DEVELOPMENT OF MUNGBEAN TILLING POPULATION AND ISOLATION OF MUTANTS AMENABLE TO MECHANICAL HARVESTING BY TARGETED SEQUENCING FOR FLOWERING CONTROL AND MORPHOGENESIS GENES

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to Tamil Nadu Agricultural University, Coimbatore

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CERTIFICATE

This is to certify that the thesis entitled "Development of mungbean TILLING population and isolation of mutants amenable to mechanical harvesting by targeted sequencing for flowering control and morphogenesis genes" submitted in partial fulfilment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY** in **PLANT BREEDING AND GENETICS** to the Tamil Nadu Agricultural University, Coimbatore is a record of *bonafide* research work carried out by **V.ANUSHEELA** under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles. However, part of the thesis work has been published in peer reviewed scientific journals of national/international repute (copy enclosed).

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(V.Anusheela)

Abstract

ABSTRACT

DEVELOPMENT OF MUNGBEAN TILLING POPULATION AND ISOLATION OF MUTANTS AMENABLE TO MECHANICAL HARVESTING BY TARGETED SEQUENCING FOR FLOWERING CONTROL AND MORPHOGENESIS GENES

By

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Degree : **Doctor of Philosophy in Plant Breeding and Genetics**

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The present investigation was carried out at the Department of Plant Genetic Resources, and Department of Pulses, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore during 2012-2014. This investigation aimed at optimizing mutagen in mungbean for saturational mutagenesis and generation of TILLING population, discovery of putative mutants from branching and flowering for robust branching type and delayed flowering type through TILLING by sequencing and biometrical characterization of the discovered mutants suitable for branching and flowering habit.

The conversion of mungbean to an entirely mechanized crop is a major breeding challenge. This endeavor will demand the development of cultivars with a combination of traits, including semi determinate growth habit, more erect plants associated with more compact (bush-type) canopy, short internodes and short secondary branches.

In this study EMS doses /genotype combination for saturational mutagenesis was identified through kill curve analysis. Genotype Co (Gg) 7 and EMS doses of 0.7%

(LD_{70}) were fixed among other mungbean genotypes *viz*., Co (Gg) 6, VRM (Gg) 1 and VBN (Gg) 1. A total of 2928 M_2 families survived till maturity. By adopting the best method *viz.,* the spin column based extraction with silica loaded binding buffer DNA from 768 families were extracted. The extracted DNA from the families was distributed across 8 numbers of 96 well microplates. Pooling and super pooling of DNA were adopted as Till *et al.* 2007 in 8*8*12 format. Since mungbean had not yet sequenced, EST sequences from model legumes *Medicago truncatula, Glycine max* and *Vigna ungiculata* were taken for designing cross species primers for amplifying the candidate genes in Green gram.

20 amplicon products were outsourced for NGS under ILLUMINA myseq sequencer. Through variant calling and SIFT analysis 8 SNP and 2 Indels were discovered across five candidate genes. By adapting SIFT analysis three mutations *viz.*, G→A, T→A and G→A residing on genes *GIGANTEA*, *RAMOSUS* and *TERMINAL FLOWERING 1b* respectively were found to be deleterious with amino acid substitution of C→Y for *GI* gene, F→L for *RMS* gene and G→S for *TFL1b* gene. The average mutation frequency for the population was found to be one mutation per 0.22 Mb. Therefore for the entire population of 2928 M_2 lines translates to an average of 13 mutations for any 1 kb DNA fragment.

The discovered mutants *viz*., MTP-399-16, MTP-134-15, MTP-134-03, MTP-134-15 and MTP-399-11 could be deployed further for delayed flowering, semi determinant type along with top pod bearing habit suitable for mechanical harvesting. MTP-580-19, MTP-134-15, MTP-580-07 and MTP-580-21 could be introgressed further for profuse branching, top pod bearing along with maximum accessible pod per plant suitable for mechanical harvesting. Further marker assisted introgression of these mutations in to a common background could result in an ideal plant type for mechanical harvesting. In spite of an intensive mutant generation and mutant discovery process undertaken in this study, mutation in the gene *CONSTANS* which can ideally contribute for flowering time control could not be recovered. Even though bushy phenotype is the implicated traits of *GI* mutants deployed, it also affects partial control on flowering time. Hence, the *GI* mutation in combination with *TFL* and *RMS* could result in desirable plant type with good scope for mechanical harvesting. The conceptual introgression programme for the phenotype modification can be achieved by deploying the mutants discovered.

