of kinetin and BAP was tested for shoot regeneration from this embryogenic callus.

3.6 Rooting of shoots

The shoots obtained from callus cultures were rooted in MS basal medium fortified with 0.2 mg/l IAA. After 5-6 weeks of culture, shoots were excised and transferred to half-strength MS medium containing 0.5mg/l, 1 mg/l and 2 mg/l IBA and 0.5mg/l, 1 mg/l and 2 mg/l NAA, 0.5mg/l, 1 mg/l and 2 mg/l IAA and 2% sucrose (w/v) for rooting. The percent frequency of root formation and the mean number of roots formed per shoot was recorded after 20-days of culture.

3.7 Acclimatization

Rooted shoots were carefully taken out of the medium and washed thoroughly in running tap water to remove traces of medium attached to roots without damaging the roots. The plantlets were transferred to plastic pots containing vermiculite and vermicomposte in 1:3 ratio. Potted plants were covered initially with polythene bags to maintain humidity. Hardening was continued for three weeks with mild irrigation during the initial period until they were successfully acclimatized. Then after, they were acclimatized to the environment and the plants were transferred to clay pots with a potting mixture of sand: soil: dry powdered cow dung (1:1:1). After 2 months, a total of 60 plants (20 from each genotype) were transferred to clay pots (15 cm in height) with fresh potting mixture for further growth.

3.8 Histological studies on embryogenesis and shoot organogenesis from immature inflorescence and shoot-tip derived calli of Pearl millet

The ontogeny of embryogenesis as well as shoot organogenesis was carried out after fixing embryogenic and shoot differentiating callus (derived from inflorescences) in acetic acid and ethanol (3:1). Histological sections were taken using a microtome and histological photographs using Nikon Eclipse E 800 Light Microscope (Japan) fitted with Nikon DXN 1200C digital camera. This was felt necessary to ascertain the origin of morphogenesis. Shoot organogenesis as well as embryogenesis were observed in the histological sections of callus derived from inflorescences of three genotypes.

3.9 Studies on calli for antibiotic sensitivity

To test the sensitivity of kanamycin, phosphinothricin and cefotaxime, callus cultures were sub-cultured onto the Petri plates containing MS media with different concentrations of kanamycin (0-300 mg/l) and phosphinothricin (0-20 mg/l) separately. Regarding phosphinothricin (PPT), the tissues were subjected to 0-20 mg/l. The lethal dose (LD) of PPT at which no calli could survive was determined.

3.10 Cloning of osmotin and chitinase double construct into pCAMBIA-2300 and Bar gene phosphinothricin marker into pCAMBIA-1300

3.10.1 Cloning of pCAMBIA2300 containing osmotin and chitinase double construct as a marker free construct

Both *osmotin* and *chitinase* genes (full-length cDNAs) were obtained from the laboratory of Prof.P.B.Kirti, Department of Plant Sciences, University of Hyderabad, Hyderabad as gifts. Earlier, both *osmotin* (isolated from

Solanum nigrum) and rice *endochitinase* genes were introduced into the vector pCAMBIA2300. This also contained a marker gene kanamycin. Since marker free gene constructs are advisable, the construct was prepared without kanamycin marker. Plasmid DNA was isolated from *E.coli* containing *osmotin* and *chitinase* genes in binary vector pCAMBIA2300 using alkaline lysis method. The binary vector was digested with Xho I to release the Npt II gene.
Digestion was performed by using the instruction manual of MBI – Fermentas company.
The reaction mixture contained plasmid DNA (3 µg) 12 µl, 10 X restriction enzyme buffer 3 µl, restriction enzyme (XhoI) (5 units/ µl) 1.5 µl, sterile distilled water 12.0 µl, total 30 µl. The reaction mixture was incubated for overnight at 37 ºC for complete digestion.

3.10.2 Self ligation of the osmotin and chitinase gene in pCAMBIA2300

Ligation of *osmotin* and *chitinase* gene without NptII after digestion was carried out separately by using the instruction manual of quick ligation kit purchased from MBI-Fermentas. The reaction mixture contained linearized pCAMBIA2300 plasmid (0.013 pmoles) 1 µl, sterile distilled water 0.5 µl, 10 x ligation buffer1.5 µl, T4 DNA ligase 2.0 µl, and the total volume was 15 µl. The above reaction mixture was incubated for overnight at 16 ºC in a water bath for ligation.

3.10.3 Preparation of competent *E. coli* cells for transformation

The protocol used for bacterial transformation is basically that of Inoue et al. (1990) with minor modifications. *E.coli* DH5α cells were cultured on Luria and Bertaini (LB) agar plates and incubated at 37 ºC overnight. Around 10 - 12 large colonies were inoculated into 250 ml SOB in a one liter flask and incubated at 19 ºC with vigorous shaking until an OD of 0.5 at 600 nm was obtained. After attaining the required OD, the flask containing the bacterial cells was placed on an ice bath for 10 min. The cells were centrifuged at4000 rpm at 4 ºC for 10 min and pelleted. The cells were re-suspended gently in 80 ml ice-cold Tris-Boric acid (TB) buffer and stored on ice for 10 min. Again the cells were centrifuged at 4000 rpm for 10 min at 4 ºC. Pellet was re-suspended gently in 20 ml ice-cold TB and 1.4 ml dimethyl sulphoxide. The cells were made into aliquots of 50 and 100 µl for transformation and stored at -80 ºC.

3.10.4 Transformation of ligation mix by heat shock method and screening of recombinants

Competent cells of *E.coli* were thawed on ice and 10 µl of ligation mixture was added to competent cells and incubated on ice for 30 min. The cells were given a heat shock at 42 ºC for 90-120 seconds and 900 µl of LB liquid medium (obtained from Hi-Media) was added and mixed well. The mixture was incubated at 37 ºC on a gyratory shaker with an rpm of 250 for 1 h. Finally, 100 µl of the culture was spread on culture plates containing selection pressure (kanamycin 50 mg/l) and incubated for overnight at 37 ºC. Colonies were selected randomly from the overnight grown kanamycin (50 mg/l) plates and each colony was inoculated into 5 ml of LB liquid medium containing kanamycin. Plasmid was isolated by using Qiagen miniprep kit. Molecular characterization of the plasmid was carried out by using restriction analysis of the plasmid isolated with restriction enzymes (XhoI, BamHI and HindIII). Digestions were performed using user manual instructions.

3.10.5 Cloning of bar gene into pCAMBIA-1300

This vector contained hygromycin marker for selection. But a *bar* gene that encodes phosphinothricin acetyl transferase (for phosphinothricin resistance) was cloned into this pCAMBIA-1300 binary vector. Then both the constructs (one without any marker and another with *bar* gene construct) were transferred into *Agrobacterium tumefaciens* GV2600 strain by using freeze and thaw method. Transformants were selected on the kanamycin containing media. This strain is used for the pearl millet transformation.

3.10.6 Cloning of *bar* gene into pCAMBIA1300 binary vector

The intermediate pPUR vector harboring the *Bar* gene was obtained and has beencloned into pCAMBIA 1300 binary vector in order to transfer the genes into *Agrobacterium tumefaciens* GV2600 strain for plant transformation. Plasmid DNA was isolated and digested with the restriction enzyme HindIII and EcoRI to release the *Bar* gene constructs along with CaMV35S promoter and NOS polyA terminator. The pCAMBIA 1300 vector was linearized with HindIII and EcoRI. The reaction mixture for this purpose contained:

***Bar* gene digestion**			**pCAMBIA 1300 linearization**		
Plasmid DNA (3 µg)	=	12 µl	Plasmid	=	12 µl
10x Restriction enzyme buffer	=	3 µl	Buffer	=	3.0 µl
Restriction enzyme (HindIII + EcoRI) (5 units/µl)	=	1.5µl	HindIII + EcoR I (5 units/µl)	=	1.5µl
Sterile distilled water	=	13.5 µl	Sterile distilled water	=	13.5 µl
Total	**=**	**30 µl**	**Total**	**=**	**30 µl**

The above reaction mixture was incubated for overnight at 37 ºC for complete digestion.

3.10.7 Elution and ligation of *bar* gene and pCAMBIA1300 by using Qiagen gel elution kit

To elute the *bar* gene insert and linearized pCAMBIA1300 vector, the digested DNA was run on the low melting agarose (FMC BioProducts, USA) and stained with ethidium bromide. After running the gel, the inserts (550 bp) and pCAMBIA1300 linearized vector (11 Kb) were excised from the gel and eluted according to the protocol supplied along with the Qiagen gel extraction kit.

Ligation of *bar* gene insert along with CaMV35S promoter and NOS poly A terminator into pCAMBIA 1300 was carried out separately by using the instruction manual of quick ligation kit purchased from MBI-Fermentas. The ligation reaction mixture contained for both sense and antisense inserts was linearized pCAMBIA 1300 plasmid (0.015 pmoles) 1.0 µl, *bar* gene insert (0.003 pmoles) 10.0 µl, sterile distilled water 0.5 µl,10x ligation buffer 1.5 µl,T4 DNA ligase 2.0 µl, in a total volume of 15 µl. The reaction mixture was incubated for overnight at 16ºC in a water bath for ligation. The recombinant vectors were transformed to *E.coli* by preparing competent cells and by heat shock method. Recombinant clones were spread in selection media containing kanamycin (50 mg/l).

Recombinant clones were confirmed by plasmid isolation and restriction digestion by HindIII and EcoRI enzyme. These *E.coli* pCAMBIA 1300-Bar clones were transferred into the *Agrobacterium* strain GV2600, confirmed by isolating plasmid and digestion by HindIII and ECoRI enzymes. These confirmed pCAMBIA1300 recombinant clones were used for plant transformation experiments.

3.11. Genetic transformation studies using *Agrobacterium*

pCAMBIA2300 double construct and pCAMBIA1300 with *bar* gene constructs were transferred into *Agrobacterium tumefaciens* GV2600 strain by using freeze and thaw method. Transformants were selected on the kanamycin containing medium.The embryogenic callus derived from 81B-P6 immature inflorescence was used as explant for genetic tranformation.

3.11.1 Transformation of pCAMBIA-2300 double construct and pCAMBIA1300 with *bar* gene constructs into *Agrobacterium tumefaciens*

The binary vector with desired gene was transformed into *Agrobacterium* by using freeze-thaw method (An et al., 1988). *Agrobacterium tumefaciens* strain GV2600 containing an appropriate helper Ti plasmid in 5 ml of YEP medium (1 g/l peptone, 0.4 g/l of yeast extract, 10 g/l mannitol and 0.2 g/l $MgSO_4$ $7H_2O$, 0.5 g/l K_2HPO_4, 0.1 g/l NaCl, 10 g/l agar if necessary) was grown overnight at 28 ºC. Overnight culture of 2 ml was added to 50 ml of YEP medium in a 250 ml flask and shaken vigorously (200 rpm) at 28 ºC until the culture reached to an OD of 0.6 at 600 nm. The culture was chilled on ice and microfuged at 3000 g for 5 min at 4 ºC. Supernatant was discarded and the cells were resuspended in 1 ml of 20 mM $CaCl_2$ solution (ice-cold). Aliquots of 0.1 ml cell suspensions were prepared into each prechilled Eppendorf tubes and 1 µg of plasmid DNA was added to the cells. The cells were immediately frozen in liquid nitrogen and thawed by incubating the tubes in a 37 ºC water bath for 5 min. About 0.9 ml of the YEP medium was added to each tube and incubated at 28 ºC for 2-4 h with gentle shaking. This time period may allow the bacteria to express the antibiotic resistance genes. The suspension was spin for 30 sec in an Eppendorf centrifuge, supernatant was discarded and the cells were resuspended in 0.1 ml YEP medium. Finally, cells were plated on a YEP agar plate containing 50 mg/l kanamycin. The plates were incubated at 28 ^{0}C overnight and transformants were screened and plated again on YEP medium containing 50 mg/l kanamycin. Colonies were selected and glycerol stocks were made with transformed *A. tumefaciens* culture and were stored at -70 ºC. They were then streaked on disposable plastic Petri plates containing YEP agar to obtain single colonies using a sterilized platinum loop and the cells were allowed to grow at 28 ºC for 24 hours. Single colonies were selected and inoculated in liquid medium containing 50 mg/l of kanamycin and grown for 24-48 h on a gyratory shaker at 28 ºC and 180 rpm in dark. The plasmid was isolated by using Qiagen mini prep kit and the insertion of the genes was confirmed with different restriction enzymes (HindIII, EcoRI, BamHI, XhoI) to release *chitinase*, *bar*, and *osmotin* genes, and NptII marker.

3.12 Constructs used for genetic transformation

Plasmids used for the co-bombardment were pCAMBIA2300 double construct rice class I *endochitinase* gene and *osmotin* gene from *Solanum nigrum* and pPUR with *bar* gene from *Streptomyces hygroscopicus* that encodes phosphinothricin acetyl transferase (PAT) which is activated by acetylation and the active component of bialaphos, phosphinothricin (PPT) was isolated with a concentration of 1 µg/µl.

3.12.1 *Agrobacterium* mediated transformation using *osmotin* and *chitinase* genes in pCAMBIA2300 and pCAMBIA1300 with *bar* gene

Single colony from freshly grown culture of *Agrobacterium* with and pCAMBIA2300 and pCAMBIA1300 vector containing osmotin and chitinase and bar genes were inoculated into 50 ml of YEP broth (kanamycin 50 mg/l and rifampacin 100mg/l) and grown for a period of 24 h (0.8 O.D.) at 28ºC using an orbital shaker set to 200 rpm. Cells were pelleted and resuspended in MS basal medium. The embryogenic calli was suspended in Ms basal medium for 3 hours using shaker at 200 rpm and placed in *Agrobacterium* suspension and shaken gently for 20 minutes at 28ºC (200 rpm). After infecting the explants with *Agrobacterium*, the calli were placed on the sterile Whatmann filter papers to blot excess bacteria from the explants and to reduce the overgrowth. The explants were co-cultivated for 2 days on co-cultivation medium (MS Basal + 100 µM Acetosyringone). After 48 hours, the explants were washed with a solution of 300 mg/l cephotaxime for one and half hour hour. The calli were then transferred to selection medium containing 3 mg/l phosphinothricin + 0.1 mg/l TDZ in Ms basal. After a week, calli that were growing on selection media were further sub cultured into regeneration media (Ms basal + 2 mg/l BAP + 0.2 mg/l NAA). The explants were subcultured for every 10 days on the same media.

3.13 Particle-inflow-gun mediated genetic transformation

3.13.1 Plant materials and establishment of embryogenic calli for bombardment

Embryogenic calli were pre-cultured in MS medium containing 10 mg/l of 2,4-D,10 mM NaCl,50 mg/l tryptophan and 0.2 M mannitol and 0.2 M sorbitol (as an osmoticum treatment) for 24 h in the dark before bombardment.

Plasmid DNA of pCAMBIA2300 containing *osmotin* and *chitinase* genes and pCAMBIA1300 containing *bar* gene were isolated from *E-coli* cultures. Spermidine 100 mM stock was prepared and filter sterilized. Calcium chloride 2.5 M was prepared and autoclaved. Gold particles of 50 mg/ml was prepared by washing with 100% ethanol first, mixed and the particles were thoroughly washed with ethanol and then vortexed for 1min and allowed to stand for 1 min. It was again vortexed for 1 min and allowed for 1 min and the vortexing was repeated third time and the particles were kept for 40 min without disturbance. They were allowed to settle down and the supernatant was removed and the particles were resuspended in 100% ethanol. The ethanol wash was repeated for two more times and then the particles were washed with double distilled H_2O. Finally the particles were dissolved in 1 ml of double distilledH_2O. Tweleve µl of the DNA-coated microcarrier suspension was loaded into the center of a macrocarrier and used for bombardment. Before bombardment the forceps, scalpels, tips, Whatman No.1 filter papers were autoclaved.

3.13.2 Establishment of parameters for particle bombardment

Optimization of the physical parameters for particle bombardment was carried out under the following conditions, acceleration pressure (900, 1100 and 1350 psi), distance from rupture disk to the macrocarrier (3, 9 and 18 mm), distance from macrocarrier to target tissue (6, 9 and 12 cm), vacuum pressure (24, 26 and 28 in Hg), particle type (gold and tungsten), particle size (gold India meters of 0.6, 1.0 and 1.6 µm), coating agents (spermidine, $CaCl_2$ and both), number of bombardments (single and double) and time of partial desiccation prior to bombardment (0, 30 and 60 min). Other DNA and biological parameters included were plasmid type (pCAMBIA 2300 and pCAMBIA 1300), DNA concentration (0.5, 1.0, 1.5, 2.5 and µg per bombardment), tissue type (embryogenic callus, somatic embryos, shoot tips), osmoticum type (mannitol, sorbitol, sucrose and glucose) and osmoticum concentration (0.0, 0.2, 0.4 and 0.6 M mannitol). Plasmid DNA was precipitated into gold or tungsten particles and bombarded according to the protocols supplied for the Biolistic PDS-1000/He particle delivery system (BioRad, USA) with minor modifications. While vigorously vortexing, 50 µl of particle solution (prepared in 50% glycerol), 10 µl of DNA, 50 µl of 2.5 M $CaCl_2$ and 25 µl of 0.1 M spermidine and 20µl of double distilledH_2O were added and the mixture was vortexed for 2 min. The microcarriers were allowed to settle for 4 min and then pelleted by spinning for 3 s in a microfuge. After removal of the upper liquid, 12 µl of the DNA-coated microcarrier suspension was loaded into the center of a macrocarrier and used for bombardment. The pellet was washed twice (without vortexing) with 140 µl of 100% ethanol. After adding 82 µl of 100% ethanol and resuspension of pellet by vortexing, 12 µl of the DNA-coated microcarrier suspension was loaded into the center of a macrocarrier, air dried and used for bombardment.

3.13.3 Shoot regeneration and selection of putative transgenics

After bombardment with pCAMBIA2300 double construct and pPUR with *bar* gene, immature inflorescence derived embryogenic calli were cultured for 2 weeks on MS basal medium supplemented with 6-benzylaminopurine (2 mg/l) and napthaleneacetic acid (NAA 0.2 mg/l) without phosphinothricin (PPT). The calli were subcultured for every 10 days on the same media. The calli were subcultured two times onto the same medium but without any selection pressure during regeneration. This is necessary to allow the calli to regenerate. Then, the selection strategy was employed for putative transgenics by selecting them on the PPT containing medium.

3.13.4 Rooting and acclimatization of transformed shoots

The shoots selected on an antibiotic medium (phosphinothricin) were transferred to MS basal medium containing 0.2 mg/l IAA for initiation of roots. Shoots with well developed roots were then transferred to pots under controlled environmental conditions. Plants that attained a height of 5-10 cm and well developed roots were removed from the bottles/tubes and washed gently under running tap water to remove adhered agar and then planted into plastic buckets/cups containing vermiculate and vermicompost in a ratio of 3:1. The cups were covered with a plastic sheet to maintain humidity and were put in a glass house. The plants were covered with poly bags for 25-30 days after which they were slowly exposed to the ambient environment and transferred to clay pots with a potting mixture of sand: soil: dry powdered cow dung (1:1:1) to the glasshouse.

3.13.5 Basta test for transgenics

One hundred µl of basta was diluted by adding 1000 µl of distilledH_2O and the leaves of transgenic and untransformed controls were collected from the net house. They were then dipped in the solution and observation was taken up to 5 days.

3.14 Molecular characterization of transformed plants

3.14.1 DNA Isolation

Total plant genomic DNA was isolated according to the protocol described by Dellaporta (1983). About 5 g of young leaf tissue was homogenized in liquid nitrogen and the powder was mixed in 20 ml of preheated (65 ºC) SDS buffer [0.1 M Tris-HCl, 0.02 M Na_2EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol, 2% hexadecyl trimethyl ammonium bromide (CTAB) pH 8.0] and 20 µl of proteinase K (10 mg/ml) in 50 ml capacity polypropylene centrifuge tubes and incubated at 37 ºC in a water bath for 1 h with occasional gentle swirling. The samples were mixed with phenol : chloroform : isoamyl alcohol (25:24:1) and the contents were centrifuged and the aqueous phase was transferred to a fresh centrifuge tube and mixed well with double volume of ethanol and 3 M sodium acetate by gently inverting the tube at least six times. This mixture was kept at -20 ºC for 30 min to allow complete precipitation of DNA and then centrifuged at 10,000 rpm for 10 min to pellet the DNA. The pellet was washed with wash buffer (70 v/v ethanol). The DNA was dissolved in 1 ml $T_{50}E_{10}$ buffer (10 mM Tris-HCl and 1 mM Na_2EDTA, pH 8.0) and the contents were transferred to 2 ml microfuge tubes. The dissolved DNA was treated with 10 mg/ml RNase A at 37 ºC for 1 h. The samples were mixed with equal volumes of chloform: isoamylalcohol (IAA) (24:1) and mixed thoroughly by inverting the tubes at least for five times and the contents were centrifuged for 10,000 rpm for 10 min. The aqueous layer was transferred into fresh tube and mixed well with double the volume of 100% ethanol for precipitation of DNA and then centrifuged at 10,000 rpm for 10 min to pellet the DNA. The pellet was washed with wash buffer (70 v/v ethanol). Finally, DNA was dissolved in 500 µl $T_{10}E_1$ buffer. The concentration and purity of DNA samples were checked by using UV-VIS spectrophotometer. The following formula was used to calculate the concentration of DNA, (A260 X 50 X dilution factory)/1000 (µg/ml). DNA from the transformed plants was isolated (from the leaf tissue) and was used as template to obtain the amplification product of *bar* gene and *osmotin* and *chitinase* genes by using corresponding primers.

3.14.2 PCR analysis

Primers List:

I). Primers for the chitinase gene (620 bp):

Chi F: 5'ACCGCAACGACCAGGCGT 3'

Chi R: 5' GCCCAGCATGTCGCAGTA 3'

II). Primers for the bar gene (530 bp):

Bar F: 5' CTACCATGAGCCCAGAACG 3'

Bar R: 5' TCAGATCTCGGTGACGG 3'.

III). Primers for the osmotin gene (750 bp):

Osm F: 5’ ATGGGCTACTTGAGATCTTCTTTT 3’

Osm R: 5’ TTACTTAGCCACTTCATCAGTACT 3’

IV). Primers for osmotin gene and chitinase gene (1.5 Kb and 1.6 Kb):

CamV 35S promoter: 5’ ATGGTGGAGCACGACACTCT 3’

Poly A terminator: 5’ ACTGGTGATTTGCGGACTCT 3’

PCR reaction was setup by using standard PCR reaction conditions by following the Molecular Cloning Manual by Sambrook and Russel (2001) and the reaction was run in a thermocycler (Perkin Elmer - 2400). The components of PCR were template DNA, gene specific primers, dNTPs, Taq buffer, $MgCl_2$ buffer and Taq DNA polymerase. The reaction volume for all PCR reactions was set to 25 µl. PCR amplifications were carried out starting with an initial denaturation at 94 ºC for 5 min, denaturation at 94 ºC for 30 seconds, annealing at 60 ºC for 45 seconds for bar primers and 59 ºC for 45 seconds for chitinase primers and 58 ºC for 45 seconds for osmotin primers and 61 ºC for 45 seconds for CMV35S and PolyA and extension at 72 ºC for 45 min. These steps were repeated for 35 cycles followed by a final extension for 5 min at 72 ºC. The reaction mixture without a template was run as a negative control. Positive controls for bar gene and chitinase were also included. Amplified DNA fragment was separated by gel electrophoresis in 1% agarose gel and images (Biorad Gel documentation) were stored after staining with ethidium bromide. Whenever required, the PCR products were purified by using QIAGEN quick PCR gel extraction kit and stored at –20 ºC until further use.

3.14.3 Extraction of total RNA

Plant material (0.2 g) was taken in a pestle and mortar (treated with DEPC) and liquid nitrogen was added and ground to a fine powder. Total RNA from transformed tissue and untransformed controls were extracted using TRI reagent according to the manufacturer’s instructions (Invitrogen, USA) or according to Sachi protocol. Finally, the pellet was resuspended in 40 µl of RNase-free water (DEPC treated) and stored at -70 °C until further use. The concentration and purity of RNA samples were checked by using UV-VIS spectrophotometer.

3.14.4 First strand cDNA synthesis and reverse transcriptase PCR of chitinase and bar genes

An amount of 5 µg of total RNA was taken for first strand cDNA synthesis using oligo-dT (20) by using M-MuLV reverse transcriptase (MBI Fermentas, Germany), following manufacturer’s instructions. After the first strand cDNA synthesis, the reaction was terminated by heat inactivation at 70 °C for 10 minutes. Polymerase chain reaction was performed for the amplification of specific chitinase gene sequence using gene specific primers. A total volume of 25 µl of PCR mix was prepared in a sterile 0.2 ml Eppendorf tube with 10 pmol/µl each of both forward and reverse primers, 0.2-2 µl of first strand cDNA as a template, 50 µM of each dNTP, 1.5 mM $MgCl_2$ and 2.5 U of Taq DNA polymerase. Each PCR aliquot was mixed and the PCR reactions were performed in Perkin Elmer machine (Waltham, Massachusetts 02451, USA) or Eppendorf Mastercycler Gradient (Germany) and / or MJ Research Inc., (USA). The standard reaction conditions carried were initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 ºC for 45 s for Bar primers and 59 ºC for 45 s for chitinase

primers and, extension at 72 °C for 90 min and a final extension of 10 minutes at 72 °C. An aliquot from the amplified PCR product was used to analyze on 1% agarose gel to check the amplification.

3.14.5 Southern blotting analysis

For Southern hybridization analysis, the genomic DNA (15-20 µg) from each of the putative transformants were separately digested with HindIII for the chitinase in pCAMBIA2300 and HindIII and EcoRI that has restriction sites for the *bar* gene which was in pPUR vector. The digested DNA was run on 0.8% agarose gel in two separate gels and the DNA fragments that were transferred onto nylon membranes (Hybond N+, Amersham Biosciences, UK) using standard protocols (Sambrook and Russel, 2001). The labeling, hybridization and detection methods were performed according to the manufacturer's instructions. The gene integration was confirmed by digesting the pCAMBIA2300 with chitinase and pPUR Bar plasmids with HindIII and EcoRI restriction enzyme, which served as a positive control. DNA was isolated from untransformed plants (tissue culture raised) and were loaded which served as negative controls.

3.15 Statistical analysis

All the experiments were repeated two times and the data represent an average of 10 replicates. Percentage frequency for each concentration was carried out with Sigma plot. The data pertaining to number of embryos, maturation and conversion were subjected to analysis of variance (ANOVA) test using M-Stat software. Mean values were calculated using the Sigma plot software.

3.16 EMS mutagenized population development and standardisaion of TILLING technique

3.16.1 Preparation of ethyl methane sulphonate (EMS)

Different concentration of EMS was prepared in milli molar concentration based on the literature search. These concentrations were used for developing Targeting Induced Local Lesions in Genome (TIILING) populations in other crops; for instance maize (Till et al. 2004), wheat (Slade et al., 2005), barley (Caldwell et al., 2004), tomato (Menda et al., 2004) and lotus (Perry et al., 2003).

3.16.2 Optimization of mutagen concentration

In order to optimize the mutagen concentration, initially 50 seeds of P1449-2-B1 genotype were treated with different concentration of EMS i.e.,5 mM, 10 mM, 15 mM, 20 mM, 25 mM, 30 mM, 35 mM, 40 mM, 45 mM, 50 mM, 55 mM and 60 mM, for a period of 16 hours. The seeds were soaked in the EMS solution and transferred to the 12" plastic pots containing soilrite and maintained in glasshouse. Survival and establishment of 50 percent seedlings (LD50 value) was chosen as a criterion to find the optimum concentration of EMS for developing TILLING population. Out of the above concentrations, 5 mM and 16 hour duration treatments were found to be lethal, hence,

different durations of treatment at 4 h, 8 h and 12 hour and concentrations of EMS at 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, and 10 mM were used for the second set of experiments. Now 100 seeds for each concentration and at different time intervals were soaked and transferred to 12" plastic pots containing soilrite and were maintained in the glass house. Based on the results of second experiment, three mutagen (EMS) concentrations i.e. 5 mM, 7 mM and 10 mM each with 4 hour treatment duration were selected for mutagenesis.

3.17 Generation of M1 lines

In order to generate the first set of TILLING lines, 2000 seeds were treated with each of three mutagen concentrations i.e. 5 mM, 9 mM and 10 mM with 4 h treatment. The treated seeds were sown in 12" diameter pots in glass house and after two weeks, the 18-day-old seedlings were transplanted in the field. A total of 4218 seedlings (1566 of 5 mM, 1385 of 9 mM, and 1267 of 10 mM) with 1721 seedlings of control were transplanted to field containing 36 ridges of 100 m long with a spacing of 30 × 75 cm. Variation for the number of mutant lines established after transplanting in the field was observed. From a total of 4218 seedlings transplanted, only 2744 M1 lines (1120 from 5 mM, 784 from 7 mM and 840 from 10 mM EMS concentrations) reached to maturity. These M1 lines were selfed and seeds were harvested at maturity.

3.18 Field arrangements

The 2 m length, with 0.5 m /0.75 m spacing between each block was made on 56 ridges of 150 m length for M1 lines and for M2, 30-50 ridges of 100-200 m long with a spacing of 30 × 75 cm was prepared to prevent cross pollination. Inially sufficient amount of DAP was added to the field before sowing. The block length varied from field to field based on the population size. The spacing between the lines was maintained in the field to reduce the cross pollination of the plants. At the time of flowering, fertilizers were added sufficiently. For every two days, watering was done with the high throughput pipes. After sowing, the seedlings were maintained carefully from rats and birds in the field.

3.19 Advancing of M1 lines to M2 generation

One M2 line from each M1 line, approximately 20 seeds from 2744 M1 lines each, were sown after taking into consideration all possible losses for e.g. embryonic lethality, sterility etc. Each individual M1 line was sown in a block of 2 m length, with 0.5 meter spacing between each block made on 56 ridges of 150 meter length. Two rows of control were taken up before and after every treatment. There was no germination in case of few M2 lines. Standard agronomic practices were followed during the crop period as per the recommendations for the crop.

3.19.1 Selfing

From each M2 line, one healthy plant was selected, tagged and labelled, from which leaf sample was collected for DNA isolation and was selfed. The rain-proof paper pollination bag (Lawson Bags, Northfield, IL) to prevent cross pollination was covered to a panicle of earlier tagged plant and labeling was carried to that bag. Selfed panicle from each M2 line was harvested separately during the second week of May. After bagging, each bag was injected with 5 ml of chloropyrifos (Dow AgroSciences) at 0.5 ml/liter to control corn earworms that could hatch within the bag and

destroy the seeds. DNA was isolated from one seedling per each M2 line out of the 20 seeds sown. For isolating the DNA from each M2 lines, leaf samples were collected from two-week-old seedlings which were tagged. A high throughput DNA extraction protocol suggested by Mace et al., (2003) was followed to isolate DNA from each individual M2 line. However, in most of the M2 lines the DNA isolated through high throughput method was found missing, may be due to the recalcitrant nature of the pearl millet. Hence, we isolated DNA from all 2,744 M2 lines following CTAB maxi preparation as per Saghai-Maroof et al., (1984). The quality of these genomic DNA was checked on 0.8% agarose gel. The quantification of individual genomic DNA was carried out using Shimadzu spectrophotometer**.**

3.19.2 DNA isolation by CTAB method

Leaves of P1449-2-P1 mutant lines were collected, frozen in liquid nitrogen, and stored at -70 ºC until use. Nearly 0.5 g of leaves was grind using mortar and pestle in the presence of liquid nitrogen. The frozen ground leaf tissue was transferred to 15 ml polypropylene centrifuge tubes. Then, 5 ml of 60 ºC extraction buffer and 50 mg polyvinylpolypyrrolidone (PVP), 0.5 g leaf tissue were taken. They were mixed by inversion and incubated in a 60 ºC oven (with shaking) for 60 minutes. It was removed from oven, and cooled to room temperature for 4 to 6 minutes. Six ml of chloroform: ethanol (24:1) was added and mixed by inversion to form an emulsion. After mixing thoroughly, it was spun at 3000 rpm for 20 minutes in a tabletop centrifuge at room temperature. The top aqueous solution was transferred to new 15-ml centrifuge tubes using wide-bore pipette tip. Chloroform-ethanol extraction was repeated to remove cloudiness (PVP) in aqueous phase. Half of the volume of 5 M NaC1was added to the final aqueous solution recovered. They were mixed well. Two volumes of cold 95% ethanol was added to this. It was mixed by inversion. If required, it can be placed in freezer at -20 ºC for 10 minutes to accentuate precipitation. The solution was left at 4 to 6 ºC to precipitate overnight. It was spun at 3000 rpm for 6 minutes. The supernatant was poured off, and pellet was washed with cold (0 to 4 ºC) 70% (v/v) ethanol. The pellet was dried in a 37 ºC oven or vacuum for ~1 hour. It was dissolved in 300 µl TE overnight at 4 ºC and transferred to 1.5-ml eppendorff tubes. Three µl RNase A (10 mg/ml) was added and incubated in a 37 ºC water bath for approximately I hour. Three µl proteinase K (l mg/ml) was added, and incubated at 37 ºC for 15 to 30 minutes. One hundred and fifty µl of phenol and 150 µl of chloroform were added to each eppendorff tube. They were vortexed briefly, and spun (in microfuge) at 14,000 rpm for 10 to 15 minutes. The upper layer was collected in new 1.5 ml tubes. Fifty µl TE was added to phenol phase. It was vortexed, spun and the upper layer was removed, and sample was added. To this 1/10 volume 3M sodium acetate and 2 volumes of absolute ethanol were added and mixed. It was left overnight in a freezer (-80 ºC) and was spun at 14,000 rpm for 10 to 20 minutes. It was drained and washed with 70% (v/v) ethanol. Ethanol was removed, and the tubes were vacuum dried. Hundred to 200 µl TE was added and the time was allowed for complete resuspension. DNA concentrations were measured using a fluorescence spectrophototmeter.DNA was isolated from all 2,744 M2 lines following CTAB maxi preparation as per Saghai-Maroof et al., (1984). The quality of these genomic DNA samples was checked on 0.8% agarose gels. The quantification of individual genomic DNA was done using a spectrophotometer and the samples were diluted to 5 ng/l.