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ABBREVIATIONS

A	:	Adenine
ABI	:	Applied Bioscience
Ac	:	Activator
ACC	:	Aminocyclopropanecarboxylate
ACO	:	Aconitase
AG	:	Agamous
AP	:	Apetala
ATP	:	Arabidopsis TILLING Project
AVRDC	:	Asian Vegetable Research Development Centre
Az-MNU	:	Sodium azide -Methyl-Nitrosourea
BLAST	:	Basic Local Alignment Search Tool
BLASTN	:	Nucleotide BLAST
BR	:	Broad Range
CDF	:	Cycling DNA binding with one finger Factor
cDNA	:	Complementary Deoxyribonucleic Acid
CETS	:	Centroradialis/Terminal Flower 1/Self-Pruning
CGs	:	Candidate Genes
CO	:	Constans
CODDLe	:	Codons Optimized to Discover Deleterious Lesions
COL	:	Constans Like
CRISP	:	Comprehensive Read analysis for Identification of Single Nucleotide Polymorphisms from Pooled sequencing
DAD	:	Decreased Apical Dominance
DET	:	Determinate
DHPLC	:	Denaturing High Performance Liquid Chromatography
DMSO	:	Dimethyl Sulfoxide
DS	:	Dissociator
DT	:	Determinant

eIF4E	:	Eukaryotic Translation Initiation Factor 4e
EMS	:	Ethyl Methane Sulphonate
FAO	:	Food and Agricultural Organization
FKF	:	Flavin-Binding, Kelch Repeat, F-Box
FMI	:	Floral Meristem Identity
FT	:	Flowering Locus T
G	:	Guanine
GA	:	Gibberellic Acid
GB	:	Gigabase
GBSSI	:	Granule Bound Starch Synthase I
gDNA	:	Genomic Deoxyribonucleic Acid
GI	:	Gigantea
GMO	:	Genetically Modified Organism
HD-ZIP	:	Homeodomain-Leucine Zipper
IAA	:	Indole-3-Acetic Acid
LD	:	Long Day
LE	:	Length
LF	:	Late Flowering
LFY	:	Leafy
LOV	:	Light, Oxygen or Voltage
LR-PCR	:	Long Range Polymerase Chain Reaction
MAQ	:	Mapping and Assembly with Quality
MAST	:	Multiple Alignment Search Tool
MAX	:	More Axillary Growth
MB	:	Megabase
MBS	:	Millet Breeding Station
MTP	:	Mungbean TILLING Population
NCBI	:	National Center for Biotechnology Information
NGS	:	Next Generation Sequencing

NMU	:	N-Nitroso-N-Methylurea
PARSESNP	:	Project Aligned Elated Sequences and Evaluate SNPS
PCR	:	Polymerase Chain Reaction
PEBPS	:	Phosphatidylethanolamine Binding Proteins
PERL	:	Practical Extraction and Reporting Language
PSSM	:	Position-Specific Scoring Matrix
QXT	:	Quark Xpress Template
RMS	:	Ramosus
RNAI	:	Ribonucleic Acid Interference
RT-PCR	:	Reverse Transcription Polymerase Chain Reaction
SAM tools	:	Sequence Alignment/Map
SAM	:	Shoot Apical Meristem
SD	:	Short Day
SIFT	:	Sort Intolerant from Tolerant
SNP	:	Single Nucleotide Polymorphism
SOC	:	Suppressor of Overexpression of Constans
SOLiD	:	Sequencing by Oligonucleotide Ligation and Detection
SYMRK	:	Symbiosis Receptor Kinase
T	:	Thymine
T-DNA	:	Transfer Deoxyribo Nucleic Acid
TFL	:	Terminal Flower
TILLING	:	Targeted Induced Local Lesions In Genome
TNGRID	:	Tamilnadu Genetic Resources Integrated Database
USDA	:	United State Department of Agriculture
WT	:	Wild type
Wx	:	Waxy

Introduction

CHAPTER I

INTRODUCTION

Pulses are an integral part of Indian Agriculture and it is the most peculiar crop facilitating more nutrients, especially proteins and vitamins to the world population. It has been shown to be an excellent source of dietary protein important for the human diet and play a key role in crop rotation due to their ability to fix nitrogen. Pulses are referred as the 'poor man's vegetable. Borlaug (1973) called for protein revolution in the developing world with pulses. He claimed that the pulses remain at low yield level and production is either static or dropping and hence he called them as 'slow runners'. This is due to the fact that pulses have been mostly grown in poor soils under rainfed conditions. Among the pulses, Greengram is one of the important pulse crops of the *Vigna* group and is cultivated in Asia, Australia, West Indies, South and North America and tropical and subtropical Africa (Ali and Kumar, 2006). India is the largest producer of mungbean in the world and accounts for 65 per cent acreage and 54 per cent production (Pratap *et al*., 2012). Being the third largest pulse crop in India, it occupies an area of 2.75 million hectares with a total production of 1.19 million tonnes and productivity of 436 kg/ha (http://www.indiaagristat.com). In Tamilnadu, it occupies an area of 796.9 thousand hectares with a production and productivity of about 241.2 thousand tonnes and 303 kg/ha respectively during 2012-13. Due to its rapid growth and early maturity, it is adapted to multiple cropping systems. Mungbean is primarily used as dried seed and occasionally as forage or green pods and seeds for vegetables (Lawn, 1995). On dry-weight basis mungbean contains 25 to 28 per cent protein, 1.0 to 1.5 per cent fat, 3.5 to 4.5 per cent fiber, 4.5 to 5.5 per cent ash and 60 to 65 per cent carbohydrate. It is an excellent and cheap source of high quality and easily digestible protein and it is comparatively rich in lysine, which is deficient in cereal grains.