3.20 Generation of new set (second set) of M1 lines

Seed germination of M2 population was not even 50 percent at 9 mM EMS. So, to increase the percentage of germination, the mutagen concentration was standardized. The seeds were treated again with concentrations 6 mM, 6.5 mM, 7 mM, 7.5 mM, 8 mM, 8.5 mM, 9 mM EMS. Based on the germination percentage results in the field, the EMS mutagen concentration for the TILLING population was decided as 7.5 mM. In order to enhance the existing mutant population, another set of 10,000 seeds of the inbred line "P1449-2-P1" were treated with 7.5 mM EMS. The mutagenized seeds were sown in pots and transplanted on to the field. Though the inbred line (P1449-2-P1), chosen for generation of TILLING population is a donor parent for downy mildew resistance for the mapping population at ICRISAT (PT 732B × P1449-2), some M1 lines were found susceptible to downy mildew. Hence, the new set of M1 lines generated will be potential candidates to look for allelic variants for downy mildew resistance genes which could help us to understand downy mildew resistance. In total, 2400 new M1 panicles were harvested. Of the 2400 M1 panicles harvested, there was no seed formation in 162 panicles. Approximately, 10 seeds per each M1 line were sown in blocks of 0.5 meter long and with a spacing of 0.75 meter between each block. Of 2248 M1 lines sown in the field, 2074 M2 lines germinated. Only three seedlings per each M2 line were retained and genomic DNA was harvested from one seedling of each M2 line using Neucleo-Spin® 96 Plant Genomic DNA Extraction Kit (Macherey-Nagel, Germany). The genomic DNA thus isolated was quantified using PICOGREEN and normalized to 5 ng/µl using TECAN robotic work station.

3.20.1 Collection of seeds from panicles

The labeled paper pollination bags containing panicles of mutant lines were harvested from the field and were taken to crop work area. Then each panicle was taken separately and crushed manually with the help of labor. The seeds collected from panicles were cleaned thoroughly and were stored in yellow packets which were used only for storage of seeds. The labeling was done properly with bold marker. These seed packets were stored in ICRISAT pearl millet cold storage room for long-term storage.

3.21 Generation of additional mutant lines (third set) to meet ideal population size for TILLING candidate genes

Assuming a population size of 10,000 M2 lines to be ideal for mining allelic variants in drought responsive candidate genes, another set of 15,000 seeds of the inbred line "P1449-2- P1" were mutagenized using 7.5 mM EMS. The mutagenized seeds were sown in pots initially and transplanted on to the field. Of the 15,000 lines, 9600 M1 lines were selfed and harvested and these 9600 M1 lines were sown for M2 population. They were sown in blocks of 0.5 meter long and with a spacing of 0.75 meter between each block in 2008 summer, 2008 Kharif and 2009 summer seasons to reach the target population size.

3.21.1 DNA isolation of M2 lines by high through put Macherey-Nagel kit

Reagents of Macherey-Nagal Kit

PE buffer, PW2 buffer, RNase A, 96 well plate containing 8 tube strips, PL1 solution, PL3 solution, PC buffer, MN

square well block, binding plate,PW1 solution,PW2 solution, PE buffer, 96 well V bottomed plate for DNA storage.

Protocol for isolation of DNA

Before starting the extraction, the incubator or oven was set to 65 ºC. The buffer PE was equilibrated to 70 ºC. Buffer PW2 was prepared and also RNaseA solution. The plant tissues were collected from the young leaves of mutant lines. The tissue was cut into small pieces and 100 mg of plant tissue was added into each 8 well tube strips of a 96 well plate. Two steel beads were added to each tube, the tubes were closed with caps provided for the strips. Cells were disrupted by vigorous shaking using a genome mixer. It was centrifuged for 5 min and the caps were removed. Five hundred µl buffer PL1 and 10 µl RNase A were added to each sample. Tubes were closed again, mixed vigorously and spun briefly for 30 sec at 2500 rpm to collect any sample from the cap strips. The samples were incubated at 65 ºC for 30 min and 100 µl buffer PL3was added and mixed thoroughly and incubated for 5 min on ice to precipitate SDS completely. The samples were centrifuged for 20 min at full speed (6000 rpm), the cleared lysate of the each sample was collected and 45 µl of binding buffer PC was added to each well of an MN square well block and mixed by repeated pipetting up and down. It was mixed at least 3 times. The samples were transferred to binding plate which contains silicon membrane and it was centrifuged at 6000 rpm for 5 min. Through the solution 400 µl PW1 was added to each well of binding plate and plates were sealed with gas permeable foil and the tubes were centrifuged at 5600 rpm for 2 min. Then 700 µl PW2 was added to each well and again centrifuged at 5600 rpm for 2 min. The above step was repeated. Hundred µl pre-warmed buffer PE was dispensed to each well directly onto the membrane. It was incubated at room temperature for 2 min and centrifuged at 5600 rpm for 5 min and the DNA was collected in 96 well V bottomed plates and stored in at -80 ºC until further use.

3.22 Agarose gel

The DNA extracted was checked on 0.8% agaraose gel to see the quality and if the DNA is missing or could not be extracted in any sample.

Gel preparation

Agarose (0.8 g) was taken in a conical flask and to this 100 ml of 1X TBE was added and boiled in a oven for 2 minutes until the powder was mixed properly in TBE. The mixture was cooled and 0.2 µl of ethidium bromide was added, mixed thoroughly and was poured in a casting tray which was sealed with tape and combs were adjusted to the marking of the plate. The plates were left for solidification under room temperature. After solidification, 5 µl of DNA samples (2 µl of DNA plus 3 µl of water) were loaded in the wells and the samples were run in gel electrophoresis unit at 90V for 20 min. Then the gel was checked in Gel documentation instrument under UV light exposure and the gel picture was saved in JEPG format in system connected to Gel doc.

3.23 Chemicals for normalization and dilution

T : E (10:1), picogreen, 96 well Tecan plates, washing reagent (hypochlorox), double distilled H_2O 96 V bottomed plates, 1 ml pipette, 200 µl pipette, multi channel pipette.

3.23.1 Normalization of the DNA by high through put quantification method

The PicoGreen stem plates were prepared. An aqueous working solution of the PicoGreen reagent was also prepared by making a 1:200 dilution of the concentrated solution in TE (1:10) buffer. It is recommended to prepare the working solution in a plastic container as the reagent may adsorb to glass surfaces. For best results, this solution should be used within a few hours of its preparation. The prepared PicoGreen working solution was poured into a block module intended for 96 well reactions which will dispense 99 µl of the PicoGreen solution into each well. The solution left over in a falcon tube was stored in the -20 ºC freezer. The method "**Magellan_2**_Files Measuring parameters were set to run and reading of the samples was started in Fluorospectro phototmeter (TECAN make). Ninty six well format page is then opened. The reading was copied and saved in an EXCEL sheet (CSV format) in folder D. The concentrated DNA was diluted in the 3:1 ratio and transferred to 1 µl of DNA to the PicoGreen module plate. It was made sure that none of the samples was empty due to evaporation. The plates were incubated for 2 to 5 minutes at room temperature, and protected from light. The samples were taken and the samples were run using the method Magellan_2. Again 96 well format pages with the concentration of DNA were opened. The reading was copied and saved in the same EXCEL sheet where the PicoGreen readings were already saved. Similarly, the normalization was carried out for all 113 plates of DNA isolated in different seasons.

3.24 Dilution of the DNA

The dilution of the samples was carried out with robotic liquid handling machince/automated pipetting station. *The Nanodrop™ II and the Safire2 on board the Genesis 200* are manufactured by TECAN company. The CSV file was converted to GWL file and the file was uploaded in LIMS, Bioinformatics tool, ICRISAT, for the concentration of 5ng/l DNA . Then, the 96 well plate containing the isolated DNA and the new plate for dilution of DNA was kept ready in liquid handling machine/automated pipetting station. First water was changed and water was filled in the system liquid container. The wash buffer hypochlorox (50 µl chlorox + 100 ml water) was filled in one basket and in the other, double distilled water was filled. The Gwl files as uploaded to the system in Folder D and software tool **Gemini** was opened. Then normalized option was selected and clicked on work list, and the GWL file was selected to carry out dilution. New page was opened for dilution, first the flush was selected and this flush was repeated 3 times so that the eight channel liquid handling arm pipette is washed and the start for dilution was selected. The DNA was diluted to 5 ng for all the 113 plates of isolated concentrated DNA to prepare the pooled plates and the plates were stored at -20 ºC. This was carried out for all the 113 normalized plates. For each plate, the source plate was changed and new plate for dilution, the GWL file was uploaded for source plate.

3.25 Phenotypic variants observed in new set of M2 lines generated during the years 2006, 2007, 2008

The mutant lines sown in field were regularly surveyed for morphologically interesting phenotypes. Although, a broad range of phenotypic variants were observed in mutant population generated during 2006, the spectrum of phenotypic variants in other set of mutagenized population was mostly confined to chlorophyll mutants.

3.26 Establishing a DNA bank of TILLING populations (DNA pools)

In order to increase the throughput during allele mining, eight-fold pooling of normalized genomic DNA from a set of 9,938 (2694 mutant lines generated in 2006, 2074 lines in summer 2007, 2791 lines in Kharif 2008 and 2379 lines in Summer 2008) M2 lines were accomplished. The strategy of DNA pooling is as depicted in the Figure 25.

3.27 Pooling

Multi channel pipette, 96 well V bottomed plates, thick films, -80 ºC Freezer are needed for pooling. Sixty-four samples are arrayed into an 8 x 8 grid on a 96-well plate (leaving the last well for control). The samples were pooled using an eight channel pipette, and the combined samples from each of the 12 rows on the plate are put into a single column of the pooled plate. The DNA from each plate (8 source plates) bearing the well ID A01 were pooled into destiny plate bearing the well ID A01. This ensures the position of A01 of the pooled plate which contains samples A01 from normalized plates 1 to 8 of individual plate. This pooling strategy produces 12 pooled columns from 8 plates. This arraying protocol allows the screening of 768 different individuals in a single assay. Tracking a putative mutation from a pool to an individual sample is straightforward. For instance, if a mutation is found in position B2 of the 96-well pool plate, the eight samples that contributed to this pool are found in samples of B2 from 1-8 plates. The missing DNA in each well was replaced by the other DNA. This DNA plate was used separately only for filling the missing samples. The gap filling was carried out before pooling and the plates were covered with thick film and stored in -80 ºC. Thus, a total of 12 pooled plates, 3 plates from the DNA isolated from the initial set of mutagenesis experiments, 4 pooled plats from mutant lines generated during 2007 and 5 from 2008 were prepared.

3.28 Laboratory Information Management Systems (LIMS)

Further, all the plate records were uploaded into LIMS (Laboratory Information Management Systems) followed at ICRISAT for data storage and retrieval. In LIMS, the data were stored in separate options like TILLING Mother Plate (TMP), TILLING Normalized Plate (TNP), TILLING Pooled Plate (TPP), and thus, the pools of DNA from mutant population is made available for international pearl millet community for allelic mining of candidate genes.

3.29 Seed storage of the population

The seeds of the 9,938 lines of pearl millet were stored in a cold storage room where the temperature was maintained at -20 ºC. M2 seeds were stored in 21 iron boxes with proper labeling and the M1 seeds were arranged in Thermocol boxes and the information was uploaded in **TWIKI** software at ICRISAT bioinformatics center.

3.30 Establishment of infrastructure for allele mining

Determination of allelic variants for a potential candidate gene of interest in a TILLING population is feasible using a LI-COR 4300 DNA analysis platform. Modules installed will provide analysis of TILLING/EcoTILLING samples.

3.31 Initiation of allele mining in *DREB2A*

For allele mining, a literature search was initiated to identify candidate genes for drought tolerance and downy mildew resistance in pearl millet. As a result of our search for drought tolerance candidate genes, *DREB2A* (>gi|77863910|gb|DQ227697.1| *Pennisetum glaucum* transcription factor *DREB2A* gene, complete cds) was chosen and sequences were downloaded from NCBI (**Error! Hyperlink reference not valid.**). A set of 8 nested primer pairs were designed to amplify the complete gene. The primers were designed using Primer 3 program22 with melting temperatures from 57 to 67 ºC and of a length in the range of 12–16 nucleotides.

3.31.1 PCR

After DNA extraction and concentration normalization, the optimal amount of genomic DNA to use for PCR should be determined empirically, by following the standard TILLING protocol with varying amounts of genomic DNA. A concentration was chosen that provides the best signal-to-noise ratio when a fixed amount of enzyme is used for heteroduplex digestion. A higher concentration is required when lower quality genomic DNA samples are used. Amplification conditions were optimized on P1449-2-P1 genotype. For these primer pairs, different picomole concentrations of DNA, different molar concentrations of dNTP's, magnesium chloride with buffer and different units of Taq DNA polymerase were used for standardization of PCR and a few TILLING lines. The nested primer pair combinations were able to generate PCR amplicons that give complete gene coverage. However, few primer (3, 8, 9 and 14) combinations did not give any amplification. The generated amplicons were sequenced and then the primer pairs have been selected to get maximum length of the gene.

3.31.2 *DREB2A* - Different primer combinations and their attachment site (bp)

Forward F1:Bases:
5'GCCATGGTGCAATTGACTGAAGT 3'
Reverse R1:Bases:
5'CCGCAAAATGACCCAACAAATGTACC3'
Forward F2: Bases:
5'CGCGTGATGGGCCGACCTC 3'
Reverse R2: Bases:
5'GCTCCATGCCGCCACCAG 3'
Forward F3: Bases:
5'TCTGGGCATCTGTGGGTACCTGT 3'
Reverse R3: Bases:
5'CCGTCTTATTCGAGCTGGATGCA 3'
Forward original (F0)
F' 5' CCGGAATTCATGCAGTCCTTGACTGATGG 3'
Reverse original (R0)

R’ 5’ CCGCTCGAGCAGTTCCCTGACTACAGGC 3’

3.13.3 DREB2A gene and designed primer details

>gi|77863910|gb|DQ227697.1| *Pennisetum glaucum* transcription factor *DREB2A* gene, complete cds

ATGCAGTCCTTGACTGATGGTGTTGTTGTGACCAGCATTCGGTTAGTTTTGTCTTGTTCATCTCCTACAT
ATTTTTACCTGCTAACTTTGTATGTGGAGTTGCCATGGTGCAATTGACTGAAGTAATATCTGTTGCTGC
TAAGATGGTTTTGTCCTTAATGTGTTCGAAACTCAATTGTAGATTGTTTAATGAGTGAAATGCTATCTG
TGCGTACGACTTCTGATCAAAACACAACTAACAGCGGGTTAGAAGCTAAATAAGTAAATAGTATCTGT
TTCTGTGGAGATGATTGGCATGTAGGAACATCTGGGCATCTGTGGGTACCTGTGTGTGAAATAGTAAC
CTGTTTCTGTGGAGATGATTGTCATGTAGGAACATCTGGACATTTGTGGCTGGTAGTAGATTGAAGTAG
TAACGTTGTTTCAATTTGGTTGAGCTTGCTCTCTACTTATTCATTAAACAGTCCCTGTTAGTCTTTTCTG
TTTAACATGGTACATTTGTTGGGTCATTTTGTTTTATTGTGTACTTGTGCTTACAAATCTGACAGAGATA
CTAAAGTGTTTCTTGTCATTTAGCAGGAAGAAGCGTCCTCGCAGATCGCGTGATGGGCCGACCTCGGT
GGCAGCTGTCATCCAGCGGTGGGCTGAGCACAACAAGCAGTTGGAGCACGATTCTGATGGCGCGAAG
CGACCAAGGAAAGCTCCTGCTAAGGGTTCAAAGAAGGGCTGCATGAAGGGAAAAGGAGGGCCTGAG
AATACGCACTGTGGATACCGCGGAGTGAGGCAGCGTACTTGGGGTAAGTGGGTTGCTGAAATCCGAG
AGCCGAATCGGGTCAACAGACTCTGGCTGGGGACCTTCCCAACTGCAGAGGATGCAGCCAGGGCTTAC
GACGAGGCTGCCAGAGCGATGTATGGAGAACTCGCCCGCACGAACTTCCCCAGTCAGAAAGCAGTGG
CCTCTAGCCAAGCTGCTCGGGTTCCAACCCCTGCCCAGGTTGCTCCAGCAGCTGTTGAAGGTGTTGTAC
CTAGCACATCATGTGAGTCAACGACAACATCAAATCACTCAGATGTTGCATCCAGCTCGAATAAGACGG
GAAGCATCTGACATGTCAAGCTCTGTGAAGGTGGAGTGCCCAGAAGCTGTGGGTGCTGGTTCACATAG
GTCTGAGATGGTATCTGGCATTTCGCATCAGCATGAAGACAGTCATCCTAGTATCCAAGCTAGCACAC
CCAATATAAGTGACAAGGAAGTATTCGAGCCACTTGAACCTATTGAAAACCTTCCAGAGGGTGATTTT
GATGGTTTTGATATTGATGAGATGTTGAGAATGATGGAAGCTGATCCGCAGAATGAAGGTGGTGCTGG
TGCTGGTGCTGGTGCTGGTGGCGGCATGGAGCAGACCTTCTTCTTTGATGGGTTGGATCCGAATCTACT
GGAGAGCATGCTCCAGTCAGAGCCAGAGCCATACTCCCTATCTGAGGAACAGGACATGTTTCTTGCTG
GCTTTGAAAGTCCTGGTTTTTTTGAGGGCCTGTAGTCAGGGAACTG

3.31.4 CODDLE

The web-based program CODDLE (http://proweb.org/coddle) was employed to select gene regions that have the highest density of potentially deleterious mutations in the *DREB2A* gene caused by treatment with chemical mutagens. Primers were designed by CODDLE using the Primer 3 program22 with melting temperatures from 67 ºC to 73 ºC and of a length in the range of 20–30 nucleotides and the order was placed for IR labeling primers to MWG. According to the COODLE results, the primers 1 and 3 combinations which was used for amplification was selected for IR labeling. A 100 mM solution of each primer was prepared in TE (10:1) buffer and aliquots were stored at -80 ºC to avoid repeated freeze thaw cycles that could reduce fluorescent activity.

3.32 Amplification of candidate gene

Optimization of PCR amplification on the pooled mutant DNA sample using IR labeled primers was accomplished using *EcoTILLING Kit*™ obtained from Frontier Genomics. Allele mining for the available mutant DNA samples was initiated.

3.33 Mutation screening of *DREB2A* gene in pooled plates

3.33.1 PCR components

Double distilled H_2O, 10X Taq buffer, 25 mM $MgCl_2$, 3 pico moles of unlabelled forward primer, 3 pico moles of unlabelled reverse primer, 10 mM deoxynucleotidetriphosphates (dNTPs), 5 units of TaqDNA polymerase, 5 pico moles of IR-labeled 700 forward primer, and 5 pico moles of IR-labeled 800 reverse primers.

Primer mix: (1:1:1:1)

5 pico moles of unlabelled F0 forward primers

5 pico moles of unlabelled R0 reverse primer

5 pico moles of IR-labeled 700 F3 forward primer

5 pico moles of IR-labeled 800 R3 reverse primer

3.33.2 Optimized PCR protocol

In step -1 PCR : PCR amplification was carried out in a 25 µl volume containing 5ng of pooled DNA, 10X Taq buffer (including 1mM $MgCl_2$), 25 mM $MgCl_2$, 10 mM deoxynucleoside triphosphates (dNTPs), 3PM unlabelled forward, unlabelled reverse with F0R0 primer combination and 1 unit of TaqDNA polymerase (Takara Biomedicals, Cambrex Biosciences, Wokingh AM, UK.).

In step -2 PCR : PCR amplification was carried out in a 25 µl volume containing PCR-1 product as template DNA, 10X SibTaq buffer (including 1 mM $MgCl_2$), 25 mM $MgCl_2$, 10 mM deoxynucleoside triphosphates (dNTPs), 5 PM primer mix (unlabelled forward, unlabelled reverse, IR-labeled forward at 700 and IR-labeled reverse at 800 in 1:1:1:1 ratio), and this primer mix was also optimized with different ratios of labeled and unlabelled combination and 1 unit of Taq DNA polymerase (Takara Biomedicals, Cambrex Biosciences, Wokingh AM, UK).PCR cycling was performed as described in Colbert et al., (2001). PCR amplification was standardized with different conditions like target PCR, touchdown PCR and touch-up PCR at different annealing temperatures and at different times in

annealing and extension steps. For *DREB2A* gene, PCR was optimized in a two step process; in the first step, pooled DNA was used as template. The first PCR amplified product was used as template in step two (PCR-2), but the component concentration used for both the reactions was similar.

3.33.3 PCR conditions

PCR amplifications were carried out starting with an initial denaturation 94 ºC for 5min and denaturation at 94 ºC for 1min and annealing at 66 ºC for 45 s and extension at 72 ºC for 2 min for first 10 cycles and the next 35 cycles with denaturation at 94 ºC for 15sec and annealing at 66ºC for 20 s and extension at 72 ºC for 30 s for first and final extension at 72 ºC for 10 min for two steps. After PCR amplification the samples were checked in 1.2% agarose gel and samples were treated according to the manufacturer's directions for the survey Mutation detection kit (Transgenomics Inc, Omaha, NE, USA).

3.34 Initiation of allele mining in *EDR2*

For allele mining, a literature search was initiated to identify candidate genes for downy mildew resistance. For downy mildew tolerance candidate genes, *EDR2* susceptible gene (NM_118022 2696bp mRNA linear PLN 21-AUG-2009, *Arabidopsis thaliana EDR2*; lipid binding (*EDR2*) mRNA, complete cds.ACCESSION NM_118022,VERSION NM_118022.4 GI:186512033, db_xref="GeneID:827642",db_xref="TAIR:AT4G19040") was chosen and sequences were downloaded from NCBI (**Error! Hyperlink reference not valid.** primer pairs were designed to amplify the complete gene. PCR amplification conditions were optimized for these primer pairs and amplification was checked on P1449-2-P1 and a few TILLING lines. The 5 degenerate primer pair combinations were able to generate PCR amplicons that give complete gene coverage. However, few primer (3, 8, 9 and 14) combinations did not give any amplification. The generated amplicons were sequenced and then the primer pairs have been selected to get maximum length of the gene.

3.34.1 CODDLE

CODDLE (Codons to Optimize Discovery of Deleterious Lesions), a web based program (http:// www.proweb.com) was employed to predict the probable regions that are more likely to be prone for mutations in the *EDR2* gene of EMS treated populations. Further primers were selected in the degenerate primer combinations that were used for amplification of the gene to amplify the region identified by CODDLE as a hot spot for the mutation. Then F1R1 and F1R3 primer combinations were obtained. Using labeled and unlabelled primers, the region of interest was amplified using two step process on few samples (eight individual pools). In the first step, complete gene of interest was amplified and in the second step, the region of interest was amplified using the first PCR product as template. Further, restriction digestion of mutant samples was carried along with the positive and negative controls using the SNiPer Eco-Mix.

3.34.2 Primer designing for *EDR2* gene

Degenerate primers were designed from consensus sequences of *EDR2*. The amplification of the gene was standardized in P1449-2-P1 genotype which was used for TILLING population. The degenerate primer sequences that were designed for the amplification of *EDR2* gene was

EDR2 F1:5'-CGGCCGGCGGAAAGATGGAGCTACG -3'

EDR2 F2:5'-GCGGAGGACCACCATHGGNAAYGG-3'

EDR2 R1:5'-GGCGGTGTGGCCGTCAGCTACCTTCCTTC-3'

EDR2 R2:5'-CGCACGATCCAGGAGCCBTTCTTSGGSAC-3'

EDR2F3:5'-GCTGCTGCGGTGCCARAAYGGNYT-3'

EDR2F4:5'-GCGGACCACCATCGGNAAYGGNCc-3'

EDR2F5:5'-GGTACTGGCGGCGGAAYGAYGAYGG-3'

EDR2R3:5'-AGGCCCAGCACGCCRTTNGCNAC-3'

EDR2R4:5'-CTGCCGCACGATCCANSWNCC-3'

EDR2 R5: 5'-TCCGCCGCCAGTACCKNACRTARCA-3'

3.34.3 Amplification of *EDR2* gene

Chemicals for PCR

For PCR, 50 ng P1449-2-P1 genotype DNA, double distilled H_2O, 10x Sib enzyme buffer, 50 mM $MgCl_2$, 10 mM dNTP's, 10 pico moles of forward primer, 10 pico moles of reverse primer, 1 unit of Sib Taq DNA polymerase, and thermocycler were used.

PCR conditions

Different conditions were used for the standardization of *EDR2* gene amplification in P1449-2-P1 genotype. The gene was amplified with target PCR in an initial denaturation step at 94 ºC for 5 min, denaturation at 94 ºC for 1 min, annealing at 61 ºC for 45 seconds, extension at 72 ºC for 2 minutes. These denaturation, annealing and extensions were repeated for 35 cycles followed by final extension at 72 ºC for 10 minutes. The components used for PCR amplification was 50 ng of DNA, 10x Sib buffer, 30 mM $MgCl_2$, 10 mM deoxynucleotide triphosphates (dNTPs), 10 pico moles of primers and 1 unit of Sib enzyme Taq DNA polymerase.

3.35 Mutation screening

PCR amplification was carried out in one step for 25 µl volume containing 5 ng of pooled DNA, 10X SibTaq buffer (including 1 mM $MgCl_2$), 25 mM $MgCl_2$, 10 mM deoxynucleotide triphosphates (dNTPs), 5 pico moles of primer mix (unlabelled forward, unlabelled reverse, IR-labeled forward at 700 and IR-labeled reverse at 800 in 1:1:1:1 ratio) and 1U SibTaq DNA polymerase (Takara biomedicals, Cambrex Biosciences, WokinghAM, UK.).The amplification of pooled samples was carried out in single step and PCR cycling was performed in two-steps as described in Colbert et al., (2001). PCR amplifications were carried out starting with an initial denaturation of 94 ºC for 5 min and denaturation at 94 ºC for 1 min and annealing at 66 ºC for 1 min and extension at 72 ºC for 2 min for first 10 cycles and denaturation at 94 ºC for 45 sec and annealing at 66 ºC for 45 sec and extension at 72 ºC for 1 min for next 30 cycles and final extension at 72 ºC for 10 min. After PCR amplification, samples were treated according to the manufacturer's directions for the survey using Mutation detection kit (Transgenomics Inc, Omaha, NE, USA).

3.36 High throughput method

3.37 Creation of heteroduplexes by programming the following thermocycler conditions

At the end of PCR reaction, heteroduplexes were created by programming the following thermocycler conditions;99 ºC for 10 minutes, held to denature the PCR product and 80 ºC for 20 seconds and 70 cycles of: 80 ºC for 7 seconds (reduced by 0.3 ºC each cycle). It was then transferred to 10 µl of the PCR product to a new plate and placed on ice or a cold block.

Similarly the control mix was prepared according to kit manufacturer's direction. Control mixes were provided for both a heterozygous and a homozygous individual. Each tube contains labeled IRDye® primers, (700 nm and 800 nm), unlabeled primers, and template. This allows the user to troubleshoot the amplification and cleavage reactions and for using GELBUDDY or an equivalent image analysis. The control PCR calculator was used to create these two master mixes. For each control reaction,

10X PCR buffer 2.5 µl
50 mM $MgCl_2$ 1.5 µl
10 mM NTPs 1.3 µl
Sharka Taq® 1.0 µl
Control mix 18.8 µl

were added. The full length control PCR product was 1600 bp. The two cleaved fragments were 1125 bp and 475 bp. PCR cycling was as follows: Initial denaturation at 94 ºC for 3 minutes and 33 cycles of denaturation at 94 ºC for 30 seconds annealing at 57 ºC for 1 minute and extension at 72 ºC for 2 minute and final extension of 72 ºC for 7 minutes and at last 15 ºC. Freezing of the PCR products overnight at -20 ºC is recommended since this improves the signal of the final product.

3.38 Cleavage of mismatch products

For every 10 reactions, the following 2X cleavage mixture is prepared. One µl SNiPer Eco -Mix is added to 125 µl of 2X reaction buffer. It was vortexed and spun in a microcentrifuge briefly.

PCR plate was removed from thermocycler and caps or film were removed. Using a multichannel pipette, or a repeat pipetter, 10 µl of the 2X cleavage mix (this should contain the 2X reaction buffer and the SNiPer Eco -Mix) was pipette into each well of the PCR plate. The plate was resealed or recaped, vortexed, and spun in a plate centrifuge. Samples were placed in a thermocycler and heated to 45 °C for 15 minutes followed by a 4 °C soak. As soon as the plate was incubated, it was placed on ice and 5 µl of stop buffer was added to each well using a multichannel pipette. This is only to inhibit further nuclease activity. The plate was kept in a cool place and protected from light. If purification is to be done the following day, the samples were frozen at -20 ºC.

3.39 Purification of the cleavage product

Chemicals for purification

For purification of cleavage product, 100% ethanol, 70% ethanol, LICOR loading dye, multi channel pipette (200 µl, 10 µl) were used.

Purification of product

To the purified product, 2/3 volume of 100% ethanol was added and mixed properly until it was precipitated at -80 ºC for 30 minutes. Then the contents were centrifuged at 5000 rpm at 4 ºC for 30 minutes. The solution was decanted and 70% ethanol was added and spun for 15 minutes at 5000 rpm at 4 ºC. Then the solution was decanted and the plate was dried in a speed vacuum for 20 minutes. Then 5 µl of loading buffer was added to each well of a clean 96 well PCR plate. The samples were denatured at 94 ºC for 5 minutes before loading onto the LICOR gel.

3.40 SDS PAGE

3.40.1 Materials

Preparation of acrylamide/bisacrylamide 29:1 (w/w), 29 g acrylamide, 1 g bisacrylamide, water (deionised distilled) to 100 ml, stored at 4 ºC, 1% (w/w) ammonium C persulphate in water (made fresh stock every week, stored at 4 ºC), TEMED (N,N,N,N-tetramethylethylenediamine, stored at 4 ºC), binding saline buffer (stored at 4 ºC), 1.5 µl binding saline, 5 ml of acetic acid, 993.5 ml ethanol.

3.40.2 Gel preparation

For 6.5% gel (LICOR plates) 25 ml of gel solution is sufficient).

25 ml acrylamide

150 µl APS

15 µl TEMED

The above chemicals were vigorously mixed and the solution was prepared properly. The acrylamide was poured into the syringe which feeds into to the glass plates and the casting comb was inserted (reverse orientation, so as to form a well) and the pressure plate was applied. Excess acrylamide solution was poured onto comb and allowed to polymerize for 30-60 min. Gels were stored overnight and as long as the plate ends are wrapped in pre-wet (1X TBE) tissue paper, and covered in cling film.

3.40.3 Cleaning of glass plates

Glass-plates are cleaned thoroughly with the soap water first and then with alcohol. If plates are not thoroughly cleaned, then bubbles will get trapped whilst pouring the gel. Few drops of Repel-Silane-ES was applied to the back-plate and rubbed over the surface (this makes it easier to separate the plate from the gel). Then plates were assembled with spacers. The plates were fixed tightly with the clips that were provided along with the LICOR instrument.

3.41 LICOR Preparation

After polymerization, the gel was setup for running. The combs were removed. The lower tank was filled with 1X TBE and approximately 250-300 ml at the back of the plate and also upper reservoir with the same quantity (approx 400 ml), to ensure that the well is covered. Before use, both the front and back plates were washed thoroughly, the casting comb was removed and all acrylamide was removed from the well. The plates were cleaned with isopropanol before placing in the LI-COR DNA analyzer, to make sure that the laser detection region is clean. The

well was cleaned by aspirating the TBE buffer using Pasteur pipette to remove small fragments of gel and tiny bubbles if any. The comb tips 1 mm in length were inserted into the gel. After the run, the plates were pulled apart carefully so that the gel is attached to one plate. The gel was then taken out with filter paper and the plates were cleaned with soap water.

3.42 Reusing membrane combs

The combs are cleaned by soaking in a tub of deionized water for at least 30 min. If many combs are washed at once, care should be taken to avoid comb damage, and water should be exchanged several times to ensure that the combs are thoroughly cleaned. The combs are air dried for several days before reusing.

3.43 LI-COR 4300

3.43.1 Loading and running gel

The gel was pre-run to warm it for at least 15 min and the gel was kept ready for run. It takes twenty minutes for pre-run and comb applications, and the 4h run may be used for running the gel. Samples are generally denatured and then electrophoresed on a LI-COR 4300 DNA Analysis System. Electrophoresis was performed through a 6.5% polyacrylamide, the gel well and glass were cleaned and the plate was assembled into LI-COR DNA analyzer. The upper and lower buffer tanks were filled with 1X TBE buffer, 15 min pre-run (recommended) ensuring that the lasers focus properly with the following parameters: 1500.0 voltage and 40 W power, 40 mA current and 45 ºC temperature on the LICOR analyzer. Then 0.5 µl samples were loaded on to the 50 tooth membrane combs (LI-COR membrane combs and dipping tray; LI-COR Biosciences, Lincoln, NE, USA) and started to run approximately for 4.5 hours. Then, 5 µl of sample was applied per membrane comb tooth starting with tooth number 4 and ending with tooth number 50. The bottom 1/3 of the tooth is stained blue after loading. To teeth 1 and 50, 2.5 µl of molecular weight ladder was applied. This asymmetry ensures that sample 1 is never confused with sample 50 in the event that the comb is inverted before application to the gel.

Samples can also be loaded directly onto the gel using a sharks tooth or a square tooth comb. Although 96-tooth shark's tooth combs are available, loading can be tricky. Loading fewer than 50 samples on a single LI-COR gel reduces throughput without providing any clear benefit for data analysis. The gels are rinsed well thoroughly using a plastic 10 ml syringe with no needle attached. Buffer was removed from the upper tank. Excess liquid is removed from the gel well by inserting filter paper into the gel well. The filter paper was removed and 1 ml of ficoll solution was added to the gel well. A small bead is formed at the edge of the front glass plate. The loaded membrane comb is inserted into the gel well at a 45º angle. Once the teeth touch the ficoll solution, the comb slides smoothly into the well. The comb should be gently inserted into the well and the teeth should not be forced into the acrylamide. The upper is filled gently with 1X TBE buffer and the top electrode is replaced. The gel was run for 4 h (40 mA, 40 W, 1,500 V, 50 ºC, image width 1,028 and scan speed 2).

Optional: After 10 min, the comb is removed and gently rinsed in the ficoll out of the gel well. The top electrode is reinserted and the LI-COR door is closed. Since it is a 4300 series machine, the run will remain paused until the

option continue is selected. Typically, the machine is started at the end of the day and the machine is left running unattended.

CRITICAL STEP: Excessive washing of the well can disturb the gel and result in lower quality gel data. Hence, care is taken.

3.44 Analysis of gel

In lanes that have a mutation in the pool, a band will be visible below the wild type band on the IRDy 700 image. A counterpart band will be visible in the same lane on the IRDye 800 image. This band is the cleavage product labeled with IRDye 800 from the complementary DNA strand. The sum of the length of the two counterpart bands is equal to the size of the amplicon, which makes it easy to distinguish mutations from amplification artifacts. After detection of a mutation in a pool (lane), the individual DNA samples in the pool are screened again to find out which of the eight pooled samples from the crossed population has the mutation. Images were analyzed visually for the presence of cleavage products using Adobe Photoshop software (Adobe system Inc, San Jose, CA, USA) positive bulks were then analyzed for single mutant identification following the same procedure as described for bulk analysis, to analyze the 400 bp fragment at the 700 channel and a 350 bp fragment at the 800 channel.