Whole genome sequencing and transcriptome analysis have provided in depth descriptions of the physical structure and the repertoire of gene expression in a growing number of eukaryotic organisms. However, to reveal the function of individual genes, genetic approaches would remain of paramount importance. Forward genetics aims to identify the causative genetic change in a phenotypically interesting mutant (i.e. mutant

first). In contrast, reverse genetics intends to assign a function to a gene of known sequence through phenotypic analysis of individuals in which the function of this gene is altered (i.e. gene sequence first). In higher plants, targeted gene disruption methods are not yet routine and alternative methods are required to obtain individuals in which the gene of interest is impaired. Random insertion mutagenesis using either T-DNA or transposons has been successfully used in *Arabidopsis thaliana* to assemble mutant libraries, which cover the vast majority of the genes of this plant (http://signal.salk.edu). Moreover, recently silencing of genes of interest using RNAi transgenesis has become a popular tool for reverse genetics (Mansoor *et al*., 2006).

An alternative and well established approach is chemical or fast neutron mutagenesis followed by identification of mutants from a suitably sized population of mutagenized individuals. Advantages of chemical or physical mutagenesis are their applicability to organisms that are not easily transformable or in which active transposons have not been characterized, and the ease of generating large independent mutant populations. Radiation typically induces deletions, which can be readily detected by PCR, using primers flanking the deletion (Li *et al*., 2001). However, their catastrophic nature limits the number of tolerated deletions per genome. Chemical mutagens, such as ethyl methane sulphonate (EMS), induce point mutations that have the advantage of being tolerated to a high density. This permits near saturation with a manageable number of mutant individuals, so that the genome is well covered (Henikoff *et al*., 2004).

In addition, allelic series with single amino acid changes and specific phenotypes bear the potential for providing more detailed information on protein function. Screening for point mutations without prior knowledge of the mutation is technically challenging. A technology based on mismatch recognition in heteroduplex DNA by endonucleases such as CEL I (Till *et al*., 2004a) provides a level of specificity that allows the detection of a single mutant allele in a pool of wild-type alleles (Colbert *et al*., 2001). When combined with chemical mutagenesis, the screening for mutant individuals in a large population using mismatch detection is referred to as TILLING (Targeting Induced Local Lesions IN Genomes) (McCallum *et al*., 2000a; Comai and Henikoff, 2006). The TILLING process involves PCR amplification with fluorescently labeled primers from pooled DNA. Mismatched heteroduplexes are generated between wild type and mutant DNA by

melting and reannealing the PCR products. Heteroduplexes are incubated with the endonuclease CEL I that cleaves mismatched heteroduplex sites, and the resulting products are separated and visualized on sequencing gels or capillaries. Subsequent sequence analysis in heteroduplex regions of individual plant DNAs identifies the mutation. TILLING platforms have been established for a variety of plants, including important crops such as maize (Till *et al*., 2004a), wheat (Slade *et al*., 2005), rice (Till *et al*., 2007), and soybean (Cooper *et al*., 2008).

The legumes possess many genes of agricultural significance that cannot be analyzed in the model plant, *Arabidopsis thaliana*. For example, *Arabidopsis* does not engage in the ecologically important arbuscular mycorrhiza (AM) symbiosis with phosphate-delivering fungi, which is formed by more than 80% of land plants (Harrison 2005). Moreover, the nitrogen-fixing root nodule symbiosis with rhizobia is almost exclusively found in the legume family (Oldroyd and Downie, 2004). In addition, certain aspects of flower symmetry (Dong *et al*., 2005) and carbon partitioning (Horst *et al*., 2007) are peculiar to legumes. This study intends to develop EMS mutagenised TILLING populations in mungbean. To overcome the limitation of Li-COR based heteroduplex analysis in detecting mutations in a high throughput fashion and high cost, the high throughput next generation sequencing technologies and bioinformatics tools to detect and analyse functional mutations in these populations for a chosen set of genes involved in flowering control time and morphogenesis gene were used.