3.45 Sequencing of the samples

Once a mutation was revealed, the eight-fold pool (samples: 1–8) was remixed into eight discrete pools consisting of two individuals each (samples: 1 & 2, 3 & 4, 5 & 6, 7 & 8, 1 & 3, 2 & 4, 5 & 7, and 6 & 8). If there is a mutation present, then the two pools containing the mutated sample will have the cleaved heteroduplexes in two separate gel lanes and thus, the individual with the mutation will be clearly revealed. Once the positives are identified, the mutant sample, P144-2-P1, and an individual sample from the positive pool was amplified and prepared for sequencing. Amplicons were sequenced either in India or sent to Macrogen, South Korean Biotechnology facility for sequencing on a 16 capillary 23 ABI 3730XLs. Prior to sequencing, samples were treated with 1 µl Exonuclease I (10 U/µl) and 1 µl shrimp alkaline phosphatase (SAP, 1 U/µl) (GE Healthcare; Piscataway, NJ) and purified with a Qiagen PCR clean up kit (Valencia, CA) to remove all excess nucleotides, primers, enzymes or other impurities. After purification, samples were sent for sequencing to Macrogen, 908 World Meridian Venture Center, #60-24, Gasan-dong, Geumchun-gu, Seoul 153-781, Korea. All samples were sequenced multiple times bidirectionally to verify the induced mutation identified from TILLING.

3.46 SNP analysis

Once the sequences were ready in the forward and reverse frame, they were aligned in forward and reverse directions to each mutant line with control sequence. Blast search was performed for the sequence to see the homology with original sequence and the complement strands of the sequence were also taken into consideration. If one of the strands (reverse or forward sequences) was reverse complement with that of original sequence, the sequence was changed to + stands with Reverse compliment tool. Now the control sequence that was taken for primer designing and the forward and reverse sequences were aligned using **MultAlin** software for SNP analysis.

The mismatch is detected in forward and reverse strands. The restriction sites was analysed between control and mutant line sequence with NEB cutter.

CHAPTER IV
RESULTS

4.1 Callus initiation and whole plant regeneration

Plant regeneration either via organogenesis or somatic embryogenesis is necessary for subsequent genetic transformation. Plant regeneration in pearl millet is highly explant and genotype dependent. Further, the frequency of plant regeneration is low and cultures often loose their ability to regenerate within 80 to 120 days. Hence, protocols for callus initiation and plant regeneration from callus cultures derived from different explants and genotypes have been standardized in the present endeavour.

For callus induction, Murashige and Skoog's media containing 2% sucrose and different plant regulators were used. Immature inflorescences measuring 8-12 cm in length were used for initiation of callus. About 50 explants were inoculated from each pearl millet line and for each concentration of the plant growth regulator used. Callus initiation was observed in immature inflorescence explant after 7-8 days of culture. Out of the six lines, 81B-P6 and 843-P2 gave 95.33% and 94% frequencies of callus induction in presence of 2 mg/l 2,4-D (Table 3) from immature inflorescence. On the other hand, dicamba at the same concentration (2 mg/l) gave 65% and 67% frequencies, but picloram induced callus with 21% and 27.6% frequencies in the above two genotypes respectively from mature embryo and immature inflorescences. However, after two subcultures, the callus turned brown and the shoot regeneration could not be seen in any of the lines used in this study. The lowest response of 2.7% was noticed in the genotypes TIFT D238 in presence of 1 mg/l dicamba from mature embryo explants and in 5 mg/l picloram in the genotypes P1449 from shoot tips. But, this callus was not embryogenic. With an increase or decrease in the auxin concentration from 2 mg/l, there was a decline in the percentage frequency of callus initiation (Table 3). Pale yellow or cream coloured embryogenic callus that is compact in nature was noticed when 2,4-D was incorporated into the medium (Fig. 1), but not in presence of dicamba and picloram. The percentage frequency of somatic embryogenesis was 17.7 in the line 843B-P2 and 3.33 in 81B-P6. In other genotypes, embryogenic callus was not observed. To improve embryogenic callus formation, antioxidants (0.1% activated charcoal, 0.1% ascorbic acid and 0.1% polyvinylpolypyrrolidone), and different concentrations of auxins alone (NAA, 2,4,5-trichlorophenoxyacetic acid and 2,4,5-trichlorophenoxy propionic acid) and also in combination with cytokinins (kinetin, BAP, MAP, adenine hemisulphate) were tried, but these attempts were not successful in the induction of embryogenic callus. However, incorporation of 10 mM NaCl along with 2 mg/l 2,4-D in the medium improved the percentage frequency of

embryogenic callus from 3.3 to 19 in the line 81B-P6 (Table 4). Callus cultures from other genotypes were also subjected to salt stress, but neither somatic embryo formation nor shoot regeneration were noticed. After three subcultures (15 days each) under salt stress, (10 mM NaCl), calli from the two lines were transferred to MS medium fortified with 0.01 mg/l or 0.05 mg/l thidiazuron. Lower concentration (0.01 mg/l) profoundly influenced the embryogenic callus frequencies with 36.7% and 37.3% in the lines 843B-P2 and 81B-P6 respectively. So, the callus derived from the genotype 81B-P6 was used for genetic transformation studies since the number of somatic embryos per callus mass was more and also the subsequent shoot forming ability of callus. Higher concentration of thidiazuron, however, decreased the frequency of somatic embryogenesis as well as shoot forming ability in both the genotypes (Table 4). Globular-shaped somatic embryos were observed in 2 mg/l 2,4-D + 10 mM NaCl containing medium (Fig. 1) in both the lines. Embryogenic callus growing in the presence of 0.01 mg/l TDZ (120-day-old) was transferred to MS medium containing BAP or kinetin along with IAA and NAA for plantlet regeneration. A combination of kinetin (2 mg/l) and NAA (0.2 mg/l) gave 97.5% (843B-P2) and 15% (81B-P6) frequency of shoot differentiation in the two lines. But 2 mg/l BAP plus 0.2 mg/l NAA stimulated the shoot differentiation in the line 81B-P6 considerably (95%), while it was 10% in 843B-P2 (Table 5 and Fig. 2). In other combinations (BAP + NAA and kinetin + NAA), shoot differentiation frequency ranged between 5 to 56.6%. The number of shoots formed per 100 mg of callus also varied depending on the genotype and growth regulator concentration. The number of shoots differentiated per 100 mg of callus varied from 2.33 to 28.5 in the genotype 81B-P6 (Table 5), while it ranged from 2.6 to 32 in the line 843-P2 (Table 5). However, if the callus was transferred directly from 2 mg/l 2,4-D medium, the frequency of shoot differentiation was only 10-15% and the number of shoots formed was 2-3 in both the lines. But the calli grown both on 2 mg/l 2,4-D plus 0.01 mg/l TDZ lost their ability to regenerate shoots after 200-days in culture. The shoots formed on kinetin plus NAA or BAP plus IAA media were green and healthy in both the lines.

4.2 Histological studies

Histology of the shoot forming callus derived from the genotype 81B-P6 is shown in the figure 3. Shoot forming calli displayed globular shaped somatic embryos, and simultaneously shoot differentiation also via organogenesis (shoot apex surrounded by leaf primordia).

4.3 Rooting of shoots and maintenance of regenerants

Shoots were excised and transferred to MS basal medium incorporated with 0.2 mg/l indole-3-acetic acid for root differentiation. Shoots developed roots with 100% frequency irrespective of the genotype within 3-4 weeks on the above medium (Fig. 2f). Well-developed plantlets were transferred to plastic pots containing vermiculite and vermicompost in 1:3 ratios. The plantlets were later transferred to clay pots containing sand and soil mixture. Plants were covered with glass beakers to maintain humidity and watered with Hoagland nutrient solution at 2-3-day intervals. Beakers were removed after two weeks of transfer to the pots. The frequency of survival was 60% in both the genotypes and morphologically the plants looked like that of seed-raised plants.

4.4 Effect of age of inflorescence

The age of the immature inflorescence and callus initiation potential showed an inverse relationship. The number of explants producing callus declined with the increase in the age of the donar plants. Explants isolated from 35- day-old plants showed the highest response (Fig. 4). Not much variation in callus initiation frequency was noticed in explants isolated from 45 to 55-day-old plants.There was a significant decline in callus differentiation with increased age (60 days and above) of the donor plants. However, callus produced from immature inflorescences isolated from 35-day-old plants was embryogenic compared to the callus initiated from 50-day-old field grown plants. These observations clearly suggest that 35-day-old plants are the best source for generating the embryogenic callus in *P. glaucum* from immature inflorescences.

4.5 Antibiotic sensitivity of callus

Calli isolated from the genotype 81B-P6 were exposed to different concentrations of the phosphinothricin in order to find out the threshold level. Calli that were exposed to 300 mg/l kanamycin survived even at this high concentration. Callus of 81B-P6 were also subjected to 0-20 mg/l phosphinothricin (Fig. 5a) to find out the threshold concentration. Nearly 100 percent of callus tissues turned dark brown and died eventually at 5 mg/l phosphinothricin. The % frequency of embryogenic calli that became dark brown in presence of different concentrations of phosphinothricin was recorded. The lethal dose (LD) of phosphinothricin (5 mg/l) at which no calli could survive was determined. Calli were able to tolerate up to 300 mg/l cefotaxime and hence this concentration was used to inhibit the excess growth of *Agrobacterium* in the shoot regeneration media.

4.6 Genes used for the transformation studies

pCAMBIA2300 and pCAMBIA1300 vectors (Fig. 6) were used for the genetic transformation studies. The *chitinase* gene was isolated from *Oryza sativa* (class I endochitinase) and *osmotin* from *Solanum nigrum* and both of them were inserted into pCAMBIA2300 because the activity of one gene may not be sufficient to impart resistance to the pathogens. The *bar* gene was isolated from *Streptomyces hygroscopicus* and introduced into pPUR vector to impart resistance to the calli for phosphinothricin. Callus initiated from the genotype 81B-P6 was used for genetic transformation studies. Since the percent frequency of regenerations from callus cultures and also the survived rate against phosphinothricin is high in the genotype 81B-P6 than 843B-P2, for subsequent studies genotype 81B-P6 was used.

4.7 Cloning of genes into pCAMBIA binary vectors

Chitinase and *osmotin* genes were put into the vector pCAMBIA2300 and *E.coli* were transformed with this vector. Digestion of *Npt*II with *Xho*I (Fig. 7) removed the selection marker and the gene was self ligated into the vector pCAMBIA2300. Now the *osmotin* and *chitinase* genes without the selection marker are ready for use. The pPUR intermediate vector harboring the *bar* gene was digested with HindIII and EcoRI along with CaMV35S and NOS PolyA. The pCAMBIA1300 vector was linearized with Hind III and EcoRI to ligate the bar gene with promoter and

terminator. The *bar* gene insert and vector were eluted after electrophoresis and purified. The concentration was checked on the gel before ligation. The ligation mix was transformed into DH5-α competent cells by giving heat shock. The transformed colonies with the recombinant plasmid were selected on LB medium containing kanamycin (50 mg/l). Recombinant plasmid was isolated and checked on 1% agarose gel. Presences of the chitinase, osmotin and bar genes in pCAMBIA binary vector were confirmed after digestion with the HindIII, PstI and HindIII + EcoRI (Fig. 8). The T-DNA maps of recombinant plasmids are shown in figure 6. pCAMBIA2300 with chitinase and osmotin and pCAMBIA1300 with bar genes were also transferred successfully into the *Agrobacterium tumefaciens* (GV2260) and the transformed colonies were selected on kanamycin (50 mg/l) and rifampicin (50 mg/l) containing media. They were checked for gene insertion by digestion, which showed 620 bp of *chitinase*, 750 bp of *osmotin* and 550 bp of *bar* gene (Fig. 9).

4.8 Transformation of pearl millet calli by *Agrobacterium* mediated method

The standardization of *Agrobacterium* mediated genetic transformation with calli as was carried out in the present study. The transformed calli were capable of producing multiple shoots on regeneration media containing phosphinothricin as a selection agent (Fig. 5b). The number of shoots formed is 2-4 and the shoots were rooted on rooting media containing phosphinothricin selection. The survival rate of the *in vitro* regenerated plantlets was over 10% and a total of 3 healthy putatively transgenic (T_0) plants were produced through *Agrobacterium* mediated gene transfer with the pCAMBIA2300 containing osmotin and chitinase and pCAMBIA1300 with *bar* gene. However, these plants did not survive in the Net house when transferred (Table 6). After co-cultivation of calli with *Agrobacterium* for 2 days, the calli were transferred onto selection media. Optimal conditions for transformation based on resistance to phosphinothricin were investigated since no GUS intron is there in the construct. Some calli co-cultivation with *Agrobacterium* was able to grow in presence of phosphinothricin. In contrast, untransformed controls did not show any resistance to phosphinothricin at 3 mg/l level. Multiple factors play a role in efficient T-DNA transfer and transformation of pearl millet. The concentration of bacterium (O.D), concentration of acetosyringone, and time mode of co-cultivation were examined. However, the three plants recovered from *Agrobacterium* mediated transformation did not survive in the Net house.

4.8.1 Addition of acetosyringone on the transformation efficiency

Acetosyringone (AS) is known to enhance the transfer of T-DNA from *Agrobacterium* to plant cells in many plants like cotton and others. The primary step in genetic transformation is the attachment of *Agrobacterium* to the host plant. This is facilitated by the genes present on the bacterial chromosome. Compounds like AS are known to induce *Agrobacterium* virulence genes that help in transfer of T-DNA to the host plant genome at the site of injury. In many cases, AS is known to induce expression of *vir* genes, which is necessary for the generation of T strands and their transfer to the plant cells. Addition of 100 µM AS increased the efficiency of *Agrobacterium transformation* (Fig. 10). This indicates that the *vir*- inducing compounds such as AS had a positive effect on the T-DNA transfer. After transforming the calli with *Agrobacterium*, calli were co-cultivated with 100 mM acetosyringone for 48 hours in dark.

4.9 Transformation by bombardment

Optimum parameters were standardized for co-bombardment mediated genetic transformation with pCAMBIA2300 containing *osmotin* and *chitinase* and pPUR containing *bar* genes. Maximum frequency (80%) of transformation was noticed when various established parameters, viz., 18 Kg/cm^2 of helium pressure; 12 cm of target distance; 1 µg of DNA; 50 mg/ml gold particles; 0.25 M sorbitol and 0.25 M mannitol as osmoticum; 8 h of pre-bombardment osmotic treatment; and 24 h of post-bombardment osmotic treatment were used. Transformation frequency was reduced to 40% when 10 Kg/cm^2 of helium pressure; 8 cm of target distance; 2 µg of DNA; 100 mg/ml tungsten particles; 0.2 M sorbitol and 0.2 M mannitol as osmoticum; 6 h of pre-bombardment osmotic treatment; and 24 h of post-bombardment osmotic treatments with 0.2 M each of sorbitol and mannitol were used. The bombarded embryogenic calli were cultured on MS medium supplemented with 0.1 mg/l TDZ and 2 mg/l BAP + 0.2 mg/l IAA but without selection pressure initially. Then the calli were transferred onto fresh regeneration medium i.e. MS medium supplemented with 2 mg/l BAP + 0.2 mg/l NAA containing phosphinothricin (Fig. 5c). Selection of calli exhibiting resistance to phosphinothricin were separated and frequently subcultured onto regeneration media. Out of 70 selected calli from 100 inoculated; only 50 resistant calli could give rise to shoot regeneration. Once the multiple shoots were regenerated successfully, they were then transferred to MS medium supplemented with 0.2 mg/l IAA (called as rooting medium). The regenerated putative transformants with well-developed roots on MS basal medium were transferred to plastic pots containing vermiculite and vermicompost (1:3) and the plants were covered tightly with beaker. The well developed plantlets were established in pots (Fig. 11) and four plants were grown to maturity in the net house (Table 7).

4.10 Molecular analysis of putative transgenic plants obtained in bombardment method

PCR was carried out on the genomic DNA isolated from the basta-tolerant putative transformants generated through bombardment (T_0 generation) and the untransformed control, using gene specific primers for *bar* gene. Upon performing PCR, various transformants and the positive control (pBar35S) showed amplification of 560 bp. PCR for the genes *chitinase* and *osmotin* was also carried out and amplification products of 600 bp and 700 bp were observed respectively. Conversely, no such band was observed in the untransformed control under identical conditions (Fig. 12). After observing the expression of bar gene in putative (T_0) transformants, four of the putative transformants were subjected to basta (0.25%) leaf dip assay which disclosed tolerance to the herbicide. The leaves of three basta-tolerant transformants retained their healthy green appearance, whereas leaves of untransformed control plants were scorched within 72 h of basta treatment (Fig. 12D). Total RNA was isolated from the leaves of Southern-positive transgenics plants and untransformed control plants were subjected to RT-PCR analysis using chtinase probe. A clear band of chitinase gene at 600 bp was observed only in three transgenics plants (Fig. 13). In contrast, no such band was observed in the untransformed control plants. Southern blot analysis was carried out using *chitinase* coding sequence as a probe on transformants that are tolerant to basta and PCR-positive for *bar*, *chitinase* and *osmotin* genes. Three transformants, showed more than one hybridizable band, while one showed only one

hybridizable band. In these transformants, however, the hybridizable bands were at 600 bp, whereas, no such band was observed in the untransformed control plants (Fig. 14).

4.11 Sterility in transgenic lines

The highest transformation frequency of immature inflorescence callus was obtained with the bacterial strains GV2260 (OD 0.3) and by co-bombarding the callus with particle gun. Such calli when exposed to phosphinothricin showed resistance with a frequency of 60%. Thus, these results establish that *A. tumifaciens* and bombardment can be employed for stable genetic transformation in immature inflorescence derived callus of pearl millet. These experiments were repeated for finding out if the protocols are reliable and repeatable. A total of 40 independent transgenic lines were developed using particle gun method. Though these plants were grown to maturity in the Net-house, they turned to be totally sterile, though pollen is produced. Hence, these transgenic lines could not be carried to the next generation for testing their resistance to fungi.

4.12 PEARL-TILL

4.12.1 Genotype background

To generate the TILLING population in pearl millet, the inbred line "P1449-2-P1" has been chosen, as it is one of the parental genotypes of a mapping population (PT 732B × P1449-2-P1) developed and maintained at ICRISAT and segregates for plant height (*d2*), downy mildew resistance, stover and grain quality. It is the tall parent, which serves as a good source of downy mildew resistance for the improvement of local cultivars. The chemical mutagen ethyl methanesulfonate (EMS) was selected to develop the TILLING population, as it generates mostly single nucleotide polymorphisms or SNPs (in genes) and can be controlled to produce a high density of point mutations causing a variety of lesions including nonsense and mis-sense mutations.

4.12.2 Current status of mutant or TILLING lines

The current status of available M1 and M2 lines is represented in Table 8 and Fig 15. A total of 9,938 M2 lines are available and DNA has been extracted from them. A total of 9928 pearl millet M2 lines were developed and DNA has been isolated from 9938 of the plants. In 2006, a total of 2581 mutant lines were generated in 2006, 2074 lines in summer 2007 and 7500 M2 lines in Kharif 2007 were generated.

4.13 Mutagenesis

The chemical mutagen, EMS, was used to generate the mutagenized population because of its high rate of success in many plant species. Previously published reports demonstrate that it is a beneficial strategy to try a range of concentrations of the chemical mutagen to evaluate the toxicity and sterility on germinal tissue before preparing large mutant populations. Therefore, as a first step, the effect of EMS on pearl millet seed germination was tested.

Initially 50 seeds were treated with different concentrations of EMS i.e. 5 mM, 10 mM, 15 mM, 20 mM, 25 mM, 30 mM, 35 mM, 40 mM, 45 mM, 50 mM, 55 mM and 60 mM, for a period of 16 hours. Survival and establishment of 50 percent seedlings (LD50 value) was chosen as a criterion to find the optimum concentration of EMS for developing TILLING population. The concentrations above 5 mM and 16 hour duration treatments were found to be

lethal, hence, different durations of treatment like 4, 8 and 12 hours (Fig. 16) and concentrations of EMS (1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, and 10 mM) were used for the second set of experiments (Fig. 17a). Based on the results of second experiment, three mutagen concentrations i.e. 5 mM, 9 mM and 10 mM each with 4 hour treatment duration were selected for mutagenesis. Treatment of pearl millet seeds with these mutagen concentrations provides 60%, 50% and 40% survival of the seedlings. In order to generate the first set of TILLING lines, 2000 seeds were treated each with three mutagen concentrations i.e. 5 mM, 9 mM and 10 mM (each 4 h). A total of 4218 seedlings (1566 of 5 mM, 1385 of 9 mM, and 1267 of 10 mM) along with 1721 seedlings of control were transplanted to field. Variations for the number of mutant lines established after transplanting in the field was observed (Table 9 and Fig. 17b). From a total of 4218 seedlings transplanted, only 2,744 M1 lines (1120 from 5 mM, 784 from 9 mM and 840 from 10 mM EMS concentrations) reached to maturity. The M1 plants were selfed and harvested. Approximately, 20 seeds from 2744 M1 lines each, were sown for M2 generation (Table 10) after taking into consideration all possible losses e.g. embryonic lethality, sterility etc. Two rows of control were taken up before and after every treatment. There was no germination in case of five M2 lines (60194, 63597, 63598, 63599, and 63600). Standard agronomic practices were followed during the crop period as per the recommendations for the crop. From each M2 line, only one earlier tagged plant was chosen, from which leaf sample was collected for DNA isolation.

In order to enhance the existing mutant population, another set of 10,000 seeds of the inbred line P1449-2-P1 were treated with 7.5 mM EMS. The mutagenized seeds were sown in pots and transplanted on to the field. This treatment had about 24% survival of seedling in the field. The survival percent was lower than expected because of the incidence of downy mildew. Though the inbred line (P1449-2-P1), chosen for generation of TILLING population is a donor parent for downy mildew resistance for the mapping population at ICRISAT (PT 732B × P1449-2), some M1 lines were found susceptible to downy mildew (Fig. 18). Hence, the new set of M1 lines generated can serve as potential candidates to look for allelic variants for downy mildew resistance genes. In total, 2400 new M1 panicles were harvested. Of the 2400 M1 panicles harvested, in 162 (6.75%) panicles, there was no seed formation. Of 2248 M1 lines sown in the field, 2074 (92.25%) lines germinated.

4.14 Generation of additional mutant lines to meet ideal population size for TILLING candidate genes

Assuming a population size of 10,000 M2 lines to be ideal for mining allelic variants for candidate genes, another set of 15,000 seeds of the inbred line “P1449-2- P1” were mutagenized using 7.5 mM EMS. The mutagenized seeds were sown in pots initially and transplanted on to the field. To avoid cross pollination in the M1 generation, plants were self fertilized and M2 population were grown. Of the 15,000 lines, 9600 (64%) M1 lines were selfed and harvested. The untreated P1449-2-P1 plants set about 20 g (2000 seeds) per panicle. After EMS treatment, not only the number of seeds per panicle was reduced but the number of panicles that set seeds also decreased with increasing concentrations of EMS treatment. At 7.5 mM EMS, about 50% of the bagged panicles set seeds. At a 9 mM EMS concentration, the set seeds were few (most of them produced less than 20 seeds). Moreover, the seeds from the 9 mM treated M1 plants had poor germination rates and very few lines produced healthy plants that set seeds in the field. Overall, a concentration of 9 mM EMS was the highest dosage at which treated seeds developed into healthy

M1 plants (40%) with M2 seeds. The germination of M2 seeds is only 20%. But in the case of 7.5 mM EMS treated plants; the percent germination of seeds was 35. EMS concentrations used to generate this mutagenized pearl millet population was relatively low compared with that used in other plants. The seeds produced by M1 plants in the different seasons were selfed (Fig. 19), harvested and collected to rise M2 generation. Ten to twelve seeds for each line at three points in each block of 2 m length with space of 0.5 meter (to prevent cross pollination) between each block made on 56 ridges of 150 m length were arranged of M1 mutant lines and two rows of control (untreated) were sown for advancement of M2 generation. To the field DAP was added initially before sowing of the seeds and at the time of flowering urea was supplemented for the healthy growth of the plant. Among three plants for each M2 line, one panicle from each row was labeled, bagged before anthesis. To prevent redundancy of mutations, only that one fertile plant from each M2 row were selected to collect M3 seeds and the leaf tissues were taken for DNA preparation from the labeled plants. This was done only to avoid sampling the same mutation in TILLING screens. To Pearl-TILL development at ICRISAT, the seed mutagenesis strategy was almost like the carried for TILLING *Arabidopsis*. In this strategy, seeds were soaked with mutagen, and each fertile seedling with the unique mutations was tagged and samples collected.

4.15 Phenotypic mutants in M2 population observed in the years 2006, 2007 and 2008

A regular and systematic phenotypic survey was conducted on M2 population and a wide range of phenotypic plasticity was observed. Strikingly, the mutants exhibited a large range of morphological modifications, such as changes in plant architecture, modified leaf morphology, or altered pigmentation. The observed phenotypic variants for instance, include: (i) the leaf color such as - dark green, pale green, yellow, albino, and variegated, (ii) leaf appearance - curly, twisted, smooth, hairy, more erect, and less erect; (iii) leaf size phenotypes- width, length, and thickness (Fig. 20A) and, (iv) shoot appearance phenotypes – fewer tillers, more tillers, branched, and thickness (Fig. 20B), (v) stature appearance- taller, shorter, and extremely short, (vi) plant development phenotypes- seedling emergence time, flowering time, and time of senescence and (vii) panicle mutants- bifurcating, partially sterile, completely sterile, long and short panicles when compared with control. All the 2744 M1 were sown after taking into consideration all possible losses e.g. embryonic lethality, sterility. There was no germination in case of five M 2 lines (60194, 63597, 63598, 63599, and 63600) (Table 11; Fig. 21). The mutant lines sown in the field were regularly surveyed for interesting phenotypes. Although a broad range of phenotypic variants were observed in mutant population generated during 2006, the spectrum of phenotypic variants in new set of mutagenized population during 2007 and 2008 was mostly confined to chlorophyll mutants (Table 12; Fig. 22). The probable reason for limited phenotypic variants can be attributed to the mutagen concentration used. The mutagenized population showed various mutant characters including albino, chlorine, and stunted plants, indicating that mutagenesis was effective. Phenotypic notes were taken on a sample of 9938 M2 plants and are summarized in Tables 11 and 12.

4.16 DNA isolation and pooling by high-throughput method

A quick DNA preparation kit was used for DNA isolation. One hundred µg of DNA concentration was ensured per µl without RNA and protein contamination. The quality of these genomic DNA was checked on 0.8% agarose gel

(Fig. 23) and measured using a fluorescent spectrophotometer to do normalization of the DNA to 5 ng/µl for pooling. DNA concentration was also checked on 0.8% agarose gel and also to see the missing samples if any (Fig. 24) using pooled DNAs from eight individuals (Fig. 25). Genomic DNA of M2 lines was isolated from the 25 day old leaves using Machery-Nagel plant genomic DNA isolation kit. DNA concentration was measured using PicoGreen reagent through Tecan spectrafluor plus and normalized to 5 ng/µl using Tecan Genesis workstation 200 (Fig. 26). Therefore, it has been chosen to pool samples eightfold for pearl millet TILLING. Prior to pooling, genomic DNAs were normalized to a standard concentration. Thus, a total of 12 pooled plates, 5 plates from the DNA isolated from the initial set of mutagenesis experiments, 4 pooled plates from mutant lines generated during 2007 and 3 from 2008 were prepared. Pooling of normalized genomic DNA from a set of 9,938 (2694 mutant lines generated in 2006, 2074 lines in summer 2007, 2791 lines in Kharif 2008 and 2379 lines in Summer 2008) M2 lines was accomplished. Eight-fold pools of genomic DNA from leaf tissues of M2 plants were used to shorten the time for validation of putative mutations. Two gene targets were selected for high- throughput TILLING (Fig. 26). With an aim to identify allelic variant for *DREB2A* transcription factor which plays a key role in drought tolerance, a set of 9,938 M2 TILLING lines have been generated using reference genotype "P1449-2-P1" with an optimized concentration of ethyl methane sulfonate (EMS). The mutagenised seeds were grown into M1 plants and allowed to self pollinate. Further population was advanced to M2 generation (Fig. 27). Further to increase throughput screening, eight-fold pooling of 9,938 M2 lines yielded 12 pooled plates. Of these, screening of *DREB2A* is completed for 12 pooled plates using standard TILLING protocol. Screening of 9938 lines yielded two positive bulk .Analysis of positive bulk for single plant identification was carried out. Figure 27 represents different stages of TILLING population (sowing, seedling, plantlet development, labeling, and collection of leaf material for DNA isolation and selfing of mutated plants) in the field.

4.17 Amplification of *DREB2A* and *EDR2* genes with CODDLE primers

The amplified fragment of *DREB2A* (Fig. 28) was single band without non-specifics when nested primers were used. The amplification of *DREB2A* for screening TILLING was proceeded in a two step process on few samples (eight individual pools). In the first step, complete gene of *DREB2A* was amplified with unlabelled F0R0 primer combination (Table 13.1) and a product size of 1.5 kb was obtained using unlabeled primers in PCR-I. In the second step, the region of interest was amplified in PCR-II with the amplified product of PCR-I as template (Table 13.2). This was carried out with unlabeled primers F0R0 and IR-labeled primers F3R3 combinations that were selected according to hotspot region of CODDLE (Fig. 29) ranging 780 bp (Fig. 30). *EDR2,* a candidate gene for downy mildew resistance was amplified in the reference genotype P1449-2-P1. The amplification of the *EDR2* with different sets of degenerate primers was carried. However, few primer sets did not work but with the primer combination F1R3, the amplified fragment size was 1.2 kb (Fig. 31). Some faint, nonspecific bands were also observed. CODDLE software was used to predict the most probable regions prone for mutations in *EDR2* gene (Fig. 32) and the primer pair used for the amplification was found in the hot spot region. So, for standardizing TILLING protocol, *EDR2* gene amplification was standardized in the pooled samples. The amplification of the target was

obtained with F1R3 primer combination in one step process (Table 14) with IR-labeled and unlabelled primers, though some non-specificity was noticed.

4.18 Mutation detection in LICOR analyzer

For TILLING, gene-specific primers were designed with the target selected regions of the genome (Table 15). Forward-strand primers were end-labeled with IRDye 700 and reverse-strand primers with IRDye 800 (Fig. 33). The standard high throughput TILLING protocol was followed for mutation discovery. After PCR amplification, products were denatured and annealed to form heteroduplexes between complementary strands. Heteroduplexes were then cleaved using SniPerase (an endonuclease from frontiers genomic kit) and the products were size-fractionated on denaturing polyacrylamide gels using a LI-COR DNA analyzer. Cleaved heteroduplexes produced two smaller molecular weight products, one labeled with IRDye 700 and the other with IRDye 800, whose sizes added up to the size of the full length product (Fig. 34). For the *DREB2A* gene according to CODDLE, the mutation was expected at 350bp in the amplified fragment of 800 bp. So, the 350 bp fragment was observed at IRDye 700 channel and the other 400 bp fragment was detected at IRDye 800 channel in LICOR gel image for analyzed mutant lines. The pearl millet population was screened for *DREB2A* mutants and two were identified. The pools containing the mutants were complex to determine the individual for the mutant genotype. PCR was performed on the template DNA of each individual of the pool, as well as on a 1:1 mixture of individual template to wild-type template (for heteroduplex formation). The products were heteroduplexed, digested, and visualized. Because the individuals digested product produced the cleavage banding pattern, it was determined that they were heterozygotes (homozygous mutants would require the addition of wild-type DNA to produce the banding pattern). Analysis of LICOR gel was based on this band and when compared with control, two mutants were found. These pools were analyzed individually to confirm the mutation in the gel. Once the *DREB2A* gene mutants were analyzed in the gel, the pooled DNA was amplified for the target and sequenced for sequencing for allele mining. After discovery on a TILLING gel, the identity of each change was determined by sequencing results.

The LICOR gel was analyzed at two different channels one at 700 and the other at 800 because of the cleavage of endonulease enzyme. This cleavage was observed after amplifying the gene with IR labeling primers forward with 700 and reverses with 800. So, the two bands of the total amplified product were analyzed in the Adobe Photoshop. To attain a higher throughput on the 48-lane LICOR global IR^2 gel amplification, mutation detection was allowed in 384 lines per run, corresponding to about 307200 base pairs. Figure 35 Shows of a true gel image where a mutation in the pearl millet *DREB2A* gene. Because the LICOR system is capable of detecting both florescent dyes fully independent, the same mutation can be detected on both complementary strands, allowing immediate visual confirmation of the mutation. In addition, the sizes of the two fragments correctly add up to full size of the PCR product. More frequent stronger bands in the gel are perhaps caused by single nucleotide polymorphism (SNPs) present in the pearl millet population.

4.19 TILLING of candidate genes - molecular mutation frequency

In order to survey the mutation frequency of the composite population at the molecular level, two genes were screened. This work was performed in parallel to the late stages of population development, so 9,938 M2 lines used for TILLING. The mutation frequency per gene fragment was estimated by dividing the total base pairs screened by the total number of mutations identified. The density of mutations was calculated in the established pearl millet population as an average frequency of all screened fragments. Scoring of mutations located either in the proximal or distal 50 bp of each amplicon was compromised because of priming and systematic artifacts on the LICOR gels (Fig. 35). Gene fragments ranging from 700 bp to 1,200 bp in length were designed to cover either whole open reading frames (ORFs) or selected coding regions of the respective target genes. The screen revealed a total of two independent mutations located in exons. The exon located mutations induced a change in the amino acid (AA). A total of two mutations were detected in DNA analyzed by TILLING in *DREB2A* gene. This was calculated by dividing the total number of mutations revealed by TILLING by the total base pairs screened, which includes the sum of the total length of the two amplicon sizes × the total number of individuals screened per run (1600 bp × 384 individuals). Previous studies have reported the difficulty of tracking mutations on the ends of the fragment (~100 bp); therefore, 200 bp was subtracted from each amplicon. To estimate the density of mutations for the population, total number of mutations identified was divided by the total base pairs screened. This technique and the protocols employed appear to have a moderate ability to detect mutations in the terminal 100 base pairs of each of the amplicon as also reported by earlier workers. Therefore, 200 base pairs were subtracted from the size of each amplicon to obtain the effective screening window size. To determine the total number of base pairs screened, the adjusted size of each amplicon was added and multiplied by the total number of samples screened. For the population, 79, 50,400 base pairs were examined for mutations (800 bp × 9938 individuals).