This study aims to apply TILLING (Targeted Induced Local Lesions IN Genomes). To generate 3000 M_2 EMS mutagenised families of mungbean for to target a set of candidate genes involved in flowering time and morphogenesis. The objectives of this investigation are follow as,

1. To produce mutant populations in mungbean with the optimal density of induced-mutations by choosing the most suitable variety and carefully adjusting mutagenesis conditions
2. To use the optimal mutagenesis protocol to treat and grow 3000 individuals, bank their DNA and seed

3. To use a high throughput sequencing technologies for detecting functional mutations of the target genes involved in flowering time and morphogenesis in TILLING populations
4. To evaluate discovered mutants in M_3 generation and record the biometrical characters to identify altered phenotype suitable for mechanical harvesting.

Review of Literature

CHAPTER II

REVIEW OF LITERATURE

2.1. Importance of pulses

Pulses are major sources of proteins among the vegetarians in India and complement the staple cereals in the diets with proteins, essential amino acids, vitamins and minerals. They contain 22-24 per cent protein, which is almost twice the protein in wheat and thrice that of rice (Yude *et al.,* 1993; Jukanti *et al.,* 2012). Pulses provide significant nutritional and health benefits, and are known to reduce several non-communicable diseases such as colon cancer and cardiovascular diseases (Yude *et al.,* 1993; Jukanti *et al.,* 2012). Major pulses grown in India include chickpea or Bengal gram (*Cicer arietinum*), pigeonpea or red gram (*Cajanus cajan*), lentil (*Lens culinaris*), urdbean or black gram (*Vigna mungo*), mungbean or green gram (*Vigna radiata*), lablab bean (*Lablab purpureus*), moth bean (*Vigna aconitifolia*), horse gram (*Dolichos uniflorus*), pea (*Pisum sativum* var. arvense), grass pea or khesari (*Lathyrus sativus*), cowpea (*Vigna unguiculata*), and broad bean or faba bean (*Vicia faba*).

2.2. Importance of mungbean

Mungbean contains a high protein content, and hence quite prevalent among poor and vegetarians. Along with protein, they are also rich in fiber, essential amino acids, fats, minerals and vitamins. It also aids in digestion and absorption of food and play a vital role in cholesterol metabolism, and thus control blood cholesterol levels. High fibre content, coupled with low sugar content, makes it a good choice for people with diabetes. In addition mungbean grains are a rich source of vitamin A (Retinol, 1 µg_ Retinol Activity Equivalent), B (Thiamine 0.16 mg, Riboflavin 0.06 mg, Niacin0.58 mg), C (Ascorbic Acid 1.00 mcg), E (Alpha-tocopherol 0.15 mg), and K (Phylloquinone 2.70 µg). (http://www.buzzle.com/articles/mung-beans-nutritional-value.html)

2.3. Production of mungbean

India is the world's largest producer as well as consumer of green gram. It produces about 1.5 to 2.0 million tons of Mungbean annually from about 3 to 4 million hectares of area, with an average productivity of 500kg per hectare. Green gram output

accounts for about 10-12% of total pulse production in the country. India is a net importer of mungbean. Imports are mainly sourced from Myanmar to meet the domestic demand. These imports have been hovering around 0.5 to 1.5 lakh tonnes during the past few years (http://www.commoditiescontrol.com/eagritrader/staticpages/index.php?id=89)

Crop production could be increased by either expanding the arable area or through intensification, i.e., using improved seeds, fertilizer, fungicides, herbicides, irrigation, etc. According to the Food and Agriculture Organization (FAO), agricultural intensification represents about 80 per cent of future increases in crop production in developing countries (FAO 2002).Based on this goal, crop breeders and scientists are focusing towards achieving improved cultivars that produce higher yields and at the same time tolerate the sub-optimal soil and climatic conditions.

Among traits that contributed to higher crop productivity in the last century, those which alter the architecture of plants rank first. Architectural changes include alteration in branching pattern and reduction in plant height. The major achievement of the Green Revolution in the 1960s was due to the development and introduction of semi-dwarf crop varieties of wheat and rice along with optimum level of inputs for crop production. These broadly adapted semi-dwarf cultivars were responding to fertilizer application and this led to tremendous increases in productivity (Esfeld *et al.,* 2013).

2.4. Concept of ideotype breeding

Every farmer dreams about ‘the perfect plant’: the one that, when grown in his farm’s environment, would give the highest attainable yield and quality. This dream has fueled the efforts to breed better genotypes, and led plant scientists (mainly geneticists and later agronomists) to coin the concept of ‘ideotype’ in order to identify the best traits to combine into such a perfect plant. Donald (1968) proposed the ideotype approach to plant breeding in contrast to the empirical breeding approach of defect elimination and selection for yield *per se*. He defined ‘‘crop ideotype’’ as an idealized plant type with a specific combination of characteristics favorable for photosynthesis, growth, and grain production based on knowledge of plant physiology and morphology.

2.4.1. Ideotype breeding approach for productivity

Light absorption is an important factor for determining crop yield, being one of the driving forces behind plant photosynthesis, and at the same time is highly dependent on plant architecture as well as on overall canopy structure (Niinemets, 2007). Plant architectural characteristics (such as the number and geometry of organs, i.e. their shape and position within the plant and the canopy), are genotype specific, while at the same time highly dependent on the climatic conditions at the time of their initiation and development (Godin, 2000).

Falster and Westoby (2003) have shown that steeper elevation angles in a number of species improve absorption at higher sun elevations and, therefore, carbon gain through assimilation as it allows more light to penetrate to the lower leaves. While the importance of leaf elevation angles for an improved light absorption strategy at the level of the whole plant has been shown in a number of studies (Pearcy and Yang, 1998; Sinoquet *et al*., 2005).

In addition to leaf angle, leaf shape and size are important aspects of leaf morphology affecting mutual shading of leaves and light absorption of the canopy (Falster and Westoby, 2003). Modifications of the arrangement and size of leaves can affect light availability, especially in the lower parts of the canopy, and alter leaf photosynthetic activity by adjusting light harvesting efficiency (Werner *et al*., 2001). A reduction in leaf clustering can increase light absorption and enhance photosynthetic productivity at canopy level (De Castro and Fetcher, 1999). Morphological characteristics such as leaf inclination and leaf shape are often inherited as simple traits (i.e. under the influence of one or a few major genes) in plants (Thurling, 1991) and can be used to create a more open canopy structure.

Tomato (*Solanum lycopersicum*) is a species that exhibits a high variability in vegetative morphology (Peralta and Spooner, 2000) ranging from small leaves with a few leaflets to big ones with many leaflets. Lately, studies of the genetic basis of this variation at the leaf level have shown that there are leaf specific genes that control its shape and morphology (Frary *et al.*, 2004). This genetic background knowledge in

combination with the detailed information on the effect of leaf topology and geometry on light absorption and photosynthesis could help to identify or approximate the theoretical optimum of plant architecture.

The quantitative exploration of the specific effects of each plant architectural characteristic on light absorption and photosynthesis was hardly possible until the introduction of spatially explicit models considering plant architecture at the organ level (Vos *et al*., 2010). General crop models are powerful tools towards a better understanding of plant processes and for testing case scenarios (Marcelis *et al*., 1998; Vos *et al*., 2007). The crop ideotype is thus an idealized crop consisting of a plant type with a specific combination of characteristics based on the detailed knowledge of morphological and physiological plant traits (Peng *et al*., 2008) as well as mutual interactions among plants of the canopy. These traits often are also contributing to plant architecture.

2.4.2. Ideotype breeding approach for pulses

Progress in breeding for high yield in dry bean, *Phaseolus vulgaris L*., has been modest (Singh *et al.*, 1991). Common bean plant architecture has attracted the attention of breeders, who have tried to find an ideotype (Adams, 1973, 1982; Kelly and Adams, 1987; Brothers and Kelly, 1993; Beattie *et al.*, 2003; Silva *et al.*, 2009).

Some morphological traits, such as plant height, internode number and length, hypocotyl diameter and length, number of pods on top, middle and bottom third of the stem, first pod insertion height, and number of pods on the main stem are part of the major studies aimed at identifying the traits that best define the plant architecture of the common bean (Izquierdo and Hosfield, 1983; Nienhuis and Singh, 1986; Acquaah *et al*., 1991, 1992; Kornegay *et al*., 1992; Brothers and Kelly,1993). Plant height, hypocotyl diameter, pod distribution in the plant's midsection and branch insertion angle have been given special consideration (Acquaah *et al.*, 1991, 1992).

In lentil, introduction of early flowering exotic germplasm such as Precoz (ILL4605) has been more successful in developing suitable plant architecture having vigourous plants, medium maturity, bold seeds and cold tolerance. There exists possibility of developing plants having stronger erect stem, fruiting mainly on secondary and tertiary branches along with some primary branches (Nadarajan and Gupta, 2010).

Satyanarayana *et al*. (1989) isolated a new plant type in urdbean, which can improve yield by 50-60% through increased number of pods per plant via more pods per node. Screening of urdbean germplasm has led to the isolation of plant with soybean type pod bearing habit (Singh, 1991). These new plant types hold immense prospects.

In mungbean for spring and summer seasons, cultivars are needed with determinate growth habit with synchronous and early maturity, longer pods with more number of bold seeds, and resistance to shattering and sprouting. The significant association of branches per plant with seed yield in mungbean has also been reported by Sarwar *et al*. (2004).