4.20 Allele mining of *DREB2A* gene

A detailed analysis was performed for the *DREB2A* gene. A 1.5 kb region covering the entire open reading frame of *DREB2A* gene was screened in 9,938 M2 lines by analyzing two overlapping fragments, giving a total 800 bp of gene sequence. In the allele mining, six missense mutations were identified. Majority of these mutations were GC to AT transitions. Four of the missense mutations were detected in the conserved region**.** The average mutation frequency was 0.3% for 800 bp fragment. One mutant identified was sequenced as a confirmation and showed a mis-sense mutation, changing asparagine to serine and from threonine to proline and histidine to asparagine. Missense mutations induced the changes in the amino acids (Table 16) with altered physicochemical properties in regards to polarity and/or hydrophobicity. In the case of chemically-induced mutations, frequent transition changes are expected because EMS alkylates predominantly G residues and result in GC->AT changes. It is concluded that most or all changes were induced by mutagenesis and detected by *MultAlin* analysis (Fig. 36). First, target amplicon lengths were trimmed by 500 bp to compensate for the ends of the PCR products where mutants are undetectable. Overlap between amplicons was also subtracted from the amplicon lengths. The trimmed amplicon lengths were multiplied by the number of M2 populations screened, and the products were summed to give the total number of base pairs screened. The total number of base pairs screened was divided by the number of mutants found. A total of 1.6 kb was screened for pearl millet. Dividing this value, i.e., 1.6 kb by a total of three mutants, gives mutation

frequency. A total of 800 base pairs were screened, and the SNP's (Fig. 36) were observed. Most of them are transition mutations from GC > AT. But some transversions, deletions were also observed (Fig. 37 and Table 16). The change in the amino acid in the mutant line and control was clear after analysis of the sequence in NEB cutter (Table 16). Because of change in the sequence, the restriction sites found in mutant lines are different from that of the control line. The conserved amino acids of the homeodomain of *DREB2A* were affected by substitutions in the phenotypic mutants analyzed. From the pearl millet population, a total of four mutants were obtained in LICOR analysis that was affected in this region; the mutant line 8408-1, contained mutations at amino acid positions identical to induced alleles. The mutation frequency of 20 bp per mutation was clearly observed. Therefore, the entire population of 9,938 M2 lines translates to an average of two to three mutations for any 800 bp DNA fragment. The mutations in *DREB2A* provide an interesting allelic series that is anchored by a predicted loss of function and contains eight other mis-sense changes, including deletions (Fig. 37). In the mutant line 84081, changes in nucleotide sequences and the corresponding amino acids were seen clearly and also in the restriction positions upon SNP analysis. Perhaps this *DREB* mutant line can be tested for drought tolerance or susceptibility. Partial knock out of the gene can also exhibit some tolerance to drought. Thus, these results contribute to a better understanding of the functionally relevant sites of the *DREB2A* protein and also for detecting a mutant plant that perhaps may be associated with drought.

4.21 Mutation detection of *EDR2* gene

Three primer sets were initially tested for amplification of a specific target. All three primer sets yielded an expected band on an agarose gel, along with three non-specific bands (Fig. 32). So, three more sets of degenerate primers were designed, and in two sets, good quality amplification was observed with one non-specific band. Of 10 primer combinations tested, 3 primer sets amplified more than one target. With the high proportion of tested primer sets that amplified more than one target, one has to wonder whether it is possible to screen for mutations in these targets by eliminating extra templates in the genomic DNA. Amplification products resulted in TILLING gels with multiple cleaved fragments in every lane, suggesting that more than one target was amplified and digested. Following this observation, subsequent primers were tested by agarose gel analysis and the bands were eluted and given for sequencing (Fig. 38). Two sequences were obtained upon sequencing the *EDR2* PCR product, one sequence corresponded to *EDR2* target but the other sequence showed homology with the unknown gene. These results point out to the fact that the primers amplified more than one target probably because of homology with other genes since it belongs to a multiple family. Multiple bands were observed when TILLING assays were performed on unpooled DNAs and unknown sequence was detected upon sequencing individuals. So, the detection of mutant alleles has become difficult as far as *EDR2* was concerned.

4.22 TILLING population resource at ICRISAT

The availability of TILLING resources in different germplasm is important while studying specific traits. A TILLING population of 9938 lines was generated in the ICRISAT pearl millet variety P1449-2-P1 and is now available at ICRISAT with Dr. R. K. V. (Fig. 39). The seeds are stored in pearl millet cold room and the seed line

numbers are stored in TWIKI software and the DNA at -80ºC and data was stored in LIMS software. The DNA plate's information was uploaded in three parts. First is the TILLING MOTHER PLATE (113 plates) which contains the DNA isolated from the field, next is the TILLING NORMALISED PLATE (113 plates) which contains the diluted DNA information and the third one is TILLING POOLED PLATE (12 plates) information. Access to TILLING resources in different genetic backgrounds could also be of importance if mutant traits have be utilized in plant breeding programs. Every individual of a TILLING population typically accumulated a high density of mutations. Thus, the results obtained demonstrated that the TILLING technique works well and can be exploited for detecting point mutations in mutagenized populations of pearl millet.

Table 3. Percent frequency of callus initiation from immature inflorescences of different genotypes of *Pennisetum glaucum (L)* on MS medium supplemented with auxins.

Growth regulators(mg/l)	Genotypes					
	81B-P6	843B-P2	863B-P3	P1449	PT732	TIFT-D238
2,4-D						
1	68.3 (+ 1.4)	77 (± 1.4)	27.7 (± 1.5)	8.0 (±1.0)	28.6 (±0.3)	10.0 (±1.0)
2	95.3 (±2.9)*	94 (±3.0)*	71.0 (±2.1)	13.6 (±3.4)	36.66 (±3.3)	19.3 (±0.6)
5	26.3 (± 2.4)	16.7 (± 2.4)	7.0 (± 2.0)	3 (±1.5)	26.33 (±1.4)	13.3 (± 0.3)
Dicamba						
1	26 (±3.0)	36.0 (±3.0)	7.0 (±0.6)	6.3 (±0.8)	16.7 (±2.0)	2.7 (±1.2)
2	65 (±2.8)	67.0 (±2.0)	26.0 (±3.0)	10.0 (±2.8)	22.3 (±1.4)	7.0 (±2.1)
5	7.6 (± 1.4)	5.6 (±2.3)	-	5.0 (±2.5)	11.7 (±1.6)	3.0 (±1.7)
Picloram						
1	8.7 (±1.3)	8.7 (±0.7)	3 (± 1.5)	5.6 (±2.3)	-	-
2	21 (±2.0)	27.6 (±1.5)	18 (±1.0)	10.6 (±0.7)	-	-
5	9.7 (±1.5)	7.7 (±1.2)	6.7 (±0.7)	2.7 (±1.7)	-	-

*Analysis of variance was performed using M-Stat software.
Values are significant among different genotypes and growth regulators.
Values are significant at $P \leq 0.6$.

Table 4. Effect of NaCl and TDZ on embryogenic callus formation in *Pennisetum glaucum* (L.) genotype 843B- P2 and 81B-P6

Media tested (mg/l)	% Frequency of embryogenic

	callus	
	843B-P2	81B-P6
2,4-D alone	17.7 (± 0.5)	3.3 (±0.7)
2,4-D + 10 mM NaCl (callus was grown for three subcultures on NaCl medium)	17.7 (±0.5)	19 (±1.0)
0.01 TDZ (callus was grown for one subculture on TDZ medium)	36.7 (±2.4)	37.3 (±2.9)
0.05 TDZ (callus was grown for one subculture on TDZ medium)	6.7 (± 1.3)	7.7 (±1.4)

*Analysis of variance was performed using M-Stat software.
Values are significant among media tested and % frequency of embryogenic callus
Values are significant (at 5% loss) $P \leq 0.10$.

Table 5. Frequency of shoot differentiation from embryogenic callus of different pearl millet genotypes (843B-P2 and 81B-P6) and the number of shoots formed per callus mass

Plant growth regulators (mg/l)		% Frequency of shoot differentiation		No. of shoots/callus mass	
		Genotypes			
		843B-P2	81B-P6	843B-P2	81B-P6
BAP	IAA				
0.5	0.1	32.5	46.7	8.5(±0.5)	11(±1.0)
1.0	0.1	42.5	51.7	9.5(±0.5)	13(±1.0)
2.0	0.1	30	46.7	6.5(±0.5)	10.5(±1.5)
Kinetin	IAA				
0.5	0.1	5	8	4 (±0.5)	2.3(±0.3)
1.0	0.1	6.7	5	6.6(±0.6)	5(±0.6)
2.0	0.1	21.7	10	9(±0.5)	4(±0.1)
Kinetin	NAA				
0.5	0.2	17.5	15	7(±0.6)	3.3(±0.2)
1.0	0.2	25	17.5	10.6(±0.7)	4.3(±0.3)
2.0	0.2	97.5*	15	32(±1.5)	5.3(±0.3)

BAP	NAA				
0.5	0.2	10	50	5(±1.0)	12.3(±0.3)
1.0	0.2	8.5	57	2.6(±1.0)	12.5(±0.5)
2.0	0.2	10	95*	3.6(±0.3)	28.5(±1.0)

*Analysis of variance was performed using M-Stat software.
Values are significant among different genotypes and growth regulators for shoot differentiation.
Values are significant at $P \leq 0.06$ for % Frequency of shoot differentiation.
Values are significant at $P \leq 0.05$ for No. of shoots/callus mass.

Table 6. Transformation of pearl millet using *Agrobacterium* strain GV2260 containing pCAMBIA 2300 with osmotin and chitinase double construct and pCAMBIA 1300 with bar gene constructs

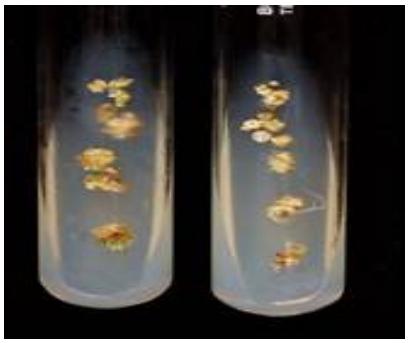
Number of embryogenic calli infected	Co-cultivation period (days)	No. of explants survived on selection media after first week	No. of explants survived on selection media after second week	% Frequency of survival of explants
100	2	30	15	10

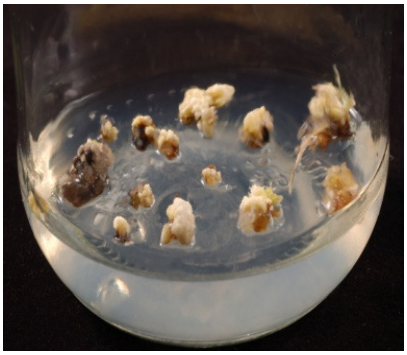
No. of calli regenerated on selection media	No. of tillers formed per plant	No. of plantlets survived on rooting media	No. of plants survived after transplantation to pots	No. of plants grown in Net House
10	4-5	8	6	6

Table 7. Efficiency of biolistic transformation of pearl millet genotype 81B-P6 using osmotin and chitinase genes in pCAMBIA2300 and pPUR with bar gene.

Parameters	Response
No. of embryogenic calli bombarded	100
No. of calli found resistant to 5 mg/l phosphinothricin	70

Total No. of shoots regenerated	60-65
No. of tillers formed per plant	10-25
Total No. of shoots rooted on rooting	50
No. of plants survived after transplantation	40
No. of plants maintained in the Net House	10
% Frequency of transformation	50





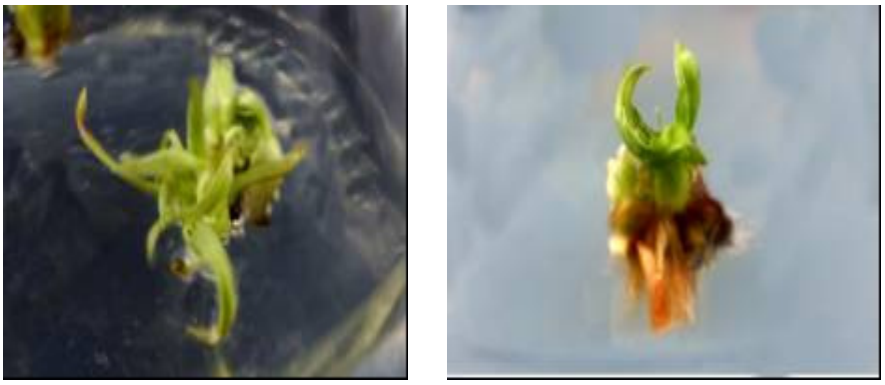
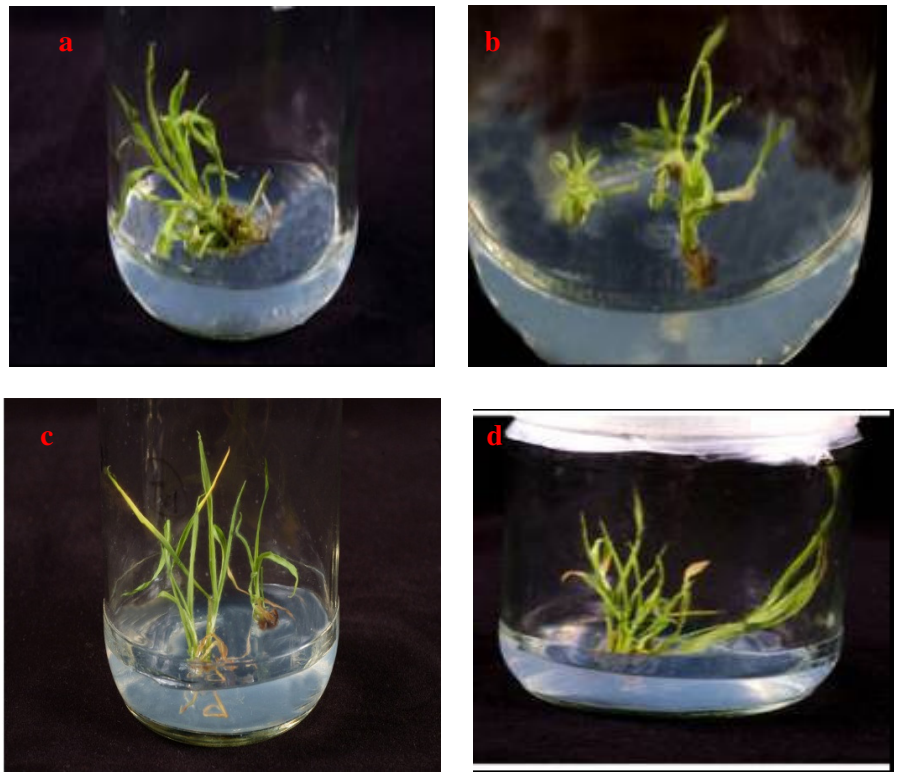


Figure 1. Different stages of *in vitro* shoot regeneration from immature inflorescence derived callus cultures of the pearl millet genotype 81B-P6.



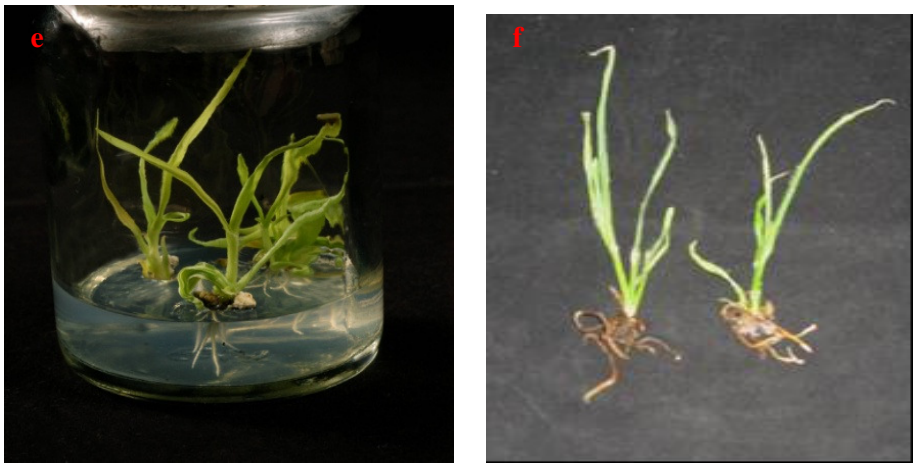
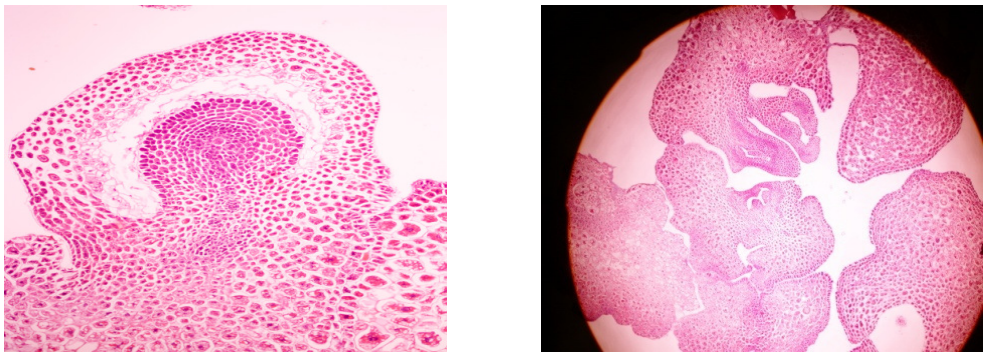


Figure 2. Different stages of *in vitro* plantlet formation from immature inflorescence derived callus cultures of the pearl millet genotype 81B-P6.



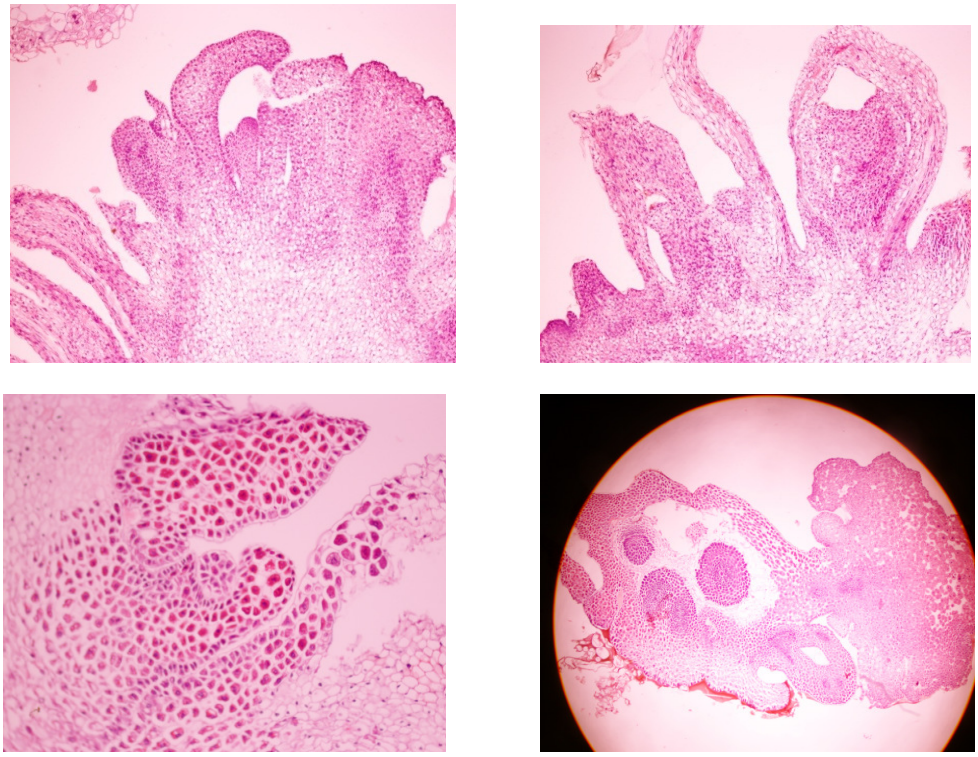
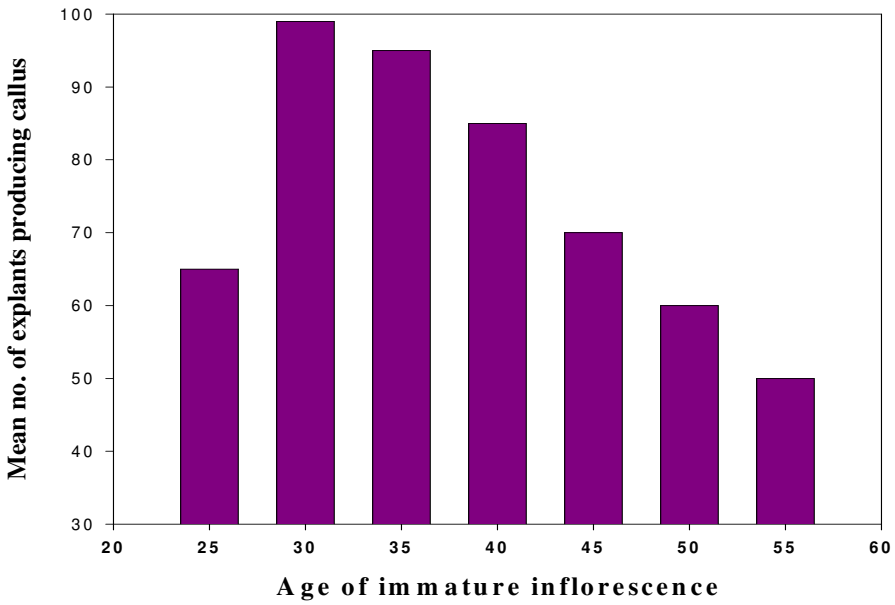


Figure 3. Histological studies on regeneration (embryogenesis and shoot organogenesis) from immature inflorescence derived callus cultures of *Pennisetum glaucum*

Figure 4. Effect of the age of the immature inflorescence



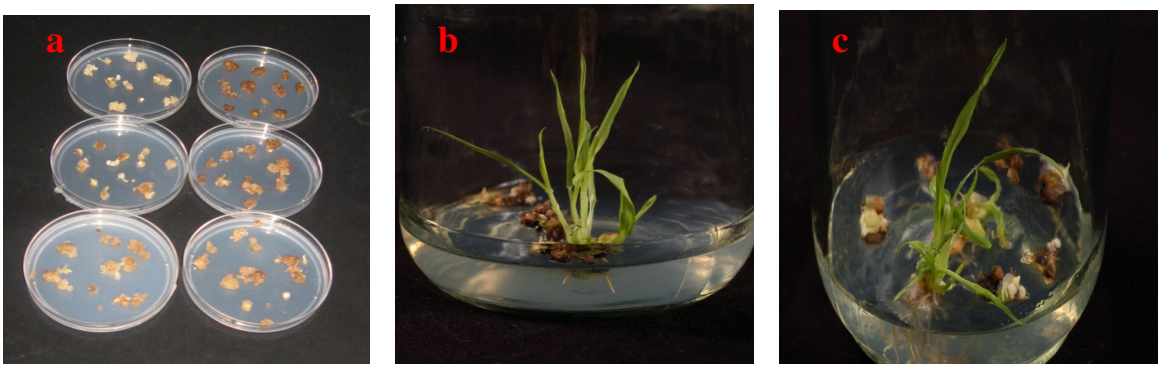


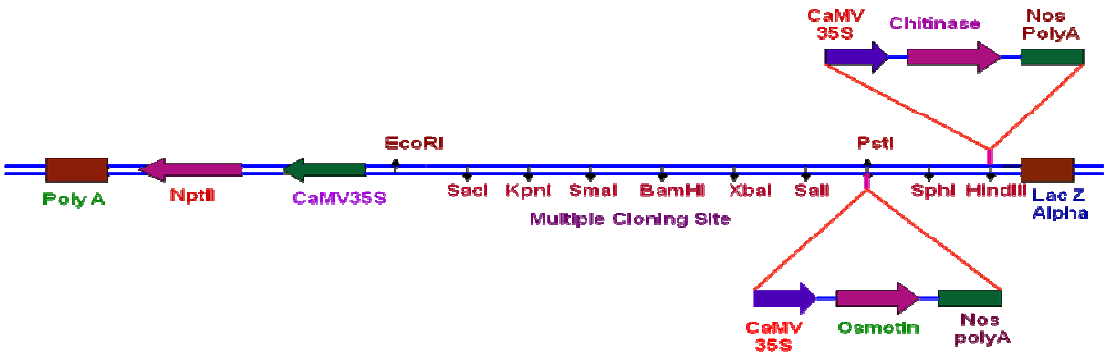
Figure 5. Effect of phosphinothricin on the survival of pearl millet (genotype 81B-P6) calli.

Fig. 5a. Calli growing on different concentrations of phosphinothricin in Petri dishes. Calli exposed to 5 mg/l phosphinothricin turned brown and died eventually. However, calli survived at lower (1, 2 and 3 mg/l level) concentrations.

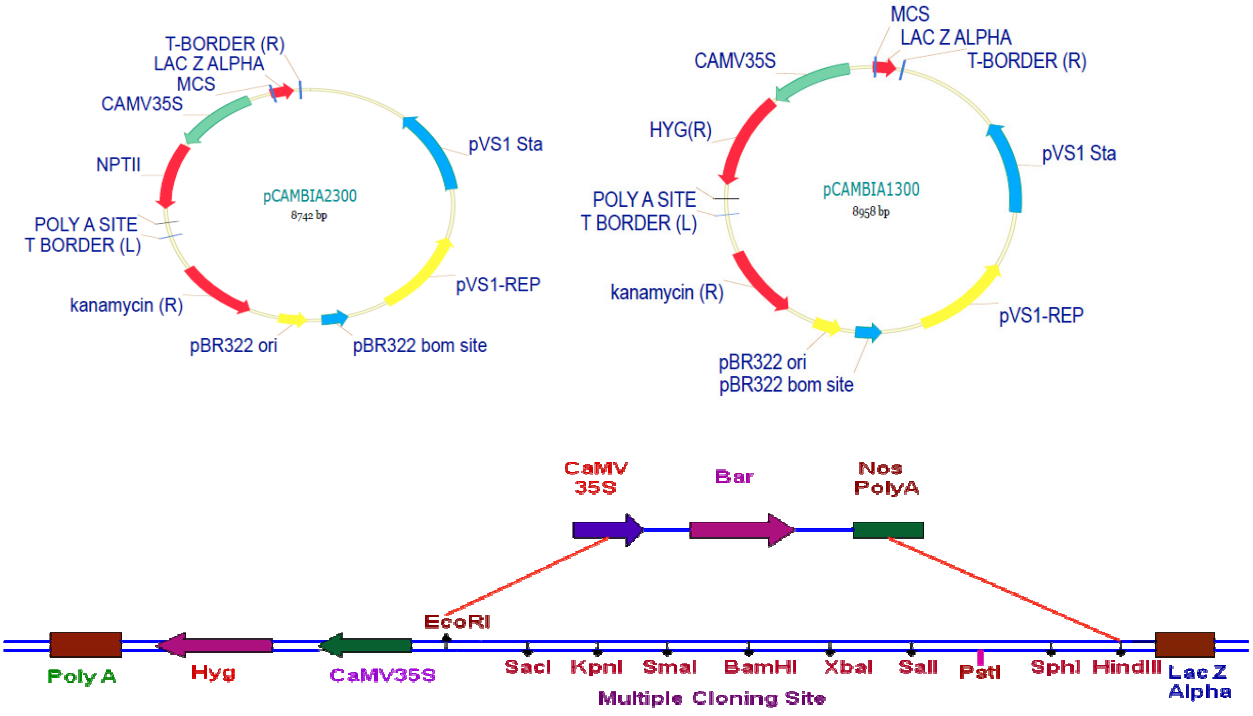
Fig. 5b. Calli growing on 3 mg/l phosphinothricin after *Agrobacterium* mediated transformation.

Fig. 5c. Calli growing on 5 mg/l phosphinothricin after bombardment.

Figure 6. Vectors and gene constructs used for genetic transformation studies



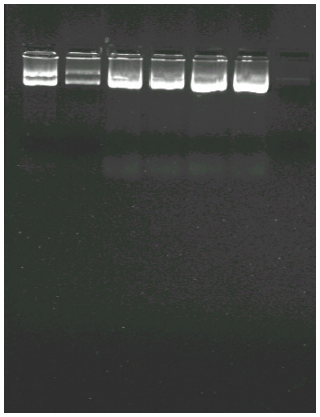
Recombinant vector map of pCAMBIA2300 - *Osmotin* and *Chitinase* genes



Recombinant vector map of pCAMBIA1300 - *Bar* gene

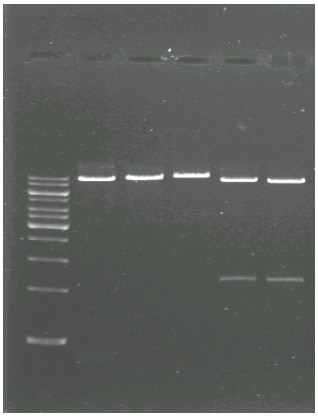
Figure 7. Plasmids of pCAMBIA 2300 containing osmotin and chitinase and pCAMBIA1300 with bar gene digestion

1 2 3 4 5 6



Plasmid DNA of pCAMBIA2300

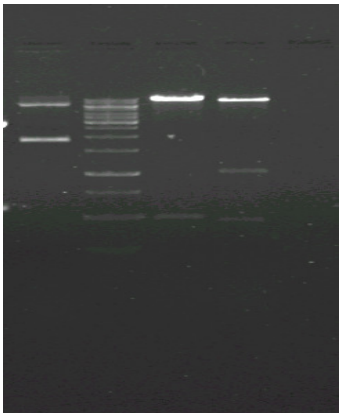
1 2 3 4 5 6



Digestion of pCAMBIA vectors

Lane 1. Ladder of 1 Kb
Lane 2. Plasmid of pCAMBIA 1300
Lane 3. Plasmid of pCAMBIA 2300 (marker free construct)
Lane 4. Plasmid of pCAMBIA 2300 (with kanamycin construct)
Lane 5. Digestion of chitinase gene (HindIII; 1.6 Kb)

1 2 3 4 5



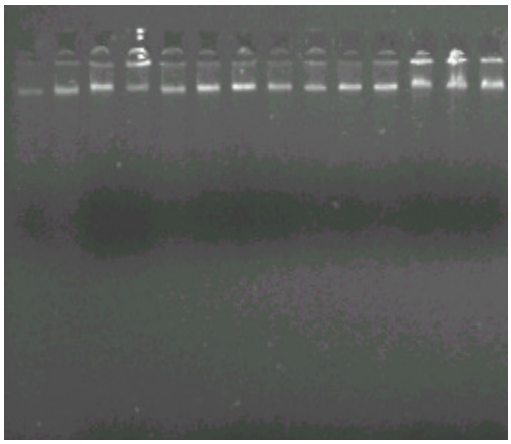
Digestion of bar gene and osmotin and chitinase genes

Lane 1. Digestion of bar gene (Hind III; EcoRI)
Lane 2. Ladder of 1 Kb
Lane 3. Digestion of chitinase gene
Lane 4. Digestion of osmotin gene (Pst I; 1000 and 500 bp)

Figure 8. Confirmation of cloning of chitinase and bar genes by restriction digestion in *E.Coli*

DNA of p2300 marker free construct and p1300 containing *Bar* gene in *E.coli*

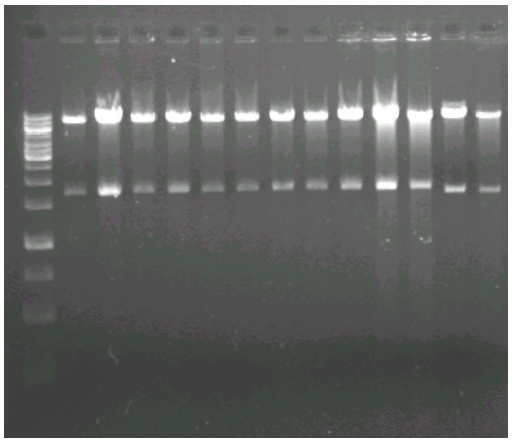
1 2 3 4 5 6 7 8 9 10 11 12 13 14



Lanes 1 to 11. Plasmid DNA of pCAMBIA2300
Lanes 12, 13, 14. Plasmid DNA of pCAMBIA1300

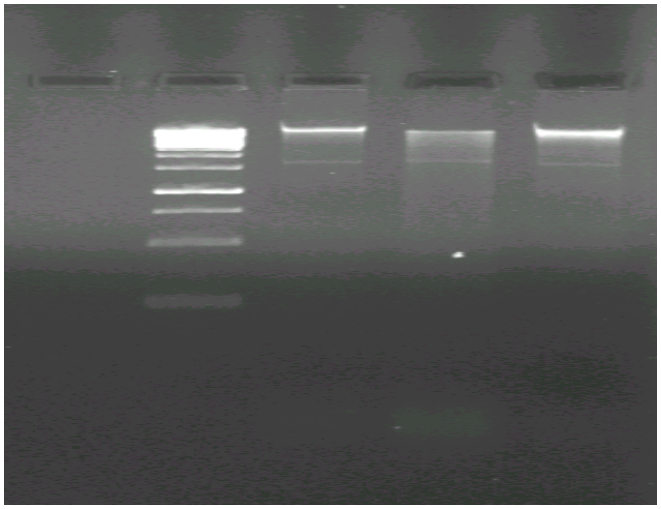
Digestion of chitinase gene in p2300 marker free construct and plasmid1300 containing *Bar* gene in *E.coli*

1 2 3 4 5 6 7 8 9 10 11 12 13 14



Lanes 1 to 11. Digestion of chitinase gene (HindIII; 1.6Kb)
Lanes 12, 13, 14. Digestion of *Bar* gene (HindIII and EcoRI; 1.6Kb)

Figure 9. Confirmation of plasmid pCAMBIA2300 with chitinase and osmotin genes and pCAMBIA1300 with bar gene insertion in *Agrobacterium*



Lane 1. Chitinase gene digestion with HidIII
Lane 2. Osmotin gene digestion with PstI
Lane 3. Bar gene digestion with Hind III and EcoRI

Figure 10. Percentage frequency of *Agrobacterim* transformation verses acetosyringone concentration in milli Molar