2.4.3. Ideotype for mechanical harvesting

Modern agriculture has brought about the handling and processing of plant and animal materials by various means ranging from mechanical, thermal, electrical, optical, to even sonic techniques. The ever increasing importance of agricultural products together with the complexity of modern technology for their production, processing and storage need a better knowledge of their engineering properties so that machines, processes and handling operations can be designed for maximum efficiency and the highest quality of the final end products (Mohsenin, 1970).

Plant architecture and fruit (or pod) setting habits of crops significantly influence the performance of mechanical harvesters. To better understand these effects and provide a methodology to breed and select horticultural cultivars suitable for once over mechanical harvesting, a multiyear study was conducted by Glancey *et al*. (2004) using 84 commercial fields. Using the harvest loss data collected, the relationship between plant architecture of four lima bean (*Phaseolus lunatus* L.) cultivars and the recovery of lima beans harvested with a pod stripper header was developed. Effect of plant architecture on header recovery was significant; cultivar M-15, which tended to set more pods in the lower zones of the plant, exhibited significantly higher harvest loss than the other cultivars. In general, greatest recovery (lowest yield loss) occurred with cultivars that set pods higher in the plant canopy. From the data collected, a mechanical harvesting index for bush type crops that relates pod setting architecture to header loss was established. The index can be used by plant breeders and equipment designers to select cultivars and machine configurations best suited for once over harvest.

Suliman *et al.* (2007) found that in snap bean 51 % of pods mostly concentrated on the crown zone of the plant, 38 % of pods concentrated in the upper canopy of the plant and 11 % of pods concentrated in furrow zone. The average height of the first pod was 6.82 cm from the soil surface The average values of pod petiole diameter and length were 0.15 cm and 1.48 cm respectively. Therefore, the optimum values according to this study help to set the suitable snap beans prototype dimensions for mechanical harvest.

Ramteke *et al.* (2012) conducted field experiments during 2008 and 2009 using 90 soyabean genotypes to screen for height of insertion of the lowest pod, the trait useful for combine harvester. Data was recorded on plant height (cm), height of insertion of the lowest pod (cm) when erect, lodging of the plant in degrees (ø), stem diameter (cm), number of nodes, number of branches and yield. Results of analysis of variance showed significant differences among genotypes in terms of traits under study, which indicate the existence of genetic variation. Correlation coefficient indicated that the grain yield was not significantly associated with all studied traits, which showed non-significant association. Plant height and nodes were negatively correlated with lodging of plants in degrees. First pod height was significant and positively associated with height as well as number of nodes/plant. Stem diameter was positively associated with the nodes/plant. Highest yield was observed in genotypes JS 95-60, Co Soya 2, JS 71-05, MACS 450, VL Soya 21, MAUS 47, KB -79, MAUS 81, PS 1225, NRC 37 and MAUS 71 and was found at par with JS 95-60. However, the genotypes JS 71-05, MAUS 47, PS 1225, NRC 37 and MAUS 71 had a yield at par with JS 95-60, with the lodging angle in degrees (> 65.00°), having pod height above 12 cm, these genotypes are recommended for mechanical harvesting.

Lather (2000) in chickpea developed promising breeding lines through recombination breeding and utilizing E100 ym, a spontaneous brachytic mutant, as donor for erectness and compactness for higher seed yield under late planting, high input conditions for rice-chickpea or cotton-chickpea sequential cropping. These progenies were erect and compact in growth habit (for higher density planting) with strong and reasonably tall stems (for better competition against weeds and lodging resistance), and few but erect secondary and later order branches (for better light interception and air exchange of canopy). These promising advanced breeding lines, particularly H96-99

(3981 kg/ha), recorded significant seed yield superiority under high density planting of 50 plants/m^2. These chickpea ideotypes are also suitable for mechanical harvesting as the fruiting zone started at about 20 cm from base.

Pulse crops are harvested by hand in the Middle-East, North Africa and West Asia. Labour is often scarce and harvesting costs are expensive; this has threatened continuity of production in some regions. However, pulses are successfully harvested mechanically in the USA and elsewhere. Introducing mechanical harvesting systems to developing countries is very difficult, because of the habit of the crop cultivars grown, the environmental conditions which prevail, and the cultural practices used by farmers (Haddad *et al*., 1988). Harvest costs decrease to about 10 percent of production costs with machine harvesting, according to the USDA Agricultural Research Service 2008.

Manual harvest of legume crops is becoming increasingly uneconomical because of the rising labor cost and shortage of labor at the peak harvest time. Delaying crop harvests leads to significant losses of legume grains and their quality. While harvesting of legume crops is mechanized in the developed world, developing countries largely hand harvest due to a lack of improved varieties amenable to machine harvesting. In order to use combine harvesters, legumes varieties need to be modified for machine harvestability. This requires development of varieties with erect and tall plants, strong stems, top pod bearing habits, synchronous maturity; and tolerance to lodging and pod shattering. Genetic variability for these traits exists in the germplasm. Mutants with upright growth habit have been identified and used for development of improved breeding lines in chickpea (Dahiya *et al*., 1990, Sandhu *et al*., 1990, Lather, 2000, Gaur *et al*., 2008) and lentil (Erskine and Goodrich, 1991).

The initial vision for the mungbean ideotype improvement came from the plant breeders and plant physiologists of AVRDC. Plant breeder David R. MacKenzie and plant physiologist Henry Wu had a healthy and constructive debate on the type of mungbean plant breeders should develop. Finally they agreed on a short, determinate plant type with all the pods on the top of the plant (MacKenzie and Shanmugasundaram 1973). In addition, it was agreed that breeders would aim for a plant with the following characteristics (Fernandez and Shanmugasundaram 1988; Shanmugasundaram and Kim, 1996) be more

compact; have a higher harvest index; be less sensitive to photoperiod; and be more determinate in growth habit compared to traditional varieties. It should have a stable potential yield of > 2 tons/ha;, have a maturity duration of around 60 to 75 days; have uniform maturity so that the harvest could be completed in one attempt; have bold seed size (50 to 60 g for 1,000 seeds instead of the 25 to 30 g for 1,000 seeds produced by the local varieties); be resistant to cercospora leaf spot, powdery mildew, bean fly, pod borer, and bruchid weevil.

2.5. Legume genomics

The study of legume genetics, genomics and biology are all important in order to understand the limitations of yield of legume crops and to support our legume breeding programs .With the advent of huge genomic resources and modern technologies, legume research can be directed towards precise understanding of the target genes responsible for controlling important traits for yield potential, and for resistance to abiotic and biotic stresses (Varshney and Himabindu, 2013).

The family Leguminosae is one of the largest families of flowering plants and includes important grain legumes such as mungbean, chickpea, pigoenpea and lentil. Legumes vary in habit from annual to perennial and in their genomes from simple diploids to large and complex polyploids. Two legume species, *Medicago truncatula* and *Lotus japonicus*, are being used as models to study legume genetics and genomics. These two species were chosen as models because of their being diploid with relatively small genomes (450–550 Mb), self-fertility, relative ease of genetic transformation, and short generation time (Young and Udvardi, 2009).

2.5.1. Mungbean genome

Mungbean (*Vigna radiata* (L.) R. Wilczek) is a fast growing warm-season legume species belonging to the papilionoid subfamily of the Fabaceae and has a diploid chromosome number of 2n=2x=22. Mungbean is cultivated mostly in South, East and Southeast Asia. Mungbean have small genome sizes estimated to be 0.60 pg/1C (579 Mbp) (Somta and Srinives. 2007)

The development of molecular markers is critical for crop improvement programmes. Although molecular marker resources are limited for mungbean, there have been several efforts to identify the genomic regions related to domestication-related traits, including seed size and seed germination (Isemura *et al.*, 2012). Moreover, molecular markers are important for integrating useful alleles of wild mungbean, such as bruchid resistance, into domesticated mungbean (Chen *et al*., 2013)

Kang *et al.* (2014) constructed 421Mb (80 per cent) of the total estimated *V. radiate var*. *radiata* genome and identified 22,427 high-confidence protein coding genes and 160 Vigna gene clusters. This is the first draft genome sequence within the genus Vigna.

2.6. Genome of plant architecture

Crop improvement has a long history as key agronomic traits have been selected over thousands of years during the domestication of crops. More recently, this progress has been accelerated as the green revolution has brought about great increases in crop yields (Khush, 2001).

Understanding the structure, organization and dynamics of genomes in plant species can provide insights into how genes have been adapted by natural and artificial selection to respond to environmental constraints and the potential for their manipulation for crop improvement. In pursuit of these goals, several plant genomes have been sequenced. Because of the high cost of Sanger sequencing and limited expertise in the plant science community, only a few plant species were initially selected as ‘models’ and targeted for both sequencing and the development of functional genomics resources. One purpose of generating genome sequences for model plant species was to better understand genome architecture and to create a ‘parts list’ for a plant (Varshney *et al*., 2015).

2.7. Genomics of flowering architecture

In an angiosperms, floral initiation is a unique and major physiological change from vegetative growth to reproductive development. Flowering is essential to the plant's survivability and reproductive success. In agricultural development, flowering is an important target of domestication. By manipulating flowering, it is possible to diversify

cultivation strategics to optimize yield in specific locations. For example, variation in flowering time is selected for in soybean. Since day length changes as locations move further and further from the equator soybean maturity groups are classifications that indicate latitudes suited for individual lines. Similarly, in wheat, the manipulation of vernalization has resulted in optimal yields based on the varying season lengths and temperatures. Winter and spring cultivars have allowed for double cropping of certain land. Highly sensitive and complex regulatory networks control the timing of flowering in response to internal cues and external environmental fluctuations. Flowering gene networks are well studied in model species, however, not in important crop species. Therefore, there is much to be known on the molecular basis of flowering for crop species (Price, 2012).