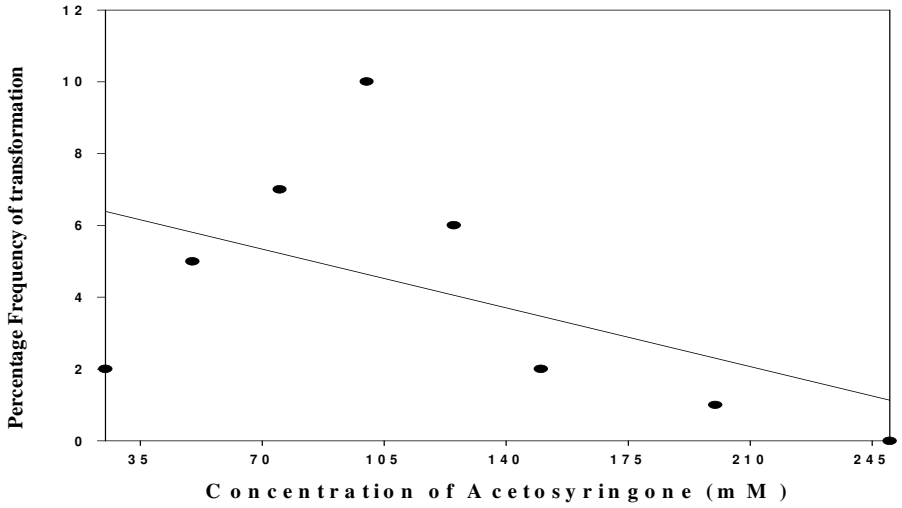


Figure 11. Transgenics generated through bombardment method growing in Net House

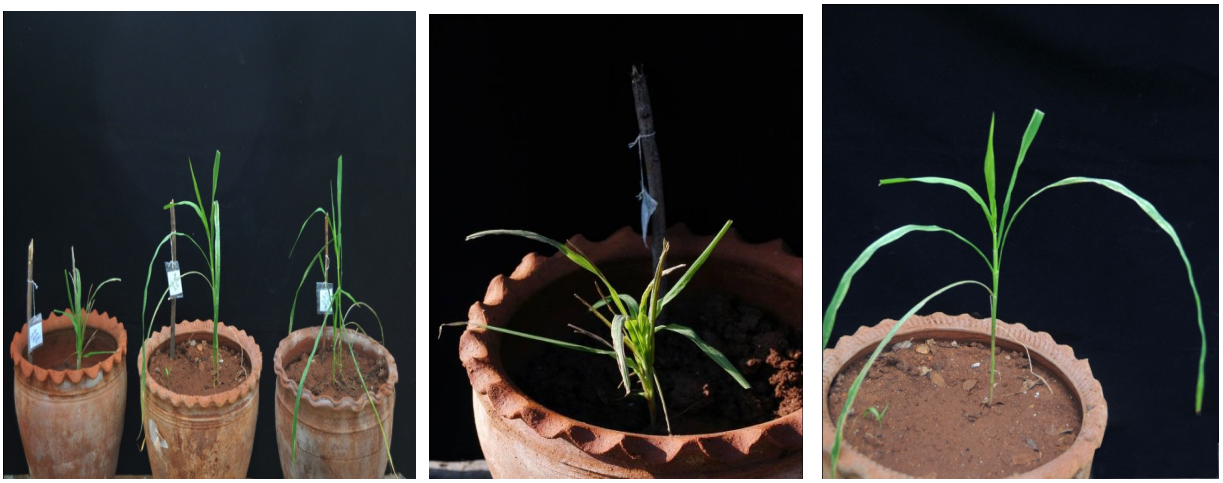
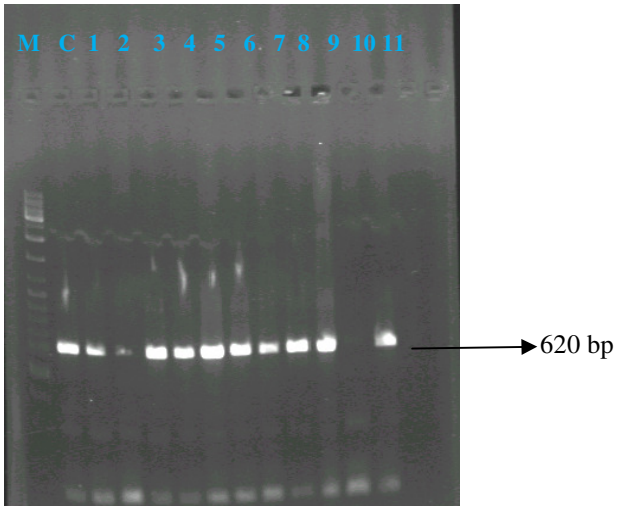
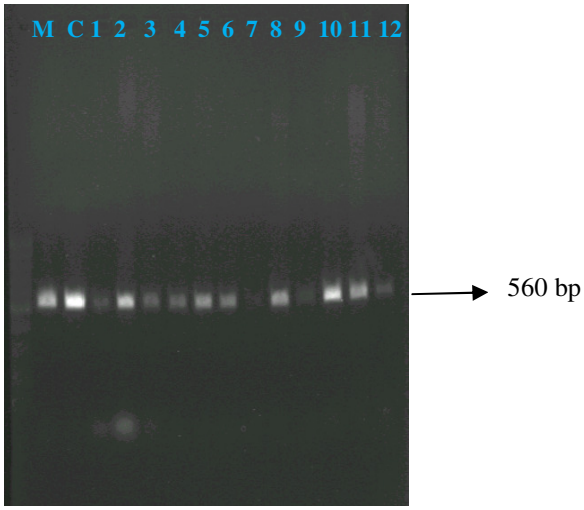


Figure 12. Amplification of chitinase, osmotin and bar genes in putative transgenics generated by bombardment

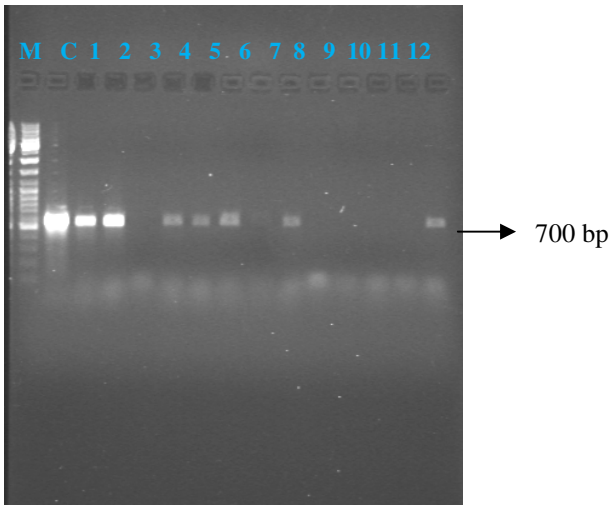
12A. Amplification of chitinase gene from putative transgenics (620 bp)



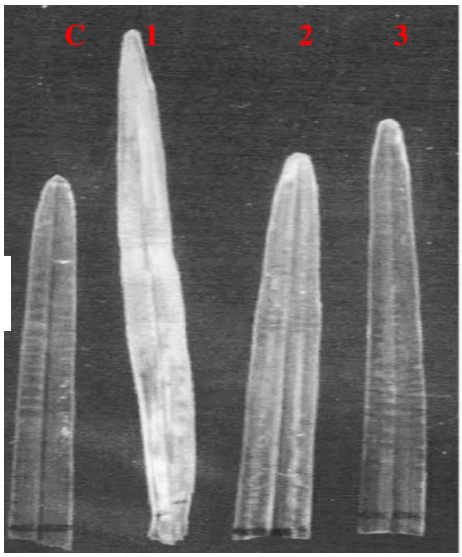
12B. PCR Amplification of bar gene (560 bp)



12C. Amplification of osmotin gene (700 bp)

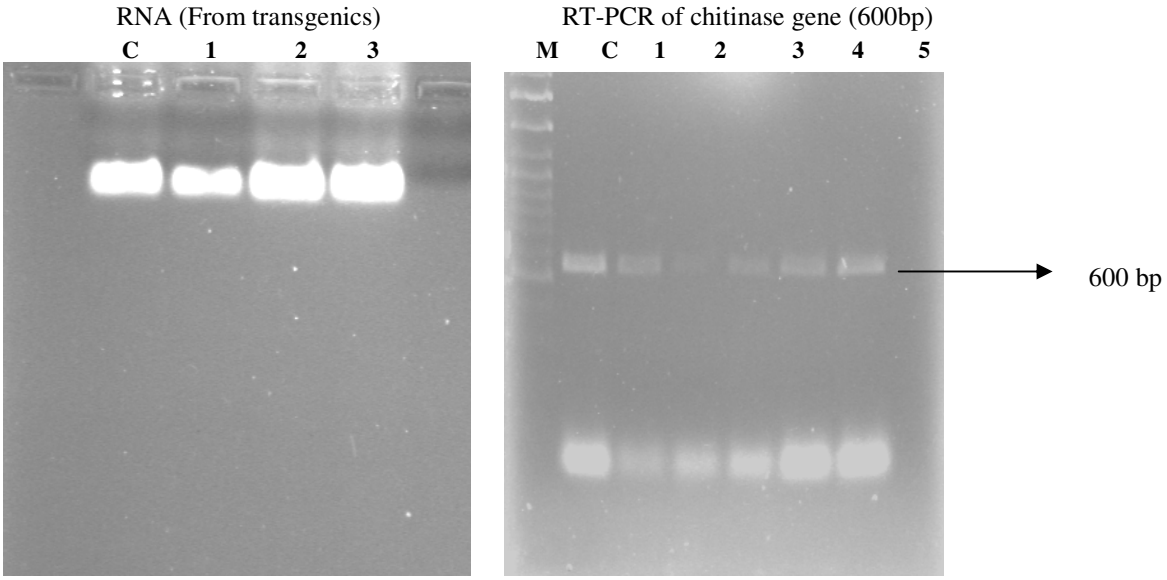


12D. Basta leaf dip assay



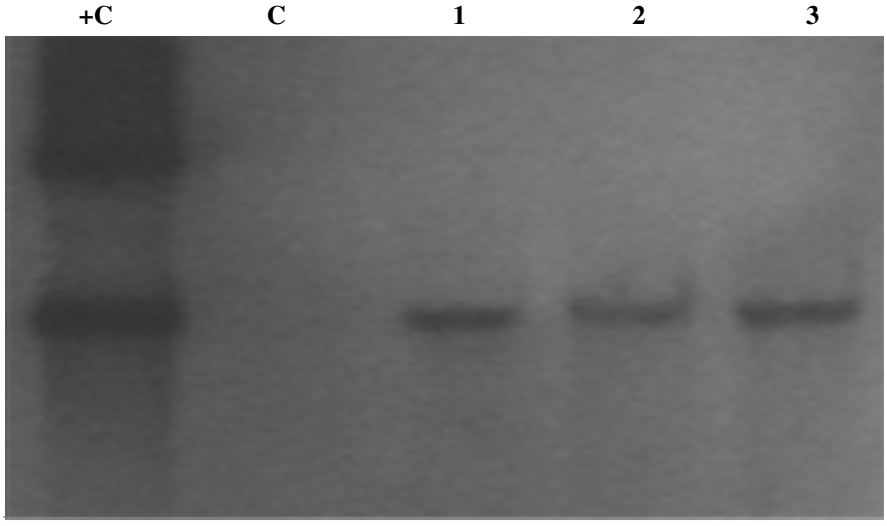
M = Marker
C = Positive control (Plasmid DNA)
1 to 12 = Transgenics

Figure 13. Extraction of total RNA and RT-PCR analysis



M = Marker
C = Positive control (Plasmid DNA)
1 to 5 = Transgenics

Figure 14. Southern blotting of chitinase gene



+C = Plasmid DNA
C = Untransformed plant
1 to 6 = Transgenics

Table 8 & Figure 15. Statistical Analysis of TILLING Population

Year	Mutagenised Seed sown	Germinated	M1 panicles obtained	M1 lines Sown	M2 DNA Isolated	M2 panicles harvested	Pooled Plates
2006-2008	31000	16218	12644	12486	9938	9938	12

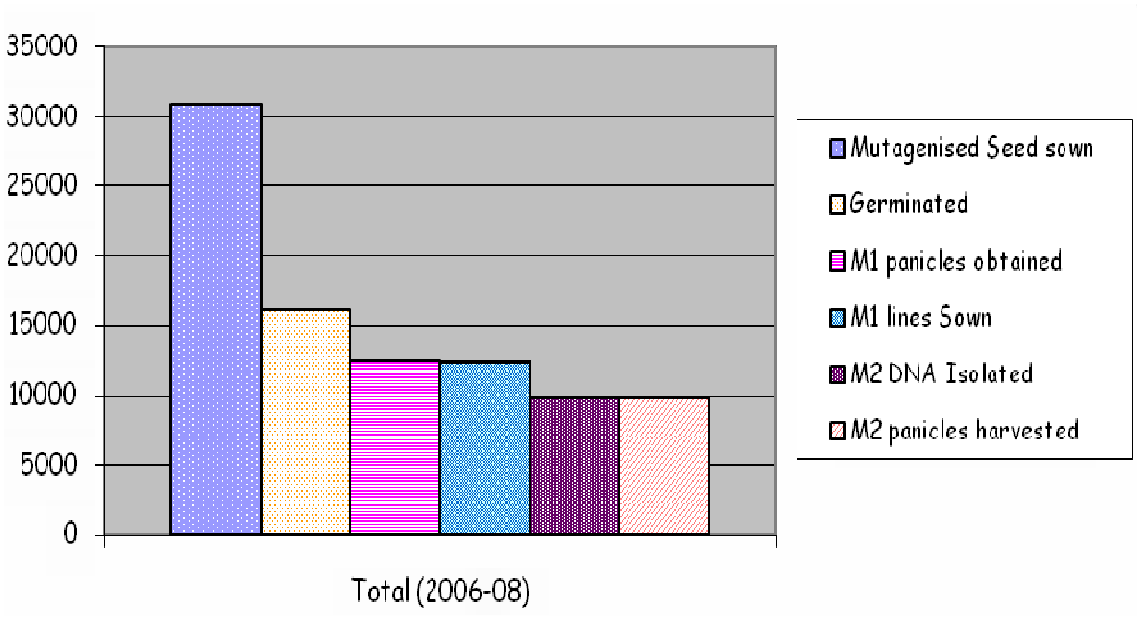


Table 9. Seedling survival and establishment status in field with different concentration of EMS at different developmental stages

Mutagen treatment (conc.)	No. of seeds treated	No. of seeds germinated	No. of seedlings survived and established in field at				
			0 week	1 week	2 week	3 week	harvested
5 mM	2000	1566	1566	1566	1487	1125	1120
7 mM	2000	1385	1385	1385	1032	786	784
10 mM	2000	1267	1267	1267	1203	841	840
Total (treated)	6000	4218	4218	4218	3724	2752	2744
Control	2000	1721	1721	1721	1721	1721	1721

Table 10. Details of M2 lines sown, germinated, DNA isolated, sterile lines and lines harvested in M2 population

Conc.	No. of M2 lines sown	No. of M2 lines germinated	DNA obtained from M2 lines	M2 lines harvested
5 mM	1118	1117	1097	1117
9 mM	784	784	781	784
10 mM	836	832	816	832
Total	2738	2733	2694	2733
Control	932	932	3	3

Table 11. Salient features of the mutants observed in M2 population generated in 2006.

Mutant line	Salient features	Mutant line	Salient features
62020	Dwarf, *DREB* relevant pathway mutant	60732	Thicker stem
62149	Half functional ovary and anthers	63817	Dark green leaves
63019	No tillering	61119	Chlorophyll mutant
63234	Semi dwarf	60157	Froked panicle
60607	Dwarf	60089	Froked panicle
63473	Early senescence mutant	62078	Partially sterile mutant
60226	Broad leave, spreading	62204	Short sterile mutant
60620	Broad leave, spreading	60156	Lesion mimic mutant
60337	Stunted growth	60153	Leaf blade lesion
60393	Early flowering	63109	Non tillering
60675	Narrow and erect leaves	63808	Vigorous
60807	Stunted growth	63736	Broad leaves
63629	Narrow and erect leaves	63043	Longest panicle
63561	Spreading	60941	Short panicle
60180	Tallest plant	63281	Lethal

Table 12. Phenotypic variant observed in M2 population generated during 2007-08

Mutant line	Salient features
70119	Chlorophyll mutant
70229	Chlorophyll mutant
70237	Chlorophyll mutant
70591	Stunted growth
70579	Chlorophyll mutant
71319	Chlorophyll mutant
71986	Chlorophyll mutant (variegated leaf)
71075	Chlorophyll mutant
81710	Chlorophyll mutant
82335	high tillering
84581	Early flowering
83438	Early flowering
81642	Early flowering
87011	Early flowering
80195	Early flowering

Figure 16. Effect of different concentrations of EMS and treatment durations on seed germination

Figure 17. Optimized mutagen concentration and field view of M1 lines

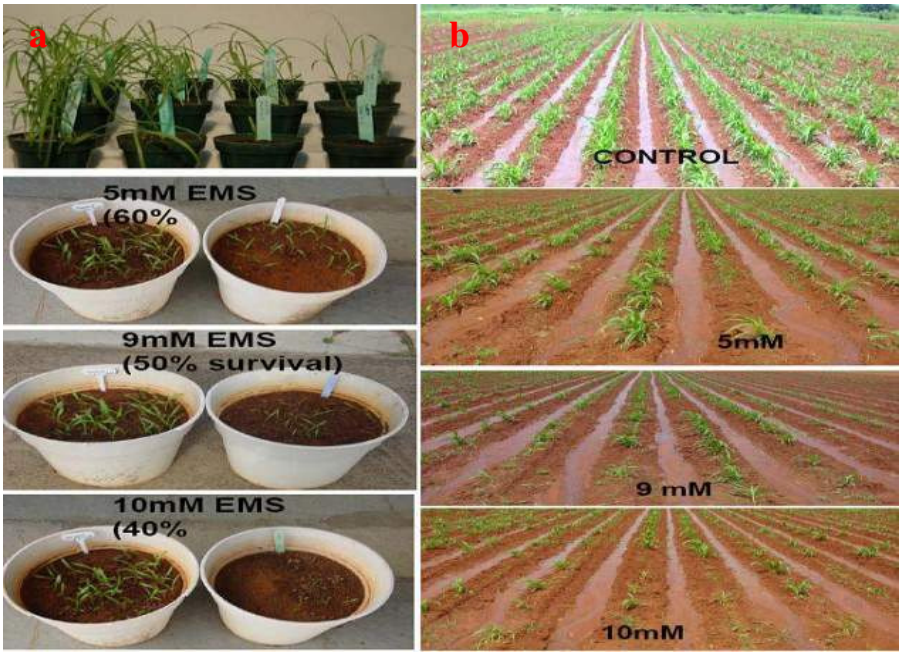


Figure 18. Occurrence of downy mildew in M1 lines, panicle reduced to leaf like structure can be visualized

Figure 19. Selfing of the mutant lines with polythene bags

Figure 20. Phenotypic variant observed in M2 population generated during 2006

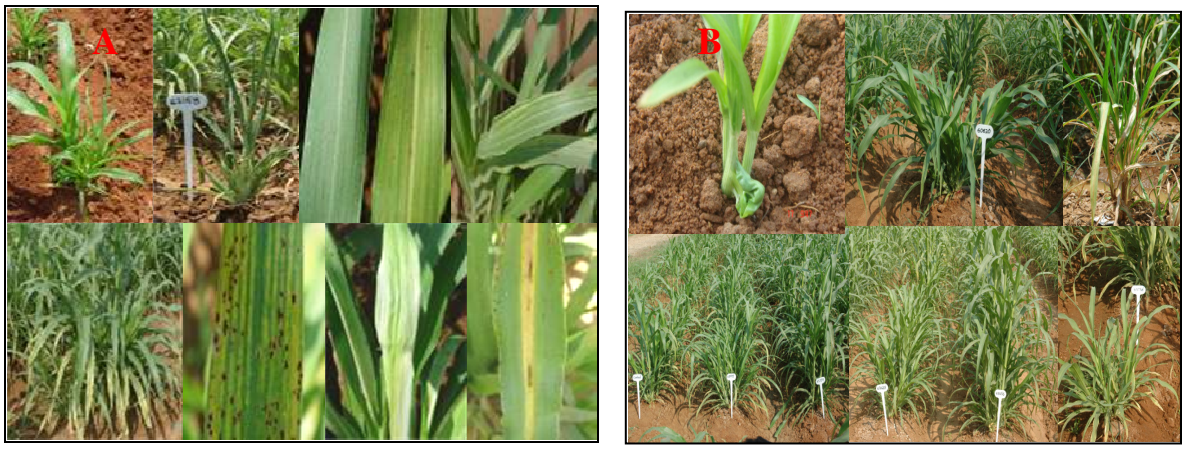


Figure A. Different form of morphological variation at early stage of plant development a) twisted seedling, b -f) variation in growth form

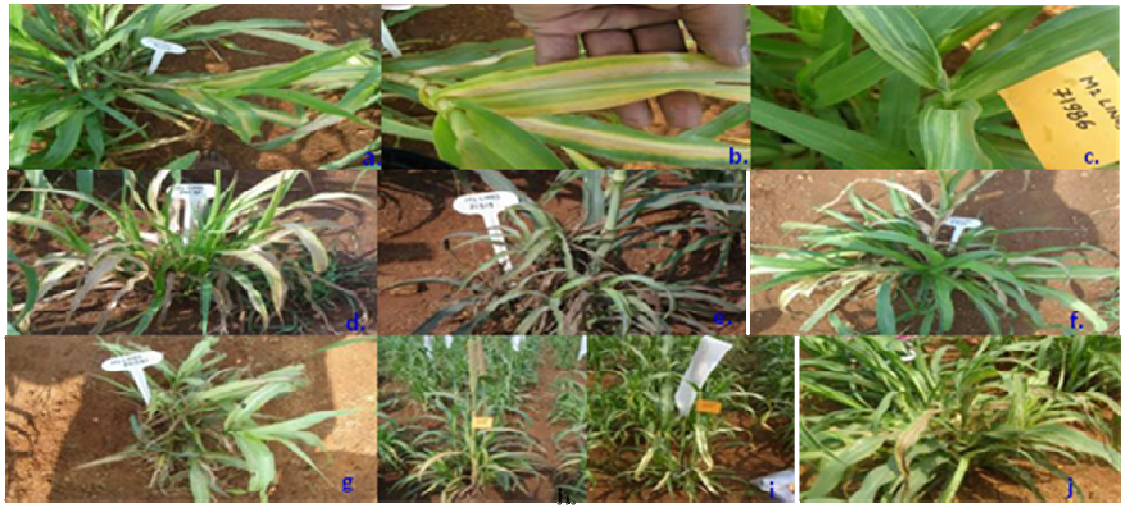
Figure B. Leaf mutants a) Broad and narrow, b) curly, c) green and yellowish and d-h) different form of chlorophyll mutants

Figure 21. Different forms of Panicles observed during M1 generation due to EMS treatment

Figure A. Different form of observed mutants at later stage of plant development, a) early flowering, b) partial grain filling, c) sterile panicle d) nil to full grain filling, e) branched, f) forked panicle, g) deformed panicle h) early senescence

Figure B. Variation in panicle size, thickness and grain fillings

Figure 22. Phenotypic variant observed in M2 population generated during 2007 and 2008



a, b, j - Chlorophyll mutants
c - Early flowering mutant
d, f - Albino mutants
e, g - Downy mildew susceptible mutant

Figure 23. Genomic DNA of M2 lines isolated using Neucleo-Spin® 96 Plant Genomic DNA Extraction Kit

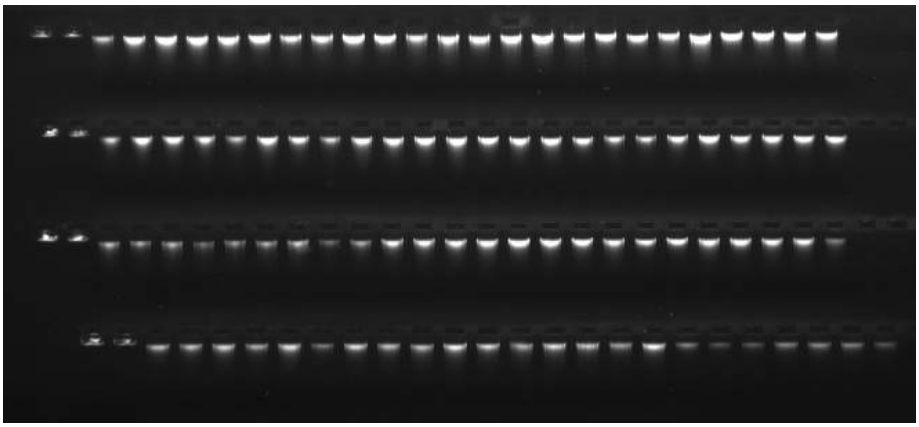


Figure 24. Normalised DNA (5ng/l) of 96 samples in a plate

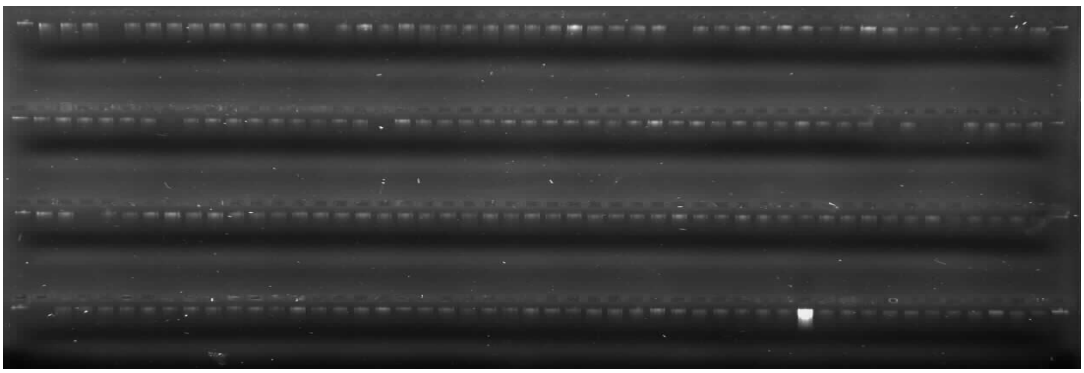
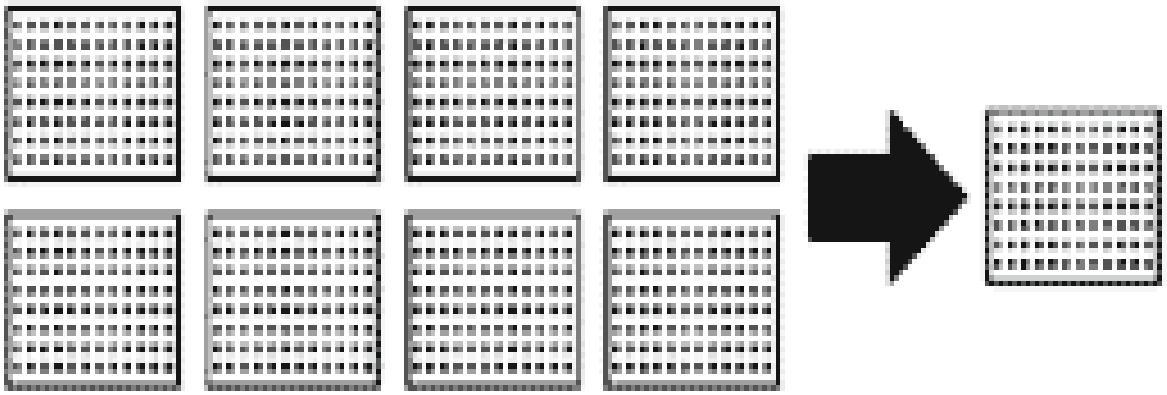
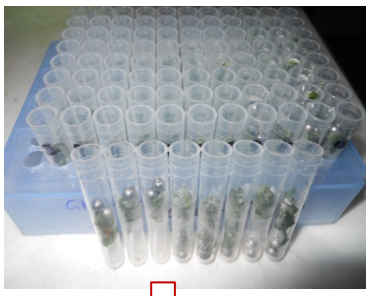


Figure 25. Schematic representation of 8-fold pooling of genomic DNA of M2 lines

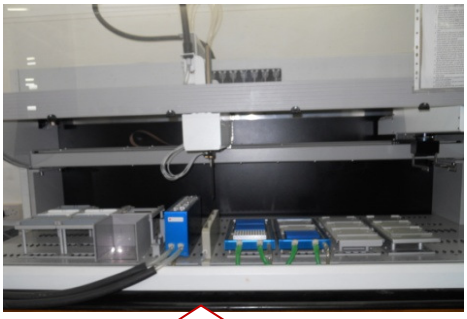


Source Plates **Destiny/ Pooled Plate**

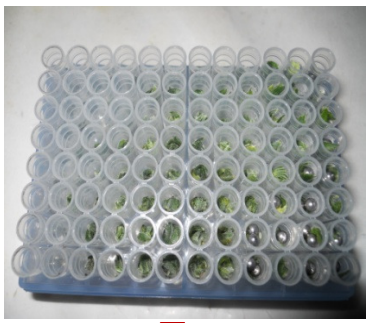
Figure 24.High throughput method



Chopping ⇩ the leaf tissue



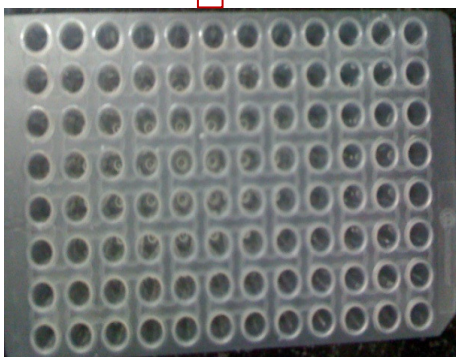
Robot machine ⇧ used for dilution



96 well plate ⇩ ready for DNA isolation

Spectrophotometer ⇧ used for Normalisation

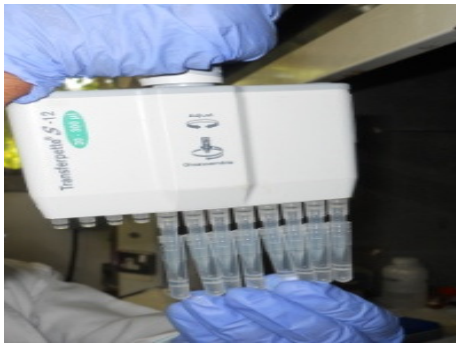
Centrifuge for ⇩ spin upto 6000rpm



DNA ⇧ storage plate

Inoculation hood for DNA isolation

⇨



Pipetting with multichannel

Figure 25. Different Stages of TILLING population in Field

Seedlings

M2 mutanted plants

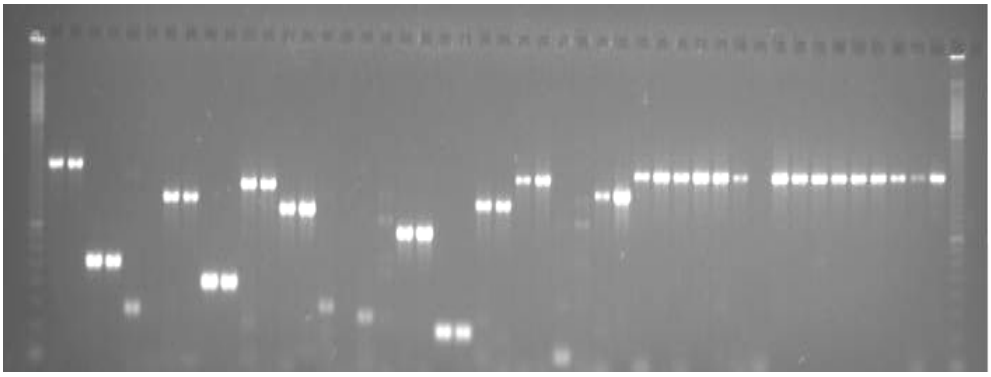
Labelling of Plants

Selfing of mutant line

Leaf collection for DNA isolation

Harvesting

Figure 28. Amplification of *DREB2A* gene with nested primers



1.F0+R0= 1-1548 (1548 bp) 2.F0+R1= 1-514 (514 bp) 3.F0+R2= 1-1397 (1397 bp)

4.F0+R3= 1-1094 (1094 bp) 5.F1+R1= 102-514 (413 bp) 6.F1+R2= 102-1397 (1296)

7.F1+R3= 102- 1094 (993 bp) 8.F2+R2= 600 -1397 (798 bp) 9.F2+R3= 600 -1094 (495 bp)

10.F3+R3= 307-1094 (788 bp) 11.F3+R1= 307-514 (208 bp) 12.F3+R2= 307-1397 (1091 bp)

13.F1+R0=102-1548 (1446 bp) 14.F2+R0=600-1548 (948 bp) 15.F3+R0= 307-1548 (1241 bp)

M = 100 bp ladder

Table 13. PCR amplification was carried out in a 25 µl volume for *DREB2A*

Table 13.1. Complete gene amplification: PCR-1: F0R0 (1.6Kb)

Components for PCR

Stock	Components	Volume 10 µl	RXN 104X
	DDH_2O	5.3	556.4
10 X	Buffer	1	104
50 mM	$MgCl_2$	0.4	41.6
10 mM	dNTP's	0.2	20.8
0.5 U	Shakara Taq	0.2	20.8
5 PM	Forward Primer	0.4	39
5 PM	Reverse Primer	0.4	39
5 ng	Template	2	

Conditions for PCR

Temperature	H:M:S	
94	00.05.00	
94	00.00.30	
65	00.00.30	
72	00.02.00	10 cycles
94	00.0015	
65	00.00.20	
72	00.00.30	35 cycles
72	00.20.00	
4	Hold	

Table 13.2. Target gene amplification - PCR-2: F3R3 (780bp)

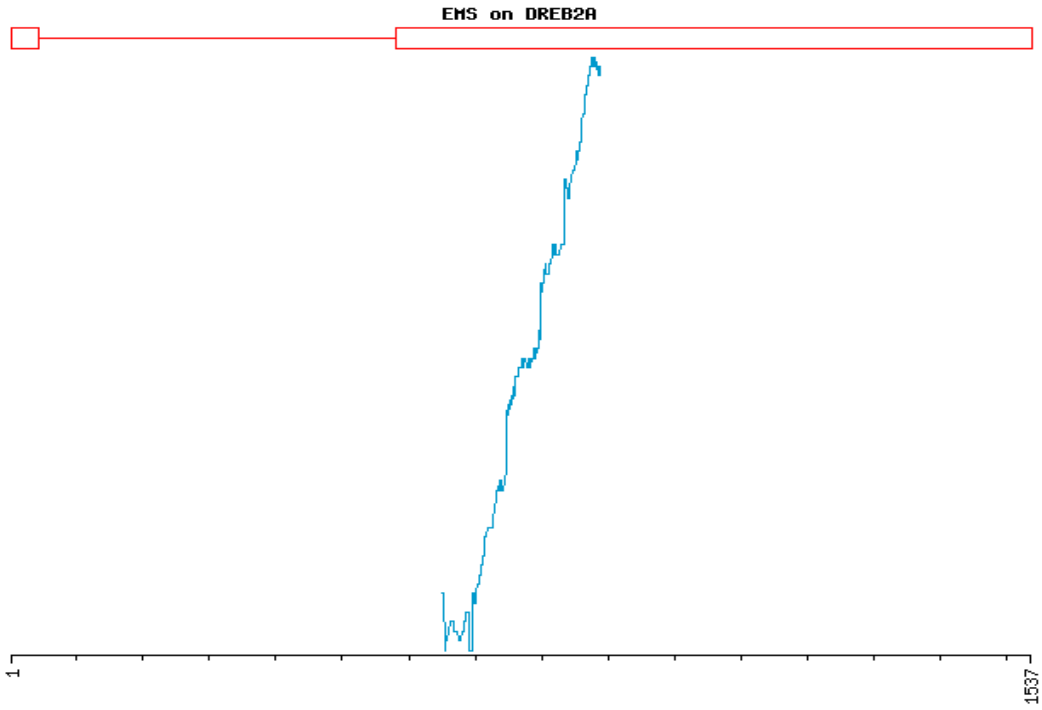
Components for PCR

Stock	Components	Volume 10 µl	RXN 104X
	DDH_2O	8.5	884
10 X	Buffer	1.5	156
50 mM	$MgCl_2$	0.9	93.6
10 mM	dNTP's	0.8	83.2
0.5 U	Shakara Taq	0.3	31.2
3 PM	Primer cocktail	1	104
10 ng	Template	2	

Conditions for PCR

Temperature	H:M:S	
94	00.05.00	
94	00.00.30	
65	00.00.30	
72	00.01.00	10 cycles
94	00.00.15	
65	00.00.20	
72	00.00.30	35 cycles
72	00.20.00	
4	Hold	

Figure 29. CODDLE



Most probable site prone for EMS mutagenesis identified using CODDLE for *DREB2A* is at 877bp

Figure 30. PCR amplification of candidate alleles prone for mutation in *DREB2A* gene using IR- labeled primers on 8-fold pooled mutant DNA samples M = 100 bp ladder

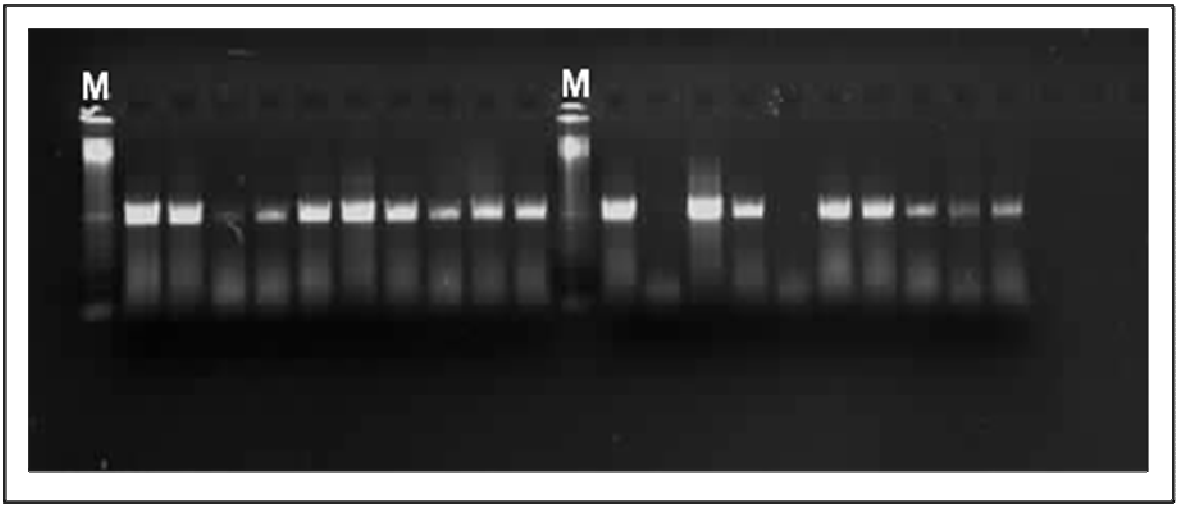


Table 14. PCR amplification was carried out in a 25 µl volume for *EDR2*

Target gene amplification: F1R3 (800 bp) (Touchdown PCR -1ºC)

PCR Components

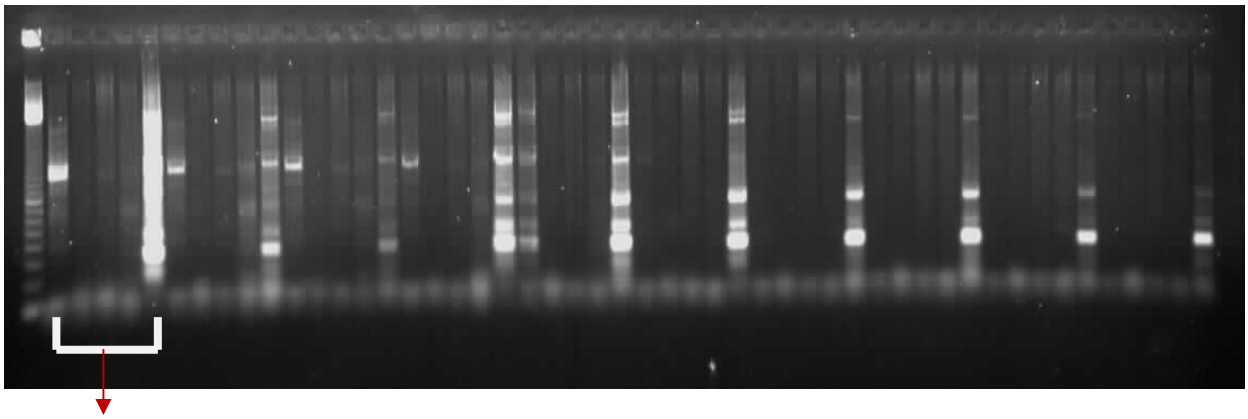
Stock	Components	Volume 25µl
	DDH_2O	
10 X	Buffer	2.5
25 mM	$MgCl_2$	1.5
10 mM	dNTP's	1.0
1 U	Shakara Taq	1.0
10 PM	Forward Primer	1.0
10 PM	Reverse Primer	1.0
50 ng	Template	3

Conditions for amplificaion

Temperature	H:M:S	
94	00.05.00	
94	00.01.00	
70	00.01.00	
72	00.02.00	10 cycles
94	00.00.45	
6o	00.00.45	
72	00.01.00	35 cycles
72	00.20.00	
4	Hold	

Figure 31. *EDR2* amplification with different degenerate forward and reverse primer combinations (1 to 5) in control unmutagenized plants

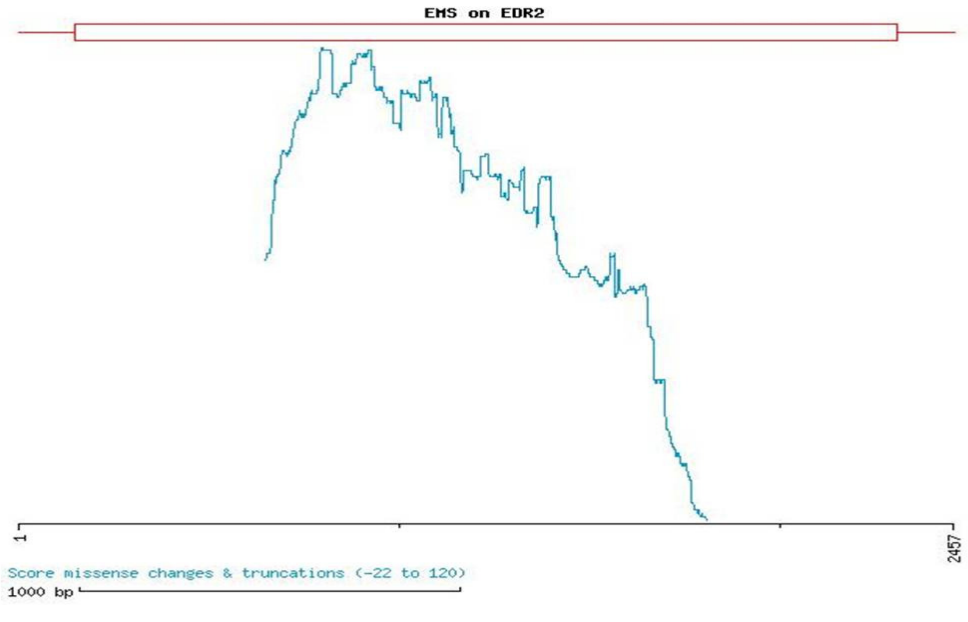
1 2 3 4 5



60.5ºC 61.5 62.5 63.5 64.5 65.0 – At different annealing temperatures

Primer combinations:
Lane: 1 F1R3-2kb Lane: 2 F2R3 Lane: 3 F3R2 Lane: 4 F4R2 Lane: 5 F5R2

Figure 32. CODDLE



Most probable site prone for EMS mutagenesis identified using CODDLE for *EDR2*

Figure 33. IR 700 and IR 800 Labelling Primers

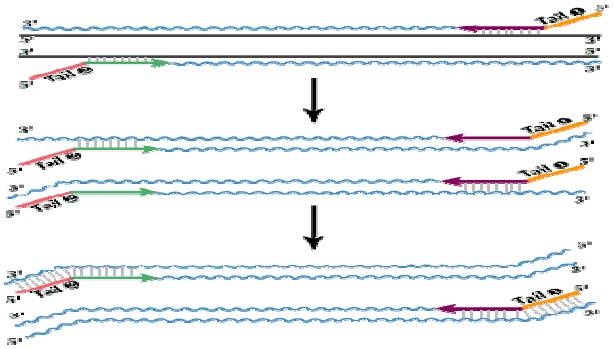


Table 15. Genes and primers used for screening the mutant population

Target name	Locus	Forward primer	Reverse primer	Amplicon size (bp)
DREB2A	>gi|77863910|gb|DQ227697.1	ATGCAGTCCTTGACTGATGG TCTGGGCATCTGTGGGTACCTGT	GCCTGTAGTCAGGGAACTG CTGGTGGCGGCATGGAGC	**1.5 kb** **780 bp**
EDR2	NM_118022.4 GI:186512033	CGGCCGGCGGAAAGATGGAGCTACG CGGCCGGCGGAAAGATGGAGCTACG	GGCGGTGTGGCCGTCAGCTACCTTCCTTC AGGCCCAGCACGCCRTTNGCNAC	**3.5 kb** **1.2 kb**

Figure 34. Protocol for TILLING Technique

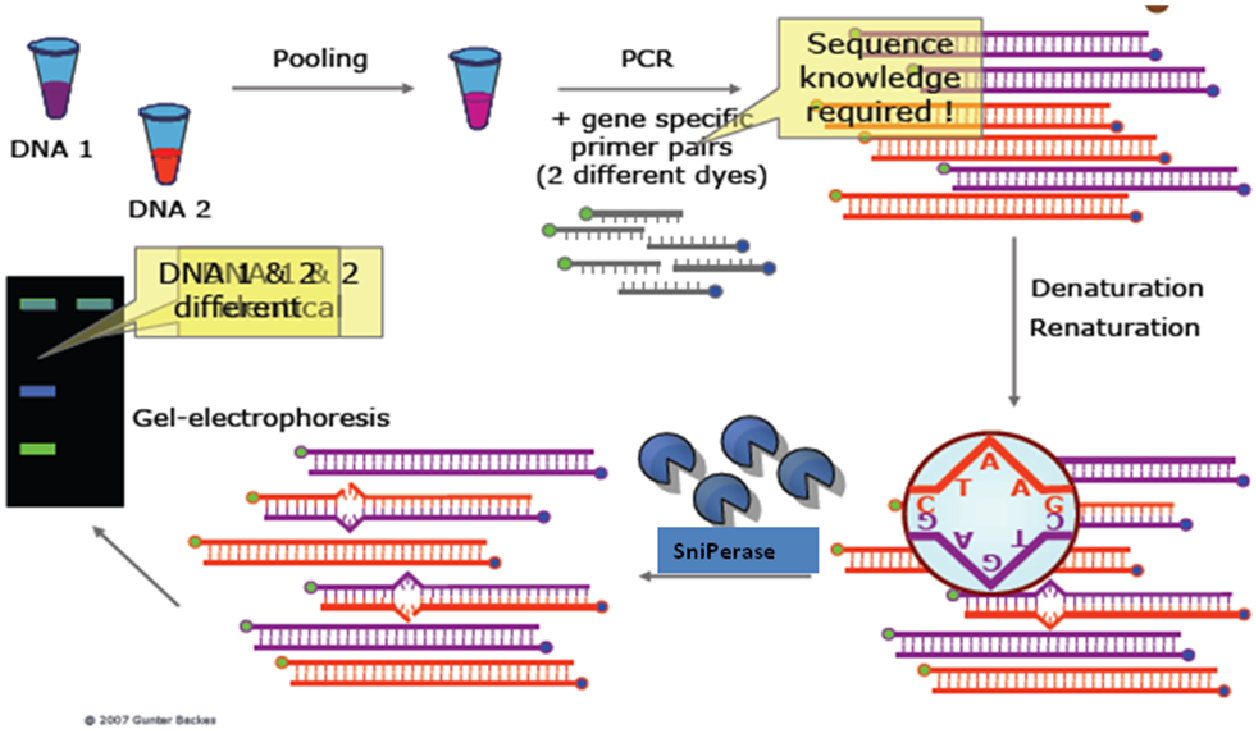
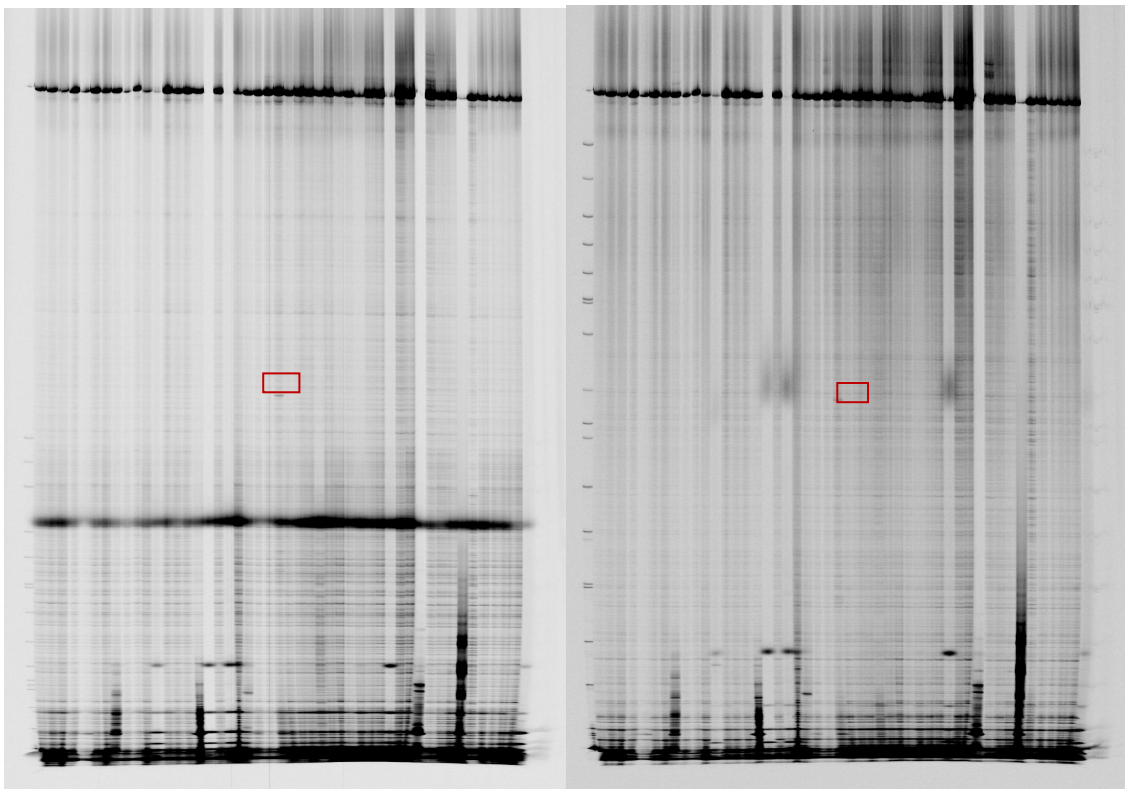


Figure 35. Identification of positive bulk for the gene *DREB2A* on mutated individuals



LI-COR TILLING Image

Even on these highly reduced gel images,bands of mutations are clearly visible(circled red). When heteroduplexes created during PCR are cleaved at base mismatches, the result is two cleavage fragments labeled with different IRDyes. With this imaging, a true mutation has two mutant bands with LICOR standards from 50-800 in the last lanes. One band is on the IRDye 700 image and the other on the IRDye 800 image. Additionally, the sum of the molecular weights of the mutant bands in a lane must equal the molecular weight of the wild type band in order for the mutation to be confirmed. Using this two-color detection method, the 4300 System virtually eliminates false positive mutation identifications.

Figure 36. SNP's in *DREB2A* mutant line by MultAlin program

```
DREB2A      AGGAACATCT GGGCATCTGT GGGTACCTGT GTGTGAAATA GTAACCTGTT
M280717R   TCCGCTTTTT  TGGTTTTTGG  GGGGACNAAT GTGTGAAATA GTAACCTGTT
M280717F   CTGTTATGCT GAGGTGACGG GGCCCCNTTT  CT...TATAA   GTAACGTGTT
Consensus    ..g...tt.T          .gG.tt.tGg          GGg.aCnt.T      gTgtgaAatA      GTAAcctGTT

DREB2A     TCTGTGGAGA TGATTGTCAT GTAGGAACAT CTGG.ACATT TGTGGCTGGT
M280717R  TCTGTGGAGA CGATTGTCAT GTAGGAAAAT CCGC.ACATT TGTGGCTGGT
M280717F   T .TGTGAA .A  TGATTGTCAT GTTGGAACAT CTGCCACATT TGTGGCTGGT
Consensus    tcTgTGgAgA    tgATTGTCAT    GTaGGAa.AT    CtGc.ACATn     tGTGGctGGT

DREB2A      AGTAG.ATTG AAGTAGTAAC GTTGTTTCAA TTTGGTTGAG CTTGCTCTCT
M280717R   AGTAG.ATTG AAGTAGGAGC GTTGTTTCAA TTTGGTTGAG CCTGCTCTCT
M280717F   AGAAACACTGAAGTAGTAAC G.GGTTTCAC  TTGGGTTGAG GGTGGACTCT
Consensus    aGtAg.AtTG      AnGtAG.Aac     Gt.GTTTCAa      tTtGGtTGAG   ..tGctCTCT

DREB2A     ACTTATTCAT TAAACAGTCC CTGTTAGTCT TTTCTGTTTA ACATGGTACA
M280717R  ACTTTTTCAT TAAACAGTCC CTGTTAGTCT GTTTTGTTTA ACATGGTACA
M280717F  AATTATTCAT AAAACGGTCC ATGTTTTTCT TTTCGGAAAATCATGGTACA
Consensus   AcTTaTTCAT  tAAACaGTcC     ctgTT.gTcT      .TtctGtttA aC     aTGGTACA

DREB2A      TTTGTTGGGT CATTTTGTTT TATTGTGTAC  TTG  ....   TAC  AAATCTGACA
M280717R   TTTGGTGGGT CATTTTGTTT TATTCTGTAC  CTGCGGTTAC  AAATATGACA
M280717F   TTAGTTGGGT CATTAAGTTT TATTGTGTAC TTGTGGTAAC AAATCTGACA
Consensus    Tn.GtTGGGT    CaTTttGTTt      TAtTgtGtAC      tTG.g.ttAC         AAATcTGAcA
```

Hot spot region for mutation according to CODDLE

```
DREB2A     GAGATACTAA  AGTGTTTCTT GTCATTTAGC AGGAAGAAGC  GTCCTCGCAG
M280717R  GAGATACTAA  AGTGGTTCTT GTCATTTAGC AGGAAGAAGC  GTCCACCCAG
M280717F  GAGATAATAA AGTGTTTATT GTCATTCAG.  AGGAAGAAGC  GTCCTCGCAG
Consensus   gAGaTAcTAA    AGTGtTTcTT    GTCATT.AGc    aGGaAGaAGn      GTCCtCgCAG

DREB2A      ATCGCGTGAT GGGCCGACCT CGGTGGCAGC TGTCATCCAG CGGTGGGCTG
M280717R   ATCGCCTGAT GGGCCGACCT CGGTGGCAGC TGTCCTCCAG CGGTGGGCTG
M280717F   ATCGCGTGAT GGGCCGAACT AGGTGGCCGC TGTAATCCAG CGGTGGGCTG
Consensus     AtcGC.TGAT    GGGCCGAcCT  cGGTGGCaGC    tGTcaTccAG      CGGTGGGcTG

DREB2A      AGCACAACAA GCAGTTGGAG CACGATTCTG ATGGCGCGAA GCGACCAAGG
M280717R   AGCACAACAA GCAGTTGGAG CACGATTCTG ATGGCGCGAA GCGACCAAGG
M280717F   AGCACAACAA GCAGTTGGAG CACAATTCTG ATGGCGCGAA GCGACCAAGG
Consensus  AGCACAACAA GCAGTTGGAG CACgATTcTG  ATGGCGCGAA  GCGAccAAGG

DREB2A       AAAGCTCCTG CTAAGGGTTC AAAGAAGGGC TGCATGAAGG GAAAAGGAGG
M280717R   AAAGCTCCTG CTAAGGGTCC AAAGAAGGAT TGCCTGAAAG GAAAAGGAGG
M280717F   AAAGTTCCTG  CTAAGGGTTC AAAGAACGGC TGCATGAAGG GAAAAGGAGG
Consensus    AAAGcTCcTG   CTAAGGGTtC    AaAGAA.Ggc     tGC.TGAAgG    GAAAAGGAGG

DREB2A     GCCTGAGAAT  ACGCACTGTG GATACCGCGG AGTGAGGCAG CGTACTTGGG
M280717R  GCCTGAGAAT  ACGCCCTGTG GATACCGCGG ACTGCGGCAG  CGTCCCTGGG
M280717F   GCCTGAGAAT ACGCACTGTG GATTCCGCGG ACTGAGGCAG  CGTACTTGGG
Consensus  GCCTGaGAAT  ACgCaCtGTG    GATaCCGCGG  AcTGaGGcAG     CGTaC.TgGG
```

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DREB2A     GTAAGTGGGT TGCTGAAATC CGAGAGCCGA ATCGGGTCAA CAGACTCTGG
M280717R  GTAAGTGGGT TGCTGAAATC CTAGAGCCGA ATCGGGTCAA CAGACTCTGG
M280717F  GCAAGTGGGT TGGTGAAATC CCAGAGCCGA ATCGTATCAA CAGTATCCGT
Consensus  GtAAGTGGGT  TGctGAAATC  C.AGAGccGa  ATcGggTCAA  CAGacTC.Gg

DREB2A     CTGGGGACCT TCCCAACTGC AGAGGATGCA GCCAGGGCTT ACGACGAGGC
M280717R  CTGGGGACCT TCCTAACTGC CGAGGATGCA TCCAGGGCTT ACGT CGAGGC
M280717F  TTGGCCAGTT TCCCCCCTGC AGATGACCCC TTCACTTTTT C .. C CGAGTC
Consensus  CTGGggAccT  TCccaaCTGC  aGAgGAtgCa  tcCagggcTT  aCg.CgAGgC

DREB2A     TGCCAGAGCG ATGTATGGAG AACTCGCCCG CACGAACTTC CCCAGTCAGA
M280717R  TGCCAGAGCG AAGTATGGAG AACTCGCCCG CACGAACTTC CCCAGTCACA
M280717F  CTCCAGAGCC CC. CATCCCA ACTTCCACCG CACCG..CTTC TCCAGTCAGA
Consensus  tgCCAnAgCg  a.gtAtggag A  acTcg.CCg  caCGaaCTTC  cCCaGTCAgA

DREB2A     AAGCAGTGGC CTCTAGCCAA GCTGCTCGGG TTCCAACCCC TGCCCAGGTT
M280717R  AAGCAATGGC CTCTC.TCAA TCTAGTGAGT TTCCACCCCA ..CCCAGATG
M280717F  CAGTC.CGGC CTCTTGCCC. . CCAGTACCT TTCCAAACCC TGCCTTGCTT
Consensus  aAGca.tGGC  CTcT.gcCaa .  Cta. T..gt  TTcCAacCCc  tgCCc.G.Tt

DREB2A     GCTCCAGCAG CTGTTGAAGG TGTTGTACCT AGCACATCAT GTGAGTCAAC
M280717R  GGTCCC.CAG CTTTTTTNGG GG.....CT CCCNCNGAAT NNNC.CCCTC
M280717F  ACATCG.... ..... .....T ACC.CTTCAT GNNGTNCAAC
Consensus  gctcC..cag ct.tt...gg .g.....cT acC.C.tcAT gnn...CaaC

DREB2A     GACAACATCA AATCACTCAG ATGTTGCATC CAGCTCGAAT AAGACGGAAG
M280717R  GATTTCCAC. .....CGCCG TNATNAAAAG NGNCN..... .....
M280717F  AACATC.... ..... .NTTTTA... .....CGAAN GGTACAGCAT
Consensus  gAcatC..c. .....c.c.g .n.Tt.aa.. ...c.cgaa. ...ac.g.a.
```

Figure 37. Genomic DNA sequence of *DREB2A* gene

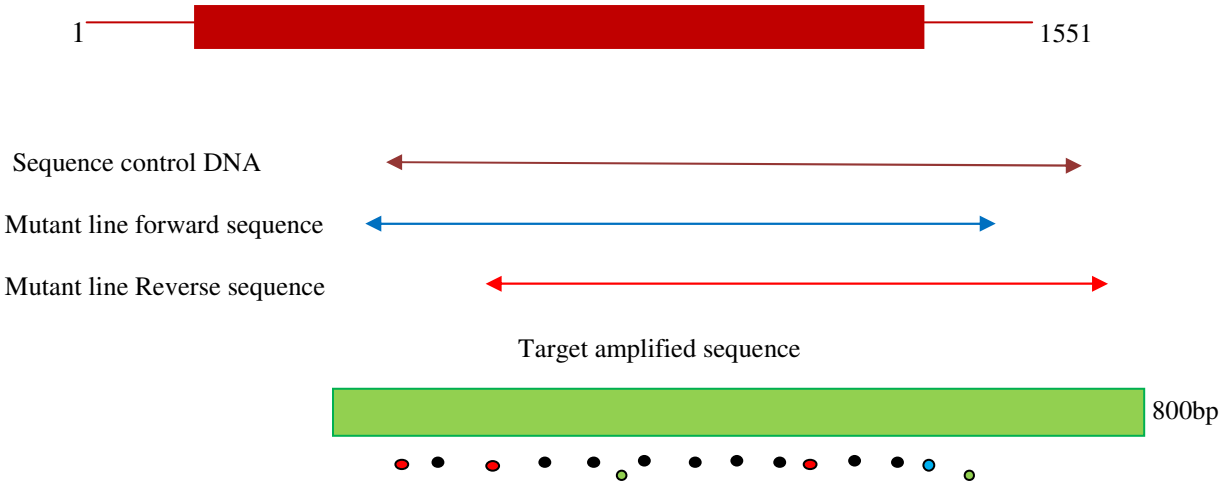


Table 16. Mutations discovered in the Pearl-Till populations mutagenized with 7.5mM EMS

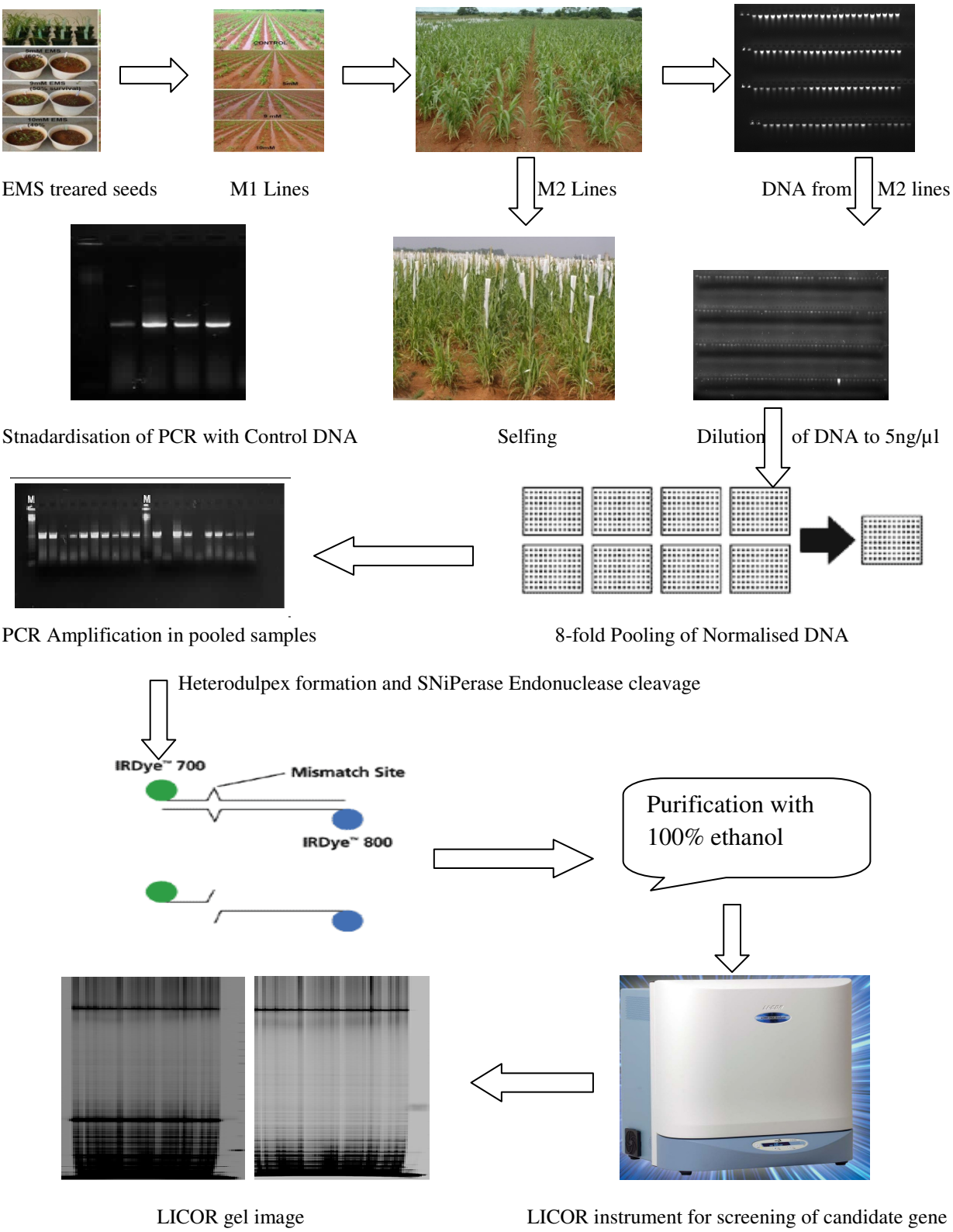
No.	Nucleotide change	Amplicon position	Aminoacid change	Restiction enzyme difference Gained in Variant	Lost from reference
1	G->T	205	G-E		
2	C->T	231	T-R		
3	A->C	245	K-T	BaeI	
4	C->A	265	E-K	BfaI	
5	C->T	276	K-Q		
6	C->A	318	P-A	BpmII	
7	C->A	328	S-R		
8	C->A	344	P-K	MmeI	BstAPI
9	G->A	374	E-K		
10	C->T	419	T-N		
11	G->A	429	K-N		BpmI
12	G->A	439	K-R		
13	C->A	465	P-H	BciVI	
14	C->A	485	A-E		
15	C->A	545	T-K		
16	G->T	550	R-R	NspI	

Figure 37. The pearl millet target *DREB2A* showing mutations discovered in test populations:
At top, the gene model is shown in red with boxes corresponding to CODDLE target of the gene. Green boxes represent target sequence. Circles indicate the location and type of mutation found. The red circles denote transversion a black transition changes a blue silent changes and green represents deletion change. The table summarizes the position and nucleotide change of each mutation and the respective effect on the protein sequence Restriction endonuclease sites that are either gained or lost due to the mutation are also listed.

Figure 38. Partial length of *EDR2* gene sequence in Pearl millet

GTAGTTAGTAGCGAGAGTAAAACCACTCAAGAGAGGAGCAGAAGGAGATTCTGACTACTTTTTCTCAA
ATGGGCTCAAAATTTAAAATGGTTCACCGTATCGAGGGGGAGGGGAAAGAGAGATGGCGAGCTCAAC
AGTGCAATGGGCCAGAAGAACTTGATCCTTGTGGTTCTTCCGACCGGCCCCCCCATGACCACGCCTTA
ACGTGTGCGTCCCCCCAAAGGGGGGTCCCGGACCGGAAGCAAATTCTGGAAAAATGGATGGGTTGAC
CCCCCTAACGAAGCTAATGAAAGGGGGGAAATTTTTTTTGGGAAGAAAAAACCGGGGAAAATAGTTC
TTTTTGCCGGTACAGAAGGGGGGGGAGAAAAAATCCCCGGGCCCCAAGGGTAATAAAGGGGCTTGGG
GAAAGGGGCTTTAAAAAACCTCGGGGGGGGGGCGCCAACCTTATTGGTTAAACCTCCCCCCCTATACC
CGGGGGGAGAAATTTCTCCCACTTTTCGCTCCCCCAATCCTGGGGGCTGCTGTCGTGATCCTATAATAC
TAGTAGATACTTGCTAGAAAAAAATACTCACAAAAAAGTGCATCACCGACGTTTGTTTATAGTTGGCG
CGCGGCAACCCATATGTATTCTCTCTCACCGCGCATAGAGATGAGAGAGAGTTGATCACAATGGTAGT
AGTGCTGCGTTTAGGAAGGAGGATACCTCTCTCACACTACTGCATCGCTACACAGAAAGCTAGTTAGT
GTCATTGCACACGCTACCATACACATCACCCGCACGAAGAAAGAATAGAAAATACAGATAGCCGTAG
CGACAGATGG

Figure 39. Pearl -TILL development at ICRISAT



CHAPTER V
DISCUSSION

Downy mildew is a major fungal disease in pearl millet caused by the fungus *Sclerospora graminicola*. There is 50-60% of the incidence of downy mildew in India depending upon the season and state and yield losses due to downy mildew disease in pearl millet are very high. Pathogensis related (PR) proteins, for example, are a group of diverse proteins whose accumulation is triggered by pathogen attack or abiotic stress. In a sense, PR proteins constitute a point where various response networks interact by reacting with different inducers such as salicylic acid, jasmonic acid, systemin and ethylene. Pathogens of plants are a significant and growing threat to crop production worldwide. The goal of producing crops with increased and durable resistance to a spectrum disease is therefore a major focus in plant research. Increasing knowledge of the plant defense has led to more sophisticated transgenic approaches to enhance resistance. The number of candidate genes put forward by transcriptomics, proteomics and protein interaction studies gives us a large choice of genes to be used. Potentially, these genes can be manipulated by over expression, induced expression and tissue specific expression.

5.1 *In vitro* plant regeneration

An attempt was made in the present study to initiate callus from the mature embryos and shoot tips of six lines of pearl millet. But the shoot derived calli from the two lines (843B-P2 and 81B-P6) regenerated shoots or plantlets with 3-5% frequency and the number of shoots formed per callus mass was only 3-4. Therefore, immature inflorescences were used as explant source for callus induction and multiple shoot formation. Immature embryos and immature inflorescences were earlier used in other cereals like *Eleusine corocana* (George and Eapen, 1990), *Paspalum* (Vikrant and Rshid, 2001; Kaur and Kothari, 2004), *Panicum maximum* (Lu and Vasil, 1982), *Pennisetum* (Devi et al., 200; Srivastav and Kothari 2002) *Setaria italic* (Vishnoi and Kothari, 1996) and *Sorghum* (Jogeswar et al., 2007). The general consensus has therefore been that immature tissues like inflorescences are most responsive for induction of embryogenic callus. However, there is no universal recipe for the good percentage frequency of callus induction and it varied depending on the genotype and explants in cereals especially with pearl millet. While 2,4-D has been the mostly used for callus induction and somatic embryogenesis in majority of cereals including pearl milet genotypes (Krishna Rao et al., 1988; Lambe et al., 1995,1999), other auxins such as para-chlorophenoxy acetic acid (Srivastav and Kothari, 2002), picloram (Kaur and Kothari, 2004), cytokinins like thidiazuron Ganeshan et al (2003), a combination of auxins and cytokinins (Devi et al., 2000; Sharma et al., 2004), ethylene inhibitors like silver nitrate (Pinus et al., 1993) also influenced significantly the formation of embryogenic callus. In the present study, though 2,4-D induced the embryogenic callus, the frequency was very less. Incorporation of NaCl into the callus proliferating medium followed by sub-culture into thidiazuron appeared essential for somatic embryogenesis in the genotypes tested in this study. The use of thidiazuron for cell and tissue cultures of monocots was rarely reported. (Ganeshan et al., 2003) used thidiazuron to test the competency of mature embryos of wheat, oat, barley and triticale for multiple shoot induction. However, this cytokinin has been bound very effective in induction of somatic embryos in pearl millet callus. Our findings are consistent with the earlier reports of Sharma et al (2005), who observed that thidiazuron is necessary to maintain long-term meristematic cultures and induction of somatic embryos in high frequency in wheat. Callus induced on 2,4-D was often found to be difficult to maintain in an

embryogenic state for long periods and also for shoot differentiation especially in *Pennisetum* (Lambe et al., 1999). While kinetin plus NAA combination was better for shoot differentiation for the genotype 843B-P2, BAP plus IAA was ideal for the 81B-P6. Similar results were reported earlier in *Pennisetum scrobicutatum* (Nayak and Sen, 1989) and *Paspalum* (Cardona and Duncan, 1997) whereas Sticklen and Oraby (2005) observed that BAP is effective in multiple shoot production but along with low concentration of 2,4-D. Long-term retention of shoot differentiating ability is desirable in cereals especially in pearl millet but is highly difficult to achieve it (Srivastav and Kothari, 2002; Kennedy et al., 2004). However, the use of NaCl for a minimum of three subcultures in the callus proliferating medium followed by thidiazuron (0.01mg/l) treatment to callus proved excellent for subsequent regeneration of shoots up to 200 days in the present study. In contrast, Srivastav and Kothari (2002) achieved it using para-chlorophenoxyacetic acid in the callus proliferating medium. The percentage frequency of shoot differentiation in 2,4-D proliferated callus was only 3 in both the genotypes (data not shown). But serial subculturing of callus into NaCl and thidiazuron media enhanced it to 95-97.5%. The number of shoots formed per culture also varied (1-52) depending on the genotype and plant growth regulator concentration in several cereals (Varshney et al, 1999; Devi et al., 2000; Ganeshan et al., 2003).In the present study, the number of shoots (25-32 per culture) formed per 100 mg of callus is high and also varied based on the growth regulator concentrations in the medium. In summary, an efficient and reproducible method for pearl millet shoot regeneration from callus cultures in two genotypes is achieved in the present study that can pave the way for genetic transformation studies. This exercise was necessary since establishment of a high frequency regeneration system is an essential pre-requisite for generation of transgenic plants.

5.2 Genetic transformation

Transfer of genes to crop plants can be achieved using several methods such as *Agrobacterium* mediated DNA transfer and particle bombardment (Christou, 1992; Sheng and Citovsky, 1996; Zhao et al., 200). Developments in genetic transformation of monocots, including pearl millet, made it possible to produce transgenic plants that express desired genes such as pathogenesis related genes to enhance resistance against diseases**.** *Agrobacterium* mediated transformation of millets has been repoted by a number of authors (Taylor, 1991; Oldach, 2001; Madhavi Latha, 2005; Lamb, 1995) but it is highly explant and genotype dependent. Further, concentration of acetosyringone and co-cultivation period plays a crucial role. The present study deals with the development of a reproducible method for genetic transformation of pearl millet by *osmotin* and *chitinase* double construct by co-bombardment mediated method and production of downy mildew resistant transgenic plants. The plant expression vector pPUR, has been used for optimising the bombardment parameters for transformation of embryogenic calli. However, <18 Kg/cm^2 helium pressure, >12 cm target distance and 2 µg DNA, 100mg/ml tungsten particles produced lesser frequency of phosphinothricin (PPT) resistant calli, probably, because of less efficient dispersion of particles into the target tissue. In Sorghum, a higher helium pressure and lower target distance was found to cause increased tissue damage leading to low frequency of transformation (Casas et al., 1995). However, >1 µg DNA/mg tungsten particles invariably increased the frequency of gene expression due to particle agglomeration, which accorded was recorded for finger millet and maize by Klein et al., (1988). The high frequency of resistant calli to PPT was

observed in the present study, by applying various pre- and post-bombardment osmotic treatments, may be ascribed to the decreased cell damage caused by increased permeability and rapid cell recovery. In earlier reports, similar osmotic treatments were found to increase the efficiency of both transient gene expression and stable genetic transformation in finger millet (Madhavi Latha et al., 2005), pearl millet (Girgi et al., 2002; Goldman et al., 2003), maize (Vain et al., 1993), wheat (Bilang et al., 1993) and tobacco cells (Russell et al., 1992). Utilizing the various optimised parameters for transient expression of the marker gene, stable transgenic pearl millet plants were successfully produced by employing pCAMBIA2300 *osmotin* and *chitinase*, CMV35S and PolyA terminator and pPUR *bar* 35S constructs. The four-step selection strategy using PPT was found effective for selecting the transformed calli and for producing stable transgenic plants. These findings are in agreement with those of Goldman et al., (2003) who could minimize the chances of escapes by applying higher concentrations of antibiotic to different transformed tissues of pearl millet. PCR analysis using *bar, chitinase and osmotin* gene specific primers revealed an amplification of 560 bp, 600 bp and 700 bp fragment in all the three Basta-tolerant putative transformants. This clearly indicated the presence of *bar* gene in various transformants similar results were reported by Madhavi Latha et al in finger millet but there is a difference in concentration of Basta used for selection. The high frequency of resistant calli to PPT observed in the present study, by applying various pre- and post-bombardment osmotic treatments, may be ascribed to the decreased cell damage caused by increased permeability and rapid cell recovery. In earlier reports, similar osmotic treatments were found to increase the efficiency of both transient gene expression and stable genetic transformation in finger millet (Madhavi Latha et al., 2005), pearl millet (Girgi et al., 2002; Goldman et al., 2003), maize (Vain et al., 1993), wheat (Bilang et al., 1993) and tobacco cells (Russell et al., 1992). Four transformants, tolerant to basta and PCR-positive for bar, were also found Southern positive for the chitinase gene, thereby suggesting the stable integration of chitinase gene into genome. HindIII digested genomic DNA with the chitinase probe disclosed the presence of hybridizable bands at 600 bp. Three of the primary transformants, exhibited the hybridizable band. Further, RT-PCR was carried out to see expression of the gene at the transcript level. The phenomenon of multiple copy integration is commonly encountered in particle-bombardment-mediated genetic transformation of plants (Finnegan and McElroy, 1994; Flavell, 1994; Kohli et al., 1996). In earlier studies, a variable number of incorporated transgene copies ranging between 1 and 20 were reported in diverse crop plants (Lambe et al., 1995; Hadi et al., 1996; O'Kennedy et al., 1998, 2004; Maqbool and Christou, 1999; Zhang et al., 1999; Lambe et al., 2000; Girgi et al., 2002; Goldman et al., 2003).

Previous research in other cereal crops such as wheat presented successful transformation and co-expression of a combination of antifungal genes such as *chi*, *tlp* and glucanase which are currently being used to breed disease resistant wheat (Chen et al., 1999; Anand, 2003a, b). Previous studies on transgenic cereals and other crops transformed with rice *chi11* or *tlp* have shown protein expression in leaf tissue (Datta et al., 1999; Chen et al., 1999; Anand et al., 2003b; Velazhahan and Muthukrishnan, 2003). Transgene silencing is an important phenomenon that frequently occurs in transgenic plants (Iyer et al., 2000) and has significant impact in transgenic research and breeding. Direct DNA transfer through bombardment frequently results in the insertion of multiple copies and rearrangement of the transgene (Matzke and Matzke, 1995; Meyer and Saedler, 1996) which can result in gene silencing. Differential transgene silencing even among progeny within a transformation event has been observed in

barley (Bregitzer et al., 2002, Kohli et al., 1999), and rice (Kumpatla and Hall, 1999). Meng et al (2003) showed that transgene silencing in barley T_3 homozygous population was related to the methylation of the first untranslated exon and 5 end of the intron in the *Ubi1* promoter complex. Moreover, there is a general concern that the Bar gene can be transmitted via pollen to wild relatives of *Sorghum* producing herbicide resistant weeds such as Johnson grass and shuttercane. Consequently, bar resistant commercial sorghum might be difficult to pass the regulatory hurdle especially in South Africa. Furthermore, the *bar* gene as a selectable marker has biosafety advantages. Transgenic pearl millet plants have been generated using *bar*, *osmotin* and *chitinase* genes in the present study and the particle gun method of genetic transformation has been found effective. For our study we used two different vectors pPUR for *bar* (marker gene) and pCAMBIA with pathogen related genes. However, the three transgenics generated in this study, did not set seed though flowered. Consequently, transgenics (T_0) could not be taken to the next generation.

5.3 Target Induced Local Lesions in Genomes

A TILLING service could provide allelic series in genes of scientific or agronomic importance. Millets have unique biological and agronomic characteristics that cannot be investigated in either *Arabidopsis* (Till, 2001) or maize model (Till et al., 2004) systems. Individual mutations may not result in phenotypic changes due to the redundant nature of the pearl millet genome. This would greatly facilitate progress in the study and breeding of pearl millet.

Two critical steps appear in TILLING: one mutagenesis and second high throughput mutation discovery. TILLING population requires eightfold pooling and amplified product size ranges from 800 bp -1.5 kb. With increasing mutagen concentration the rate of mutation also increases (Greene, 2003) but developing a well-mutagenized population is also challenging because diverse species and even varieties of the same species display a different response to mutagenic treatments (Wu, 2005). Doses of mutagen that result in high lethality of the treated seed in species such as barley (Caldwell, 2004) and rice (Till et al., 2007), may not yield the high mutation density that is seen with much less lethality in another species, such as Arabidopsis (Till, 2003) or wheat (Slade, 2005). In the present study also, we experienced such a diffiulty and showed low mutation densities (Till et al, 2003). Phenotypic analysis of M2 individuals can estimate the mutation rate. In *Arabidopsis,* it is possible to reliably score embryo lethals, a phenotype resulting from deficiency at any of hundreds of genes. This measure has been incorporated in routine mutagenesis for TILLING (Henikoff, 2004). Comparison of phenotypic versus molecular mutation rates in these populations should provide phenotypic markers for assessing future TILLING populations.

The success of chemical mutagenesis in plants depends on the seed set of the mutagenized plants and the frequency of induced mutations. The factors that affect the induced mutation rate are mutagen concentration, length of mutagen application, wash length, treated organs (pollen or seeds), and chosen ecotype (or genotype) of the target species. For example, pearl millet seeds are small and seed coat is a very thin layer. This may be one reason why pearl millet seeds need a lower dosage for mutagenesis than *Arabidopsis*, *Barley* and *Sorghum*. The concentration of 7.5 mM EMS was used in our pilot TILLING study as pearl millet appears to be very sensitive to EMS treatment. Only about 30% of the M1 plants set seeds even at 5 mM EMS concentration. At 0.3%, the commonly used concentration in *Arabidopsis*, very few M1 pearl millet plants set seeds. Moreover, the seed from fertile M1 plants

had poor germination rates. The highest concentration of EMS that produced an acceptable number of fertile plants was in 7.5 mM. Embryo lethality, however is not easily ascertained in pearl millet. The frequency of albino mutants is surprisingly high (8%) in pearl millet populations this optimal mutation rate was determined by TILLING (Wu, 2005). Through optimization of mutagenesis treatments and by choosing the lower mutagen concentration compatible with acceptable seed survival, a satisfactory mutation rate has been achieved in the present study. Nipponbare populations treated either with 1.5% EMS or by sequential soaking in 1 mM sodium azide and 15 mM MNU, showed a density of putative mutations in TILLING, ~1/300 kb. This mutation density is well suited to high-throughput mutation discovery.

Additional mutations were discovered in the present study by digesting the template DNA to eliminate an extraneous amplicon that was hampering mutation identification. An adequate mutation frequency is required to keep the number of PCR reactions associated with TILLING to a manageable number. In Till et al. (2007), the authors argued that efficient TILLING requires a population with mutation frequency of $\geq$ 1 mutation/500 kb to ensure that at least one mutation is found per gel run in the LI-COR DNA analyzer. Achieving this goal requires a high concentration of mutagen or special treatments. The experience of the Seattle TILLING Project is that, a library of mutagenized individuals is optimally suited for TILLING when in average at least one mutation is found per LI-COR gel run. Till et al., (2007) have also tested the ability to detect mismatches in pools of germplasm. Pooling is an approach that is routinely used in TILLING and may be advantageous for EcoTILLING as a first-pass screen among lines that are considered closely related. Our results indicate that pools of up to 8-fold are possible, which will allow 768 accessions to be screened in a single run for preliminary analysis.

EMS mutagenesis of pearl millet seed resulted in populations with mutation frequencies that are feasible for use in a high-throughput TILLING operation. The mutation frequencies in this pearl millet population are similar or lower than have been found in our *Arabidopsis* populations. Although the population was treated with the same concentration of EMS, the resulting mutation frequency is lower. It is possible that the genetic background could have an effect on the efficiency or toxicity of the mutagen, as has been observed in rice (Till, 2007), but differences due to other environmental or experimental conditions cannot be ruled out. It is noted that treatment of *Arabidopsis* seed batches with the same concentration of mutagen can vary in mutation frequency from experiment to experiment, probably because of the effect of environmental conditions on the plant response. So, it is expected that mutagenesis experiments performed at different locations with different mutagen concentrations may result in very different mutation frequencies. About 20% will be un-compatible mutations, and perhaps one fourth of these will be homozygous. Therefore, each M2 plant will theoretically contain at least ten profoundly affected genes per genome. This background of mutations is seen to be a major obstacle for utilizing TILLING-derived mutant alleles in plant breeding. The background mutational load needs to be removed by recombination in multiple generations of backcrossing or cross-breeding. After backcrossing for six generations, theoretically only 1.5% residual heterozygosity will remain in a mutant, so the true efficiency of removing linked, undesired mutant variation may differ significantly depending on the region of the genome (Slade and Till, 2003).

A TILLING service is currently available for *Lotus japonicus* (Perry, 2003). While much knowledge will be gained using *L. japonicus* as a model system for legume gene function, the application of that knowledge to modification of cereal (Bidinger, 2005; Hash, 2001) traits remains difficult. Given the limits of other functional genomics approaches in pearl millet (Paterson, 2008) a TILLING service could provide allelic series in genes of scientific or agronomic importance. However, the high mutation frequency combined with the ability to screen individual targets allows one to screen homeologs or gene family members individually and then combine the mutant alleles through breeding. This would greatly facilitate progress in the study and breeding of pearl millet and other polyploids in which the efficiency of mutation breeding might otherwise be low. One public service may be developed in the future at ICRISAT. A TILLING service for the pearl millet community is planned and will be accessible at the following URL: http:/ /pearltill.icrisat.org.

5.4 Collection of germplasm and maintenance

Several factors impact the establishment of useful TILLING populations in plant system such as Pearl millet and sorghum Cross pollination must be vigorously controlled to produce a high quality mutagenized population. Under normal growth conditions, pearl millet is predominantly self-fertilized with cross-fertilization (Bidinger and Hash, 2005). In Sorghum mutagenesis attempt was unsuccessful when cloth bags failed to prevent cross pollination (Zhanguo Xin, 2008). Cross pollination was prevented by covering the panicles of each mutant generation with rain-proof paper pollination bags (Lawson Bags Northfield) before anthesis. This approach effectively minimized cross pollination. In the present study, it has been found that the mutagenized pearl millet population has an adequate mutation density and low cross-fertilization, providing a useful community resource for functional analysis of pearl millet genes. The variety of visible phenotypes observed in the mutant population is a good indicator of the depth of the genetic lesions, strongly suggesting that the mutant population is altered for multiple traits of agronomic importance.

5.5 Pre-testing the amplification

Pre-testing unlabeled primers by amplifying DNA followed by agarose gel electrophoresis and sequencing should reduce the number of primer sets chosen for TILLING that amplify more than one target. It has ben found that pre-testing was successful for pearl millet targets which are known to be members of gene families. The maize TILLING service, which faces a similar problem, has successfully implemented such pre-testing in a high-throughput manner (Till, 2004). In this study, it has been found that only 80% of pearl millet primers passed pre-testing and of those, only 60% produced high quality TILLING data (similar resuls have been found in soyabean). Our observation that amplification of multiple products derived from homeologous templates reduces the ability to detect mutations agrees with that of Slade and colleagues, (2005). Clearly, robust amplification of a single target will be a requirement for pearl millet TILLING. Sequence information from homeologous or paralogous genes could be used to direct primer design toward less conserved regions. In cases, where a primer set that only amplifies one target cannot be identified, it is possible to use sequence information gathered while testing the primers to find a restriction enzyme that digests only one homeolog or paralog thus eliminating amplification from the corresponding

template DNA. Restriction digestion adds an extra step and requires larger amounts of template DNA. The step, however, can easily be done in a high throughput manner by digesting templates in 96 well formats prior to PCR and even the additional amount of DNA required would allow the many genes to be screened with the present DNA yield (1 µg/µl individual plant). To determine whether pearl millet is suitable for high-throughput mutation discovery, two targets were screened in mutagenized populations. The incompletely sequenced genome makes it difficult to define primers specific for a single gene, so that amplification of multiple products becomes a significant issue for a high-throughput pearl millet TILLING service.

5.6 IR-labelling of primers

The PCR part of the tailed primer is designed following standard rules for primer design. Having established the sequence of the PCR primer, a standard primer sequence is added. Any sequence can be used as a tail, but generally established sequencing primers are commonly employed. Typical tailed primers are 36-45 bases in length. The tailed primer method provides high quality sequence data from PCR products while using standard labeled primers. This method employs a two-part primer where a standard sequencing primer sequence or "added to the 5'-end and 3'-end of the PCR primer sequence. Such a primer essentially contains two primer sequences on one oligonucleotide. The tailed primer sequence is incorporated into the PCR product during the amplification reaction. Following purification, the PCR product is sequenced with the appropriate sequencing primer using standard labeled-primer protocols. Bi-directional sequencing strategies can be run with IR^2 systems (dual dye systems) to obtain the sequence of both strands in the same set of reactions. This approach is particularly useful in mutation analysis. In this case the forward and reverse PCR primers would contain different tail sequences. The tracking of probes labeled with IRDye 800CW has become popular for optical imaging (Fig. 33) because near-infrared fluorophores minimize the optical challenges of detecting photons in tissues. A fundamental consideration in optical imaging is maximizing the depth of tissue penetration, which is limited by the absorption and scattering of light. Scattering decreases as wavelength increases. Dyes and fluorescent proteins absorbing below 700 nm are difficult to detect in small amounts at depths below a few millimeters. In the NIR region (700 - 900 nm), the absorption coefficient of tissue is at its lowest, and light can penetrate to depths of several centimeters. Above 900 nm, light absorption by water begins to cause interference. The IRDye 680LT is highly soluble in water and is significantly brighter and more photostable than many other 700 nm near-infrared dyes tested. The spectral characteristics of IRDye 680LT are well suited for use on LI-COR imaging instruments with absorbance and emission maxima in aqueous solution and methanol of 676 and 693 nm, respectively.

5.7 Mismatch cleavage with endonuclease

The hydrophobic IRD dyes at each 5'end perform an inadvertent end-blocking function, stabilizing perfectly base-paired heteroduplexes to end-cleavage activities, whereas imperfectly base-paired PCR failure products that constitute the background are not as well stabilized and so are digested. No such improvement was seen when using endonuclease (Oleykowski, 1998) for TILLING, because binding of Taq polymerase to ends of DNA duplexes acts to protect them, and the presence of Taq in a standard TILLING reaction carried over from the amplification step.

Nibbling would not be a limiting factor when SNiPerase are used to cleave heteroduplexes that are detected on agarose electrophoretic gels by ethidium bromide staining, because the slight shortening that removes the terminal 5' base will be almost imperceptible. In higher concentrations of enzyme are needed to cleave both strands simultaneously, which increases both the rate of nibbling and the number of ends that would be attacked during the reaction. It has been found that SNiPerase (From Frontiers Genomics kit) are also capable of double-strand cleavage when assayed by agarose gel analysis (data not shown), an activity that has been documented for the Surveyor™ preparation of SNiPerase. Their model can also account for nicking of supercoiled plasmids, for cleavage at sites of Y junctions and damaged base pairs and for digestion of looped-out regions by these enzymes. Single base pair mismatches are the smallest bulged-out regions and are the least susceptible to cleavage. Unless conditions are optimal for SNiPerase to bind and cleave these small bulges, nibbling of end-labeled duplexes predominates, decreasing sensitivity. The ability of a variety of SNiPerase to cleave mismatches in our TILLING assay has implications for genetic technologies. At present, high-throughput TILLING appears to be the method of choice for mutation scanning and has potential for SNP discovery applications. By default, our TILLING project uses 8-fold pooling and obtains consistent detection of heterozygous bands present at 1/16th the level of wild-type; higher levels of pooling would increase efficiency and lower the cost of the overall process. Higher efficiency might be achieved in part from continuing improvements in PCR technology that would reduce the intensity of the nuclease-resistant background banding pattern. As has been noticed in the present study, optimization of SNiPerase conditions can also improve detection by increasing mismatch cleavage relative to end. SNiPerase from kit of choice for TILLING and EcoTILLING of highly polymorphic regions. It is anticipated growing understanding of the mechanism by which these enzymes discriminate between mismatches and duplexes will result in improved genomic methodologies.

5.8 LICOR

The strength of the 4300 System for TILLING is being proven every day in multiple labs around the world.When heteroduplexes created during PCR are cleaved at base mismatches, the result is two cleavage fragments labeled with different IRDyes. With two color imaging, a true mutation has two mutant bands below the wild type band in the same lane. One band is on the IRDye 700 image and the other on the IRDye 800 image. Additionally, the sum of the molecular weights of the mutant bands in a lane must equal the molecular weight of the wild type band in order for the mutation to be confirmed. Using this two-color detection method, the 4300 System virtually eliminates false positive mutation identifications. TILLING can be used as a high throughput screening technique. With the 4300 System as the enabling technology, the following TILLING performance results can be achieved with a single instrument.

• Up to 750,000 base pairs can be screened per run.

• Up to 2000 samples can screened per day.

• Up to 2 million base pairs can be screened per day.

• 1000 base pairs can be screened per sample.

Results are dependent on species and other factors.

• The 4300 System is fast and efficient.

• It takes 2-4 hour run time (dependent on the size of the amplicon).

• Pre-mixed acrylamide gel formulations are available.

• Gels can be reloaded.

LI-COR sequencers can routinely provide 800-1100 nucleotide one-pass reads with accuracy levels better than 99% on single and double stranded DNA templates. The four lane presentation format also permits the rapid review and editing of sequence reads, however, this format allows the analysis of fewer reactions on a single gel.

5.8.1 Requirements for TILLING in LICOR

Point mutations of genes making discovery a challenge for the researcher and the detection instrument. Sensitivity of the instrument is critical for detection. The ability to distinguish false positives is of equal importance. Gel images from slab gel electrophoresis have emerged as a data format well suited for TILLING. On gel images produced by an instrument with high sensitivity, new bands resulting from mutations are easily identified.

5.8.2 Generating high quality TILLING images

The LI-COR4300 DNA Analysis System is uniquely suited for TILLING because it uses two color infrared fluorescence detection to generate two true gel images during electrophoresis. LI-COR is a leading innovator in the development of infrared fluorescence labeling and detection systems for proteomic, molecular imaging, and genomic research.

5.8.3 Infrared detection for highest sensitivity

The high sensitivity of infrared detection in LI-COR4300 System is critical because endonuclease activity in TILLING results in some lost signal. The inherent low background of infrared detection compared to visible detection, combined with LI-COR IRDyes, results in another key advantage wide dynamic range. Wide dynamic range is critical for resolving weak mutation bands along with strong wild type bands. Low infrared background from amplification artifacts makes it easy to resolve mutations with the 4300 System.

5.9 CODDLE

The Codons Optimized to Detect Deleterious Lesions (CODDLE) program facilitates the choice of the gene regions more suitable for TILLING of genes by assessing the probability of obtaining deleterious lesions and then interpreting the results obtained, which is an important part of the TILLING procedure. CODDLE identifies the gene region with the highest frequency of potential G to A changes that are likely to produce truncations of the gene product of missense alleles predicted to be damaging to the gene product. These predictions are based primilarly on the asssumption that blocks of conserved amino acid sequences are functionally important and the non-conserved amino acid changes within these blocks are more likely to be severely deleterious. A key component in TILLING has been the bioinformatics tool CODDLE, developed by the proWeb group (http://proweb.org), which is used to select regions of a gene most likely to yield useful mutations.

5.10 SNP in DREB in pearl millet TILLING

An adequate mutation frequency is required to keep the number of PCR reactions associated with TILLING to a manageable number. In Till et al. (2007), the authors argued that efficient TILLING requires a population with mutation frequency of ≥ 1 mutation/500 kb to ensure that at least one mutation is found per gel run in the LI-COR DNA analyzer. Achieving this goal requires a high concentration of mutagen or special treatments. In the present study 4 differen mutant lines were observed under LI-COR ou of 9938 mutant lines. This means, detectable mutattion rate is 0.04%. The estimated mutation density was reported for *Arabidopsis* is 1/300 kb (Greene, 2003), but it is slightly higher in barley (Caldwell, 2004) or rice (1/Mb) (Wu, 2005), but similar to the mutation density reported in soybean 1/550 kb (Cooper, 2008) and pea 1/669 kb (Triques, 2007). Given an average mutation frequency of 1mutation/0.5 Mb, a haploid genome size of 5,000 Mb for barley, and genes with an average ORF length of 1.5 kb at an average G/C content of approximately 80%, the presence of approximately 100 induced SNPs that affect ORFs can be assumed for every plant of the TILLING population. Of these, about 20% will be non-synonymous mutations, and perhaps one fourth of these will be homozygous. Therefore, each M2 plant will theoretically contain at least ten profoundly affected genes per genome. This background of mutations is seen to be a major obstacle for utilising TILLING derived mutant alleles in plant breeding. The background mutational load needs to be removed by recombination in multiple generations of backcrossing or cross-breeding. After backcrossing for six generations, theoretically only 1.5% residual heterozygosity will remain in a mutant, so the true efficiency of removing linked, undesired mutant variation may differ significantly depending on the region of the genome.

In tests of *DREB2A* on TILLING population, two putative SNP have been detected in 9938 pearl millet mutant lines. Many SNPs was detected in these distant contrasts lines, that appears to be absent from most wild species but present in mutant lines were found in our pearl millet *DREB2A* analysis has been found similar wih that of rice *OsDREB* sequence analysis in mutant population (Till, 2007). Till et al., have also tested the ability to detect mismatches in pools of germplasm. Pooling is an approach that is routinely used in TILLING and may be advantageous for EcoTILLING as a first-pass screen among lines that are considered closely related. Our results indicate that pools of up to 8-fold are possible, which will allow 768 accessions to be screened in a single run for preliminary analysis. By improving the throughput of the system, we will be in a position to identify variants in the large collection of germplasm accessions in the International Pearl millet Genebank Collection held at ICRISAT.

EMS is reported to have a mutational bias for 5'-PuG-3' sites or a middle G base in a stretch of three or more G bases (Till, 2001). Therefore, TILLING gene targets with an elevated G/C content may yield higher mutational densities. The mutational density may change after additional screening of other genes and/or more mutant lines from the population or by changing the pooling strategy. In these systems, EMS-induced changes were mostly GC>AT transitions, as expected from the frequent alkylation by EMS of guanine residues (Sega, 1984; Greene, 2003). In our EMS-treated pearl millet population, we found 80% GC->AT, 3% AT->GC, 10% GC->TA, and 10% AT->TA in the analysed *DREB2A* sequence. Pearl millet, therefore, might differ from Arabidopsis, maize, rice and wheat in its mutagenic response to EMS. Both transitions (GC->AT, and AT->GC) and transversions were observed

after sodium azide treatment in barley (Olsen, 1993). In Arabidopsis, maize, and wheat, more than 99% of EMS-induced mutations are G/C to A/T transitions (Greene, 2003; Comai, 2006). In contrast, the percentage in rice, barley, and Drosophila ranges from 70-84% (Vogel, 1995; Wienholds, 2003; Perry, 2003). In EMS-treated soybean populations, the percentage of G/C to A/T transitions was in the range of these previously published frequencies (A = 92%; B = 75%; C = 92%). Given the sequences of the *DREB2A* targets, the distribution of mutations was as expected. The majority of induced mutations were G/C to A/T transitions. But, one can discover additional mutations by digesting the template DNA to eliminate an extraneous amplicon that was hampering mutation identification.

Stress-related genes are found in all plant species; earlier studies have shown that the over-expression of *AtDREB2A* and *OsDREB2A* in *Arabidopsis* was not sufficient for the induction of target stress inducible genes (Liu et al., 1998; Dubouzet et al., 2003) and it was assumed that some post-translational modifications, probably phosphorylation and/or dephosphorylation events, may be necessary to play a role in activating the expression of stress-responsive genes. In eukaryotes, reversible phosphorylation is one of the common mechanisms through which extra-cellular signals regulate transcription of genes (Hunter and Karin, 1992). Reversible phosphorylation can modulate transcription factor activity by affecting their translocation from the cytoplasm to nucleus, their transactivation capacity and/or their DNA-binding activity (Hill and Triesman, 1995; Hunter and Karin, 1992).

The Arabidopsis TILLING project has provided the most comprehensive set of data available upon which to assess the spectrum of mutations induced by EMS. In 99% of cases, EMS generated G to A and C to T transition point mutations and its effectiveness in inducing a high frequency of point mutations in a wide range of organisms in the absence of gross chromosomal abnormalities, as expected from its established mode of action and induced changes in genes that were randomly distributed across the *Arabidopsis* genome. Mutations in DNA can be categorized as silent, missense or truncation depending upon how they affect the encoded protein. Across all 1890 mutations in *Arabidopsis*, the observed distribution of silent, mis-sense and truncation events was close to the expected distribution, as was the overall heterozygous: homozygous ratio of 2:1. The one clear exception was that truncation events were detected 3.6 times as often in heterozygotes, suggesting that such severe lesions are frequently deleterious to plants in the homozygous or haploid condition.

5.11 *EDR2* gene amplification

EDR2, a candidate susceptible gene for downy mildew resistance was amplified in the reference genotype P1449-2-P1. With the assumption that *EDR2* acts as a negative regulator of cell death, it is known to activate the salicylic acid signal transduction pathway in adjacent living tissue in a positive feedback loop that amplifies signal transduction via this defense pathway. Because loss-of-function mutations in the *EDR2* gene confer enhanced disease resistance to powdery mildew, EDR2 probably functions as a negative regulator of downy mildew resistance. The amplification of the *EDR2* with different sets of degenerate primers was carried. Some faint nonspecific bands were observed along with the target band amplification. So, it became difficult for standardizing TILLING protocol in control and pooled DNA samples, because after digestion with endonuclease, more bands

were observed in the pooled samples (mutant lines) than in the control. So, the target bands in LI-COR gel could not be detected. However, the amplified product was sequenced, which showed 20% similarity with pathogen related protein sequences of rice and sorghum.

5.12 High-throughput large-scale applications of TILLING

TILLING is a high-throughput, low-cost method that is suitable for a large-scale production operation. The first production-scale TILLING service to be offered was the Arabidopsis TILLING Project (ATP; http://tilling.fhcrc.org:9366/). Several computational tools were adapted to provide a completely web-based system for target selection, primer design and evaluation of sequence-verified mutations. Mendelian ratio of heterozygous to homozygous mutations in samples pooled eight-fold in *Arabidopsis*. Approximately 90% of orders supplied by users are successful, with occasional failures usually attributable to primer design. ATP was used as a model to create maize (http://genome.purdue.edu/ maizetilling/) and Drosophila (Fly-TILL, http://tilling.fhcrc.org:9366/fly) TILLING services. There are alsoseveral independent TILLING services, including a service for lotus (http://www.lotusjaponicus.org/tillingpages/homepage.htm) and barley which illustrates the value of the method for production scale reverse genetics (http://www.scri.sari.ac.uk/programme1/BarleyTILLING.htm). The high throughput TILLING in pearl millet TILLING population of 9938 lines at ICRISAT (http/www. pearltill. icrisat.org /stauts.html) will be developed in future. This should serve the scientists across the globle who are interested in mutation screening and subsequent utilization in breeding programs that are aimed at crop improvement.

Taking cognizance of the entire work, following conclusions are drawn.

5.13 Genetic transformation studies in pearl millet

- Both organogenic and embryogenic calli from immature inflorescences of the genotypes 81B-P6 and 843B-P2 was initiated in MS medium containing 2,4-dichlorophenoxyacetic acid. This was confirmed by histological studies of the callus.

- Shoot formation from callus cultures was obtained with different frequencies. Whole plant regeneration was achieved and plantlets were successfully transferred to the pots with 60% frequency.

- Different parameters such as concentration of DNA, microcarrier size, vacuum pressure, distance from rupture disk to the microcarrier and to the target tissue were standardized using callus for *Agrobacterium* mediated genetic transformation in the genotype 81B-P6.

- Concentration of acetosyringone versus the percent frequency of transformation was standardized. Putative transgenics were selected on phosphinothricin selection media and whole plants were regenerated and

transferred to net house, though the percent frequency is low. A total of 44 plants were generated from the line 81B-P6 using microprojectile bombardment and *Agrobacterium* methods.

- T_0 generation plants were confirmed by PCR amplification (using gene specific primers) of *osmotin*, *chitinase* and *bar* genes and RT-PCR (chitinase). But the plants could not be taken to the next generation since all the transformants were found to be sterile. But the experiment was repeated and the transgenic nature of these plants was confirmed with PCR analysis, RT-PCR and BASTA test. Putative transgenics obtained from the second batch of callus tissue also appeared to be sterile**.**

5.14 TILLING population and screening for point mutations

- A mutant population in pearl millet genotype P1449-2-P1 was generated using EMS-mutagenesis. Different concentrations of EMS were standardized and all seeds were treated with 7.5 mM EMS for M1 germination in two batches to reach the target population.

- M1 lines were grown and selfed and seeds were collected for developing M2 generation. M2 lines were sown and phenotyping was performed in the field.

- A total of more than 9938 pearl millet M2 lines were developed and DNA was isolated from 9938 lines and selfing of the plants was carried. Seeds were collected for raising M3 populations and for phenotyping analysis after the confirmation of mutants by screening M2 plants.

- The DNA was quantified on agarose gel and fluorescence spectrophotometer and the DNA was normalized to 5 ng /µl in liquid handling robotic machine and eight-fold pooling was prepared.

- The DNA information from the 9938 lines was kept in LIMS, a bioinformatics tool for data storage and retrieval. The DNA plate's information was uploaded in three parts. First, it was the TILLING MOTHER PLATE (113 plates) which contains the DNA isolated from the field, next was the TILLING NORMALISED PLATE (113 plates) which contains the diluted DNA information and the third one TILLING POOLED PLATE (12 plates) information. Standardization of TILLING protocol and screening for mutants in *EDR2* and *DREB2A* genes in TILLING population was carried out subsequently.

- *EDR2* and *DREB2A* gene amplifications were standardized using forward and reverse primers and heteroduplexes were formed by denaturing and annealing and the mismatch cleavage was performed with SniPerase endonuclease and purification was continued after cleavage and samples were loaded onto the SDS page gel and analyzed in a LICOR4300 machine.

- Once the mutants were confirmed, samples were given for sequencing for SNP analysis. The forward read and reverse read and the mutant line were aligned in MultAlin online software for SNP detection.

- From the pearl millet populations, a total of two mutants were detected for *DREB2A,* a gene that is associated with drought stress. In the mutant line 84081, a change in nucleotide sequence and the corresponding amino acid change showed clearly in the restriction positions upon SNP analysis. Perhaps this DREB mutant line can be tested for drought tolerance/susceptibility and can be utilized to analyze the loss of function of this gene.

- The present research covers practical aspects of the technology, ranging from building the mutagenized population to mutation discovery, and discusses the possible improvements to current protocols and the impact of new genomic methods for mutation discovery in relation to the future of the TILLING approach. The high mutation frequency combined with the ability to screen individual targets allows one to screen homologs or gene family members individually and then combine the mutant alleles through breeding.

CHAPTER VI

SUMMARY AND CONCLUSION

6.1 Regeneration and transformation

Pennisetum glaucum L. (pearl millet or cattail millet) is an important cereal crop of India. It is the fifth most important cereal crop and the most important millet crop occupying 55% of global millet production. It is grown by poor farmers on marginal lands. It is grown over 40 countries, predominantly in the continents of Africa and Asia. It is the staple diet for the vast majority of poor farmers and also forms an important fodder crop for livestock population in arid and semiarid regions of India and elsewhere in the world. It is cultivated in 29 million, hectares of land supporting more than 100 million people – the poorest of the poor. India is the largest producer of this crop, both in terms of area (9.1 million hectares) and production (7.5 million tons) with an average productivity of 780 Kg per hectare.

Pearl millet is an important cereal crop of India. As the world population continues to increase, food supplies must also grow to meet nutritional requirements. One means of insuring stability of food maintenance is to limit yield loss caused by plant pathogens mainly fungus, bacteria and virus. Downy mildew is a major fungal disease in pearl millet caused by a fungus. Millets rank as the world's sixth most important food crops among cereals and are primarily grown in Asia and African countries. Plant breeding efforts over the past six decades have contributed tremendously to the genetic improvement of cereals in terms of yield and quality. However, traditional approaches to crop improvement have several limitations. Therefore, yield and productivity cannot be sustained indefinitely (Vasil, 1994) so as to meet the food demands of growing population. Tissue culture and genetic engineering techniques can supplement traditional methods and thus help to enhance the yield.

Different genotypes of pearl millet that are susceptible and tolerant to fungal pathogens especially to downy mildew have been collected from ICRISAT, Hyderabad and the germplasm was maintained in the green house/field. Callus cultures were raised from immature inflorescences on Murashige and Skoog's media containing different plant growth regulators. Somatic embryogenesis and organogenesis was achieved from callus cultures and these cultures were used for regeneration of whole plants. Rooting of the shoots was observed and whole plants were recovered. Regenerated plants were later transferred successfully to the pots. Embryogenic callus was subjected to transformation using *Agrobacterium tumefaciens* and by micro projectile bombardment methods.

Downy mildew is a major fungal disease in pearl millet caused by the fungus *Sclerospora graminicola* (Sacc.) Schroet. Other major diseases affecting pearl millet are smut (*Moeszimyces penicillariae*), ergot (*Claviceps fusiformis)* and rust (*Puccinia substriata*). There is 50-60% of the incidence of downy mildew in India depending

upon the season and state and yield losses due to downy mildew disease in pearl millet are very high. Pathogen related (PR) proteins, for example, are a group of diverse proteins whose accumulation is triggered by pathogen attack or abiotic stress. In other words, PR proteins constitute a point where various response networks interact by reacting with different inducers such as salicylic acid, jasmonic acid, systemin and ethylene. Pathogens of plants are a significant and growing threat to crop production worldwide. The goal of producing crops with increased and durable resistance to a spectrum of diseases is therefore a major focus in plant research. Increasing knowledge of the plant defense has led to more sophisticated transgenic approaches to enhance resistance. The number of candidate genes put forward by transcriptomics, proteomics and protein interaction studies gives us a large choice of genes to be used. Potentially, these genes can be manipulated by over expression, induced expression and tissue specific expression.

Some transgenics have been developed in pearl millet, but no single gene is capable of giving complete resistance to the fungi and on the other hand, multiple genes if transferred can effectively control this fungus or multiple fungi. In the present study, we introduced two genes namely osmotin and chitinase into pearl millet. Immature inflorescence derived embryogenic calli were co-bombarded with plasmids containing osmotin and chitinase in pCAMBIA 2300 and bar gene in pPUR vector driven by CaMV35S promoter. Bombarded calli were cultured on MS medium with phosphinothricin as a selection agent. Transgenic pearl millet lines (primary transformants) expressing *osmotin* and *chitinase* genes have been recovered (T_0) in the present study. These transgenics were characterized by gene specific amplification of *osmotin*, *chitinase* and *bar* genes and also by RT-PCR method for *chitinase* gene expression. Southern blotting was performed in the untransformed controls and also in putative transgenics to ascertain gene integration into host genome. However, these transgenics appeared totally sterile and hence, second generation (T_1) plants could not be recovered.

6.2 Target induced local lesions in genomes (TILLING)

Induced variation caused by artificial mutation or genetic engineering technique, shown by single nucleotide polymorphism (SNPs), insertions and deletions (InDels), can be indentified and discovered using small-scale to high throughput systems. TILLING (Target Induced Local Lesions in Genomes) is a method to detect and discover new mutations, insertions and deletions in DNA. TILLING is a technique which can be used in chemically induced mutation populations with high throughput genome-wide screening for point mutations in genes of interest. TILLING will be comparably efficient for plants with larger genomes because ethyl methanesulfonate (EMS) toxicity is expected to scale with the number of functional genes, which is likely to be similar for all higher plants. This method effectively complements classical forward mutation screening based on phenotypes. TILLING has several advantages as a general reverse genetic tool, especially for organisms for which other options are limited. The high density of mutations resulting from chemical mutagenesis means that, relative to insertional or deletional mutagenesis, far fewer plants are required for screening and much smaller genes can be effectively targeted. TILLING provides an allelic series of mutations, and is the only method that can focus the search for missense mutations to just part of a protein, such as in a single domain of a multidomain protein.

Mutation detection in large populations, were mainly discovered by DNA sequencing, as the cutting efficiency of the endonuclease enzyme allows for the discovery of multiple mismatches in a DNA duplex, and also specific PCR primers must be developed for the screening of any polymorphism. This method is both quick and cheap as it is based on PCR reactions, followed by digestion and subsequent fragment analysis, and allows for the screening of many individuals simultaneously. Thus, this technique can further be used to construct linkage map for group genes involved in biotic/abiotic stresses.

It is known that EMS is a nearly perfect mutagen for inducing G to A and C to T mutations and that all classes of mutations can be recovered at the expected frequencies. All other reports, although less comprehensive, generally support and extend this conclusion. Given the high regulatory and intellectual property costs associated with transgenics and the current concerns about genetically modified crop plants, there is likely to be agricultural interest in producing phenotypic variants without introducing foreign DNA of any type into a plant's genome.

A mutant population in pearl millet was generated using EMS as a mutagen. Phenotyping performed in the field, combined with TILLING, demonstrated that chemical mutagenesis is an effective approach to generate mutants with altered agronomic traits for genetic studies and to predict the gene function through identification of an allelic series by TILLING. EMS mutagenesis of pearl millet seed resulted in populations with mutation frequencies that are feasible for use in a high-throughput TILLING operation. The mutation frequencies in this pearl millet population are lower than what has been found in *Arabidopsis* populations. Although the population was treated with the same concentration of EMS, the resulting mutation frequency was lower. It is possible that the genetic background could have an effect on the efficiency or toxicity of the mutagen, as has been observed in rice (Till, 2007) but differences could be due to other environmental or experimental conditions.

Assuming a population size of 10,000 M2 lines to be ideal for mining allelic variants in candidate genes, a set of 10,000 and 15,000 seeds of the inbred line “P1449-2-P1” were mutagenized using 7.5 mM EMS. The mutagenised seeds were grown into M1 plants and allowed to self pollinate. The population was advanced to M2 generation. In each M2 line, one panicle from each row was labeled, and bagged before anthesis. To prevent redundancy of mutations, only one fertile plant from each M2 row were selected to produce M3 seeds and the plants leaf tissues were sampled for DNA preparation for the labeled plant. From mutagenized populations, DNA samples were isolated using high-throughput method and pooled and arrayed in microtiter plates. Genomic DNA of M2 lines was isolated from 25-day young leaves using Machery-Nagel plant genomic DNA isolation kit. DNA concentration was measured using PicoGreen reagent through Tecan spectrafluor plus and normalized to 5 ng/µl using Tecan Genesis workstation 200. Further to increase throughput screening, eight-fold pooling of 9,938 M2 lines was carried which yielded 12 pooled plates. Two gene targets were selected for high throughput TILLING.

Phenotypic evaluation of portions of the M1 and M2 generations revealed the presence of a wide spectrum of morphological diversity, which is a further indication of the forward genetic potential of this resource. The phenotypic variants observed in 2006 included: the leaf color- dark green, pale green, yellow, albino, and

variegated, leaf appearance – curly, twisted, smooth, hairy, more erect, and less erect; leaf size phenotypes- width, length, and thickness and shoot appearance phenotypes like fewer tillers, more tillers, branched, and thickness, stature appearance phenotypes such as - taller, shorter, and extremely short, plant development phenotypes - seedling emergence time, flowering time, and time of senescence and panicle mutants - bifurcating, partially sterile, completely sterile, long and short. The mutant lines sown in field were regularly surveyed for spotting interesting phenotypes. Although a broad range of phenotypic variants were observed in mutant population generated during 2006, the spectrum of phenotypic variants in new set of mutagenized population during 2007 and 2008 was mostly confined to chlorophyll mutants.

The screening of *DREB2A* and *EDR2* was done for 12 pooled plates using standard TILLING protocol. The pools were then amplified by PCR using gene-specific primers. PCR amplification products were incubated with the SNiPerase endonuclease, a member of the nuclease family of single strand-specific nucleases. SNiPerase cleaves to the 3´side of mismatches and loop outs in heteroduplexes between wild-type and mutant DNA while leaving duplexes intact. Cleavage products were electrophoresed using the LI-COR gel analyzer system, and a standard commercial image processing program, to examine the gel readout. Differential double-end labeling of amplification products allows for rapid visual confirmation because mutations are detected on complementary strands and so can be easily distinguished from amplification artifacts. Upon detection of a mutation in a pool, the individual DNA samples were similarly screened to identify the individual carrying the mutation. The double-end labeling strategy provides confirmation within the pool screen, and further confirmation comes from identifying the same fragments in tracking down individuals. Therefore, sequencing was carried out with near certainty that a mutation exists within a small interval.

With an aim to identify allelic variant for dehydration responsive element binding factor (*DREB2A*, a transcription factor), which plays a key role in drought tolerance, a set of 9,938 M2 TILLING lines have been generated using reference genotype “P1449-2-P1” with an optimized concentration of EMS. Screening of 9938 lines yielded two positive bulk DNA. Analysis of positive bulk for single plant identification was later carried out. A detailed TILLING analysis was performed for detecting the point mutation. In the mutant line 84081, a change in nucleotide sequence and the corresponding amino acid change showed clearly in the restriction positions upon SNP analysis. Perhaps this DREB mutant line can be tested for drought tolerance and can be utilized as a drought tolerant line. We were able to detect the change in one of the amino acids, i.e. arginine to serine in the DREB2A protein. These results contribute to a better understanding of the functionally relevant sites of the *DREB2A* protein and also for detecting a mutant plant that would be drought tolerant. *EDR2*, a candidate gene for downy mildew resistance is amplified in the reference genotype P1449-2-P1. The amplification of the *EDR2* with different set of degenerate primers was carried. However, the target band was observed along with some non-specific bands. Multiple bands were observed when TILLING assays were performed on unpooled DNAs and multiple heterozygous sites were detected upon sequencing individuals. These results point out to the fact that the primers amplified more than one target, probably because of homology with other genes since it belongs to a multigene family. Two sequences were obtained upon cloning the *EDR2* PCR product, one sequence corresponded to the

EDR2 target but the other sequence showed homology with an unknown gene. So, the detection of mutant alleles has become difficult as far as *EDR2* was concerned.

Conclusions

- ✓ Protocols for *in vitro* plantlet regeneration in pearl millet and genetic transformation using inflorescence derived callus cultures via *Agrobacterium* and microprojectile methods were standardized. Transgenic pearl millet plants were confirmed by gene specific amplification of chitinase and osmotin and also the bar genes. However, the transgenics turned out to be sterile, though pollen was produced.

- ✓ Mutagenized population of pearl millet from inbred line P1449-2-P1 was produced using the chemical mutagen ethylmethane sulfonate and M2 generation was raised. Phenotypic analysis was carried out in M2 generation. The mutagenized population was screened for point mutations using the TILLING technique.

- ✓ The mutant lines (9,938 lines) were screened for two candidate genes, one associated with water stress (dehydration responsive element binding factor, *DREB2A*) and the second associated with pathogen resistance (enhanced disease resistance, *EDR2*).

- ✓ Allele mining was carried out for DREB2A gene; amino acid arginine was changed to threonine and serine to proline in the hot spot region that was predicted by the CODDLE software. It is hoped that these amino acid changes would bring loss of function of this gene and thus can help to find out the function of the gene under water stress conditions.

- ✓ TILLING was performed for EDR2 gene; but because of non-specificity, the mutations could not be detected in the LICOR gel.

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1. **N. Jalaja**, V.L. Sirisha, S. Prashant, P.B. Kavi Kishor and Amita Jain.
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   Effect of growth regulators, carbon source and cell aggregates size on berberine production from cell cultures of *Tinospora cordifolia* Miers.
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3. Maruthi Rao. I. Sampath Kumar, **N. Jalaja**, D. Madhavi, P. Srilaxmi, P. Nataraj Sekhar, P.S. Reddy, Rathnagiri Palavarapu and P.B. Kavi Kishor
   High frequency plant regeneration from callus cultures of two finger millet (*Eleusine coracana*) cultivars.
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4. M. Muralidhar Rao, Amita Jain, A. Maruthi rao, M.S.L. Sunita, **N. Jalaja** and P.B. Kavi Kishor
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   Advances in Plant Science. 22 : 323-329 (2009).

5. V.L. Sirisha. S. Prashnat, D. Ranadheer Kumar, **N. Jalaja**, M.S.L. Sunita, Pramod Sivan, Preeti Mishra, K.S. Rao, N. Sreenivasulu, B.M. Khan, S.K. Rawal, P.B. Kavi Kishor.
   Cloning, characterization and impact of up and down regulating subabul cinnamyl alcohol dehydrogenase (*CAD*) gene on lignin profiles in transgenic tobacco.
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APPENDIX

Composition of Solutions, Reagents and media

LB media

Tryptone - 10 g

Yeast extract - 5 g

Sodium chloride - 10 g

Make up the volume to 1000 ml by adding distilled water

Agar (if required) - 15 g/l

YEM medium

Yeast extract - 0.4 g

Mannitol - 10 g

$MgSO_4$ $7H_2O$ - 0.2 g

K_2HPO_4 - 0.5 g

NaCl - 0.1 g

pH - 7.0

Make up the volume to 1000 ml by adding distilled water

Agar (if necessary) - 15 g/l

YEP medium

Peptone - 10 g

Yeast extract - 10 g

NaCl - 5 g

pH - 7.0

Make up the volume to 1000 ml by adding distilled water

Agar (if necessary) - 15 g/l

Induction medium

2 mM NaH_2P0_4 - 2 ml

75 mM MES - 10 ml

1% Glucose - 1g

100 µM Acetosyringone - 100 µl

1X AB salts - 5 ml

Make up the volume to 100 ml with distilled water.

Composition of AB salts

373 mM NH4Cl - 20 g

$MgS0_4.7H_2O$ - 6 g

40 mM KCl - 3 g

0.1 mM $FeSO_4.7H_2O$ - 0.05 g

Make up the volume to 1litre with distilled water.

ABG media

0.5% Glucose - 5 g

1X AB salts - 50 ml

1X AB buffer - 50 ml

Make up the volume to 1 litre with distilled water

Agar - 15 g

Composition of AB buffer

K_2HPO_4 - 78.6 g

NaH_2PO_4 - 23 g

Make up the volume to 1 litre with distilled water

Co-cultivation medium

MS basal + 2% sucrose and 1% agar containing 100 µM acetosyringone

Selection medium

MS + 4 mg/l BAP + 500 mg/l cephotaxime + 5mg/l phosphinothricin+ 3% sucrose and 1% agar- for pearl millet.

Osmoticum medium

MS basal medium + 36.4 g/l mannitol and 36.4 g/l sorbitol + 30 g/l sucrose (pH 5.8) and 1% agar

PM Callus M medium

MS medium+ 2.0 mg/l 2,4-D + 100mg/l NaCl + 3% sucrose (pH 5.7)and 1% agar

PMRM medium

MS+ 2.0 mg/l Kinetin + 0.1 mg/l NAA + 3% sucrose (pH 5.7) and 1% agar

0.1M $CaCl_2$ Solution:

Dissolve 1.47 g of calcium chloride in 100 ml of distilled water and store at 4 ^oC.

20 mM $CaCl_2$ Solution

Dissolve 294 mg of calcium chloride in 100 ml distilled water and store at 4 ^oC

RNase (10 mg/ ml)

Dissolve Rnase in water, place in a tube in a boiling water bath for 10 minutes. Allow this to cool on a bench and store at -20 ºC.

Tris HCl (1 M, pH 8.0)

Dissolve 121.1 g Tris in 800 ml of water. Adjust pH to 8.0 with conc. HCl make volume up to 1 liter and autoclave.

EDTA (0.5 M, pH 8.0)

Dissolve 186.1 g $Na_2EDTA.2H_2O$ in 800 ml water. Adjust pH to 8.0 with sodium hydroxide pellets. Make up volume to 1 liter and autoclave.

$T_{10}E_1$ buffer

1M Tris HCl pH 8.0 - 10 ml

1M EDTA pH 8.0 - 1 ml

And make up to 1 liter with sterile distilled water.

10X Tris-Borate Buffer (TBE)

Tris buffer - 108 g

Boric Acid - 55 g

EDTA - 9.3 g

Distilled wate made up to 1 litre.

pH - 8.3

Plasmid Isolation Solutions

Solution-I

15%Glucose - 7.5 g

25 mM Tris-HCl pH 8.0 - 1.97 g

10 mM EDTA - 1 ml

100 µg/ml RNase A - 2 µl

Make up the volume to 50 ml with distilled water

Solution-II

0.2N NaOH - 0.4 g

1% SDS - 0.5 g

Make up the volume to 50ml with distilled water

Solution-III

3M Potassium acetate pH 4.8. - 14.72 g

Dissolve 14.72 g of sodium acetate in 25 ml water. Adjust pH to 4.8 with glacial acetic acid and make volume up to 50ml.

6X Gel loading buffer (0.25 % Bromophenol blue, 40 % sucrose) (10 ml)

Sucrose	- 4 g
Bromophenol Blue	- 2.5 ml
dH_2O	- up to 10 ml

Store at 4 ^oC.

Ethidium bromide (10 mg/ml)

Dissolve 100 mg ethidium bromide in 10 ml of distilled water; wrap tube in aluminium foil and store at room temperature.

Caution: Ethidium bromide is extremely mutagenic.

CTAB (Cetyl Trimethyl Ammonium Bromide) (2 %) buffer

CTAB	- 20 g
1 M Tris	- 200 ml
5 M NaCl	- 280 ml
0.5 M EDTA	- 40 ml
Na_2SO_3	- 2.5 g
pH	- 8.0
Distilled water	- 460 ml

Add mercaptoethanol (0.1 %) fresh while using CTAB (2 %) solution.

Chloroform: isoamyl alcohol (24:1)

Chloroform	- 240 ml
Isoamyl alcohol	- 10 ml

Store in dark at room temperature. Make up and dispense the solution in a fumed cupboard.

Ethanol (70 %)

Absolute alcohol	- 70 ml
Distilled water	- 30 ml

100 base pairs/1kb ladder (50 ng/ml)

Ladder (stock conc. 1 µg/µl) - 50 µl

Blue (6X dye) - 165 µl

$T_{10}E_1$ buffer - 785 µl

Protein extraction buffer

50 mM NaH_2PO_4 - 6.5 ml

50 mM Na_2HPO_4 - 93.5 ml

Solutions used for Southern blotting

Denaturation solution:

1.5M NaCl - 43.83 g

0.5M NaOH - 10 g

Make up the volume to 500 ml with distilled water.

Depurination solution:

0.2 N HCl - 4.38 ml of HCl

Make up the volume to 200 ml with distilled water.

Neutralisation solution:

1 M Tris-HCl pH 8.0 - 60.55 g

1.5 M NaCl - 43.83 g

Make up the volume to 500 ml with distilled water

20x SSC

3 M NaCl - 86.75 g

0.5 M sodium citrate pH 7.0 - 44.1 g

Make up the volume to 500 ml with distilled water.

Primary wash buffer (per liter)

2 M Urea - 120 g

0.5 M NaH_2PO_4 pH 7.0 - 7.8 g

150 mM NaCl - 8.7 g

1 M $MgCl_2$ - 1 ml from1M stock

2% Blocking reagent - 2 g

Secondary wash buffer: (20x stock)

1 M Tris Base - 121 g

2 M NaCl - 112g

pH - 7.0

Make up the volume to 1 liter with distilled water

Working Stock: Dilute 1:20 and add 2 ml/l of 1 M $MgCl_2$ to give a final concentration of 2 mM.

Preparation of EMS

Formula of EMS: $C_3H_8O_3S$

Molecular weight of EMS is (3×12) + (8×1) + (3×16) + (1×32) = 124 g EMS/mole

5mM EMS:

For 1Litre solution:

(5 mMoles EMS/l) × (1 mole/1000 mMoles) × 1L = 0.005 moles EMS

124 g EMS/mole × 0.005 moles = 0.6 g/l = 600 mg/l

Like this calculation for all the concentrations 5 mM EMS,10 mM EMS,15 mM EMS, 20 mM EMS,30 mM EMS,40 mM EMS,50 mM EMS,60 mM EMS was done and prepared the mutagen chemical.

DNA isolation Solutions

Chloroform:ethanol 24:1 (v/v),5 M NaC1, TE buffer: 10mM tris-HC1, 1mM EDTA, pH 8.4, RNase A: 10 mg/ml, Proteinase K: 1 mg/ml ,Chloroform:Phenol: (25:25:1) , Extraction buffer:100 mM Tris, 1.4 M NaC1, 20 mM EDTA, pH 8.0, 2%, CTAB, 0.3% Mercaptoethanol

CTAB (Cetyl Trimethyl Ammonium Bromide) 2 % buffer

CTAB - 20 g

1M Tris - 200 ml

0.5M Nacl - 280 ml

0.5M EDTA - 40 ml

Na2So3 - 2.5 ml

Distilled water - 460 ml

Add Mercaptoethanol (0.3%) fresh while using CTAB (2%) solution.

RNase (10mg/ml)

Dissolve RNase in water, place in a tube in a boiling water bath for 10minutes. Allow this to cool on a bench and store at -20 ºC.

Chloroform: Ethanol (24:1)

Chloroform - 240 ml

Ethanol - 10 ml

Store in dark at room temperature. Make up and dispenses the solution in a fumed cupboard.

Ethanol (70%)

Absolute alcohol - 70 ml

Distilled water - 30 ml

NaCl (5 M)

Dissolve 292.2 g NaCl in 750 ml water.Make up to 1 liter with water, filter and autoclave.

Phenol/ Chloroform

Mix equal volume of the buffered Phenol: Chloroform: Isoamyl alcohol (24:25:1).Store at 4 ºC

Sodium acetate (2.5 M, pH 5.2)

Dissolve 340.2 g sodium acetate in 500ml water, Adjust pH to 5.2 with glacial acetic acid and make volume up to 1 liter and autoclave.

Tris HCl (1 M, pH 8.0)

Dissolve 121.1 g Tris in 800 ml water. Adjust pH to 8.0 with Conc. HCl make volume up to 1 liter. and autoclave

EDTA (0.5 M, pH 8.0)

Dissolve 186.1 g $Na_2EDTA.2H_2O$ in 800 ml water. Adjust pH to 8.0 with Sodium hydroxide pellets. Make up volume to 1 liter and autoclave.

$T_{10}E_1$ buffer

1M Tis HCl pH 8.0 - 10 ml

1M EDTA pH 8.0 - 1 ml

And make up to 1 liter with sterile distilled water

$T_{50}E_{10}$ buffer

1M Tris HCl pH 8.0 - 50 ml

0.5M EDTA pH 8.0 - 20 ml

Make volume upto 1 liter with sterile distilled water.

10X Tris-Borate Buffer (TBE) (per liter)

Tris buffer

Boric Acid

EDTA

108 g Tris base, 55 g Boric acid and 9.3 g EDTA, Add deionised H_2O to 1 liter. The pH is 8.3 and requires no adjustment.

6X Gel loading buffer (0.25% Bromophenol blue, 40% sucrose) (10ml)

Sucrose - 4 g

Bromophenol Blue - 2.5 ml

dH_2O - 10 ml

Store at 4 ºC

Ethidium bromide (10 mg/l)

Dissolve 100 mg ethidium bromide in 10 ml of distilled water; wrap tube in aluminium foil and store at room temperature

Caution: Ethidium bromide is extremely mutagenic.

Acrylamide / bisacrylamide 29:1 (w/w)

Acrylamide - 29 g

Bisacrylamide - 1 g

Water (deionised distilled) up to 100 ml

Store at 4 ºC for 1month

Acrylamide/bisacrylamide 29:1 (v/v)

Acrylamide - 87 ml

Bisacrlamide - 3 ml

Add deionised water to 300 ml. Solution can be stored up to 1 month at 4 ºC

10% (W/V) Ammonium per Sulphate

Ammonium per Sulphate - 1 g

Water (deionised distilled) - 10 ml

Make fresh stock every week and store at 4 ºC

Binding silane

0.15 ml Bind silane

0.5 ml acetic acid

99.35 ml ethanol

Mix the ingredients and store at 4 ºC

100 base pairs ladder (50 ng/l)

100 bp ladder (stock conc. 1 µg/µl) - 50 µl

Blue (6X dye) - 165 µl

$T_{10}E_1$ buffer - 785 µl

Repel silane

Readymade, store at 4 ºC

Components of Frontier Genomic Kit

10X PCR Buffer

50mM $MgCl_2$

10mM dNTPs

Shakara Taq

2X Reaction Buffer

SNiPerase

Eco-Mix Buffer

Stop Buffer

Loading Buffer

Homozygous Control Mix

Heterozygous Control Mix

Primers Suspension

All stock primers are suspended to a final concentration of 100 µM in TE pH 7.4. The volume required to achieve this concentration varies between primers and is listed on the MWG datasheet under Volume for 100 pmol/µl.

Double and triple check the correspondence of the name on the lid of a stock tube to the name on the side. Check the appearance of the lyophilized primers before suspension.

Add $T_{10}E_1$ to primer stock tubes or a final concentration of 100 µM. Do this at a genomic DNA/non-PCR product bench with the lights dimmed or off. Use PCR-clean tube racks and pipettes. Let TE stand in primer tube for several numbers to allow primers to go into solution. Thoroughly vortex primer tubes until primers are in solution. This may take some time for IRD 700 and IRD 800 primers. Repeat vortexing and centrifuge tubes for 10sec and prepare aliquots.

Aliquots: Make 3 sets of 20 µl aliquots of forward and reverse primers. The more aliquots the better, but try to leave 50 µl of master stock tube. Store 2 sets of primer aliquots at -80 ºC in a box.

Primer Mixtures

Primer mixtures should be made fresh whenever possible. The fluorescent dye is unstable in the mixture and the breakdown products may be be inhibitory to PCR annealing and elongation.

Mix primer cocktails in a 1.5ml microfuge tube as follows.

Unlabelled forward Primer (100 µM)

IR 700 blue forward Primer (100 µM)

Unlabelled reverse primer (100 µM)

IR 800 green Reverse Primer (100 µM)

Primers of *DREB2A* gene

1 Forward Primer 1 (F1)

Name: 54 deg 102-124 Len:23 Score:93

Predicted Melting Temperature: 54 degrees Celsius

GeneTool Score: 93

Start: 102 End: 124 Length: 23

Bases:5'GCCATGGTGCAATTGACTGAAGT 3'

2 Reverse Primer 1 (R1)

Name: 55 deg 306-328 Len:23 Score:91

Predicted Melting Temperature: 50 degrees Celsius

GeneTool Score: 91

Start: 492 End: 514 Length: 23+3=26

Bases:5' CCGCAAAATGACCCAACAAATGTACC 3'

3 Forward Primer 2 (F2)

Name: 59 deg 20-38 Len:19 Score:94

Predicted Melting Temperature:59 degrees Celsius

GeneTool Score: 94

Start: 20 End: 38 Length: 19

Bases: 5' CGCGTGATGGGCCGACCTC 3'

4 Reverse Primer 2 (R2)

Name: 56 deg 800-817 Len:18 Score:91

Predicted Melting Temperature: 56 degreesCelsius

GeneTool Score: 91

Start: 800 End: 817 Length: 18

Bases: 5' GCTCCATGCCGCCACCAG 3'

5 Forward Primer 3 (F3)

Name: 56 deg 97-119 Len:23 Score:90

Predicted Melting Temperature: 56 degrees Celsius

GeneTool Score: 90

Start: 97 End: 119 Length: 23

Bases:5' TCTGGGCATCTGTGGGTACCTGT 3'

6 Reverse Primer 3 (R3)

Name: 57 deg 862-884 Len:23 Score:91

Predicted Melting Temperature: 57 degrees Celsius

GeneTool Score: 91

Start: 862 End: 884 Length: 23

Bases:5' CCGTCTTATTCGAGCTGGATGCA 3'

7 Forward original (F0)

F-5'CCGGAATTCATGCAGTCCTTGACTGATGG 3'

8 Reverse original (R0)

R- 5' CCGCTCGAGCAGTTCCCTGACTACAGGC 3'

Preaparation of PicoGreen

No. of Samples or Plates	**$T_{10}E_1$ in µl**	**PicoGreen in µl**
1	99.5 µl	0.5 µl
1 Plate or 96 samples	10,000 µl	50 µl
2 Plates	20,000 µl	100 µl

Quantification of DNA using PicoGreen

Switch on the TECAN SPECTRAFLOUR PLUS machine.

Switch on the respective computer

- Press Ctrl+Alt+Del
- Press enter (No password)
- Double click 'Magellan2" software icon
- Press cancel
- In the menu bar: Click the Insert → Measurement Parameters.

Do the following changes in Measurement Parameters

- **General**: Fluroscence icon is already selected (Keep the default settings)
- **Meas. Params**:
  - **Others**: Excitation Wavelength = 485
    Emission Wavelength = 535

  Gain: Manual = **103**

  Number of Flashes **= 10**
- **Shaking:**

  Shaking before measurement: Duration = **15** seconds

Moving Plate (FLUOTRAC200) into the Machine:

Instrument → Move Plate & filter → Move Plate **In →Ok**

Prepare your plate (FLUOTRAC 200)

1. First with Picogreen and $T_{10}E_1$ solution without DNA for taking RFU reading **"Blank reading"**

2. Next add DNA and take the RFU reading **"Sample reading or DNA reading"**.

Blank Reading:

Instrument → Move Plate & filter → Move Plate **Out Ok** → Keep the plate (FLUOTRAC 200) on the tray → Instrument → Move Plate & filter → Move Plate → **In Ok**

Instrument → Start Measurement → Start → (After measurement is finished) Edit → copy to Excel sheet andsaveitwithrelavantname.(Ex.File→Saveas"Plate1_Project1")

Remove plate (FLUOTRAC200) Instrument → Move Plate & filter → Move Plate → **Out Ok**. Then add DNA.

Sample Reading:

Instrument → Move Plate & filter → Move Plate **Out Ok** → Keep the plate (FLUOTRAC 200) on the tray → Instrument → Move Plate & filter → Move Plate **In Ok**

Instrument → Start Measurement → Do you want to overwrite **Yes** (Note: Check if you saved earlier reading or else overwriting will erase earlier data) → (After measurement is finished) Edit → copy →Paste it below the Blank reading in the same excel sheet → Save it in excel →Output is in CSV format containing both PicoGreen and DNA in PicoGreen.

Preparing your plate (FLUOTRAC 200)

1. For Blank Reading:

- Take a washed and dried MG plate
- Prepare the $T_{10}E_1$ & Picogreen mix according to the number of samples.
- Take the solution in a tray.
- Into each well of MG plate add 100 µl of "$T_{10}E_1$ & PicoGreen" mixed solution.
- Then take the "Blank reading"

2. Sample Reading or DNA reading:

Add 1 µl of mother DNA into respective wells of MG plate.

Formula= -2.78273+0.002019*(DNA reading – Blank reading)

If the concentration of Mother DNA is expected to be more than 110 ng/ul

- Then dilute the Mother DNA 3 times in a PCR plate. i.e. 4 µl of DDW and 2 µl of Mother DNA.
- From this diluted DNA solution take 1 µl DNA and add in the respective wells of MG plate with the $T_{10}E_1$ & Picogreen solution.

Formula= 3{-2.78273+0.002019*(Blank reading – DNA reading)}.

Dilution of DNA using Gemini software in Tecan machine

- Convert CSV files of Normalised data of DNA to GWL files
- Switch on the TECAN machine.
- Switch on the respective computer
    - Press Ctrl+Alt+Del
    - Press enter (No password)
    - Double click Gemini" software icon.

Preparing plates (TECAN machine)

- In Two 96 well stands place the source plate (in first stand toward left) and the empty plate (next stand at right side).
- Fill the double distilled autoclaved water in one container and the florox for washing the needle in other container.
- Fill the can with double distilled water that was provided along with the machine.

Gemini software for Dilution of DNA

Open Gemini tool ⟶ Upload the GWL file ⟶ **Strat Dilution** ⟶ new page opened For Dilution Click wash the neddle ⟶ Repeat the step for 3 times ⟶ Click **Run** ⟶ Dilution of samples was started for 96 well plate of DNA isolated (Mother Plate).