The shoot apical meristem (SAM) is a set of indeterminate stem cells, located at tip of the shoot axis. Meristems which grew up in shoot apex can generate either shoots or flowers. This achievement depends on expression of two sets of meristem identity genes: first, floral meristem identity gene like *LEAFY (LFY*), *APETALA 1 (AP1)* and *CAULIFLOWER (CAL)* that appoint lateral meristem in Arabidopsis to convert into flowers rather than shoots or leaves (Bowman *et al.*, 1993). Second, shoot meristem identity genes, like *TERMINAL FLOWER* 1*(TFL1)*, appoint inflorescence shoot apical meristem as indeterminate and inhibit flower creation from shoot (Bradley *et al.*, 1997). Researchers have shown that there are two basic types of flowering architecture in plants: namely, determinate and indeterminate. In first case, growing of shoot is indefinitely and flowers are only produce peripherally. In second case, growth of apical meristem cease when a terminal flower is created on apex (Ratcliffe *et al.*, 1999).

2.7.1. Flowering pathways of long day model *Arabidopsis thaliana*

Transition to flowering is an important ecological trait in *Arabidopsis thaliana* in response to various endogenous and environmental cues, such as photoperiod, light quality, ambient temperature, and vernalization, as well as biotic and abiotic stresses. Long day flowering plant *Arabidopsis thaliana* is a model for understanding a complex genetic network by forward and reverse genetic approaches (Srikanth and Schmid, 2011). In *Arabidopsis thaliana*, there are six major genetic pathways that control flowering;

photoperiod, vernalization, autonomous, gibberellin (GA), ambient temperature, and age pathway. These pathways control a shift from vegetative development to reproductive development by regulating floral meristem identity genes in the shoot apical meristem (SAM), which results in flower organ development.

2.7.2. Internal signals of floral initiation

Plants require many internal, or developmental, factors to promote flowering in conjunction with external, or environmental, cues. Gibbereilic acid (GA) is capable of inducing flowering during non-inductive photoperiods and without vernalization (Langridge.1957; Chandler and Dean.1994). In long day growth conditions, GAs have little influence on flowering time, but the GA pathway in short day growth conditions becomes the major player in initiating flowering, where the plant is deprived of photoperiod induced flowering (Gottgens and Hedden, 2009). However, GA is not universal in inducing flowering, and, therefore, is not a true flowering hormone (Zeevaart 1983). In some species, GAs have little effect or inhibit floral initiation (Zeevaart 1983), Nevertheless, GA plays a significant role in the control of flowering in many species.

The cumulative effect and ultimate redundancy of these pathways in controlling flowering is predicated on many early observations of a diverse array of plants without the application of modern plant genetics and molecular biology. However, since the advent of modern genetics the use of *Arabidopsis thaliana* as a model plant due to its small genome, out-crossing ability, large seed count, and short-life cycle has illuminated a much deeper understanding of plant life processes, such as flowering, at the molecular level (Laibach. 1943). The resulting paradigm can serve as the foundation for the study of more complex and useful plant species.

2.7.3. The photoperiod pathway

Central genes involved in the downstream of the photoperiod pathway in *Arabidopsis thaliana* are *GIGANTEA (G1), CONSTANS (CO),* and *FLOWERING LOCUS T (FT)* (Redei, 1962; Koornneef *et al.,* 1991, 1998). These genes are well conserved across many species of flowering plants (Hecht *et al.,* 2005). *CO* encodes a putative zinc finger transcription factor, and plays a central role in photoperiodic flowering control in Arabidopsis (Putterill *et al.,* 1995). *CO* is expressed predominantly

in the phloem (An *et al.*, 2004). The quantity of *CO* expression has a rate-limiting effect upon flowering time, with greater *CO* accumulation resulting in earlier flowering (Samach *et al*., 2000). Overexpression of *CO* results in an early-flowering phenotype and increased expression of *FT*, a floral integrator controlling flowering.

CO transcription is under the control of the circadian clock. In *Arabidopsis thaliana*, *CO* expression was reported to be regulated by *GI. FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1),* and *CYCLING DOF FACTOR (CDF)* genes, which are also regulated by the circadian clock (Fornara *et al*., 2009; Imaizumi *et al*., 2005; Sawa *et al*., 2007). In long day, *FKF1* and *GI* accumulate as their expression is in phase, but under non-inductive short day *GI* and *FKF1* peak out of phase from one another (Sawa *et al*., 2007). When in photo-inductive long day, the LOV(Light, Oxygen, or Voltage) domain of *FKF1* protein binds to the N-terminus of *GI* in the presence of blue light, which is sufficiently abundant only in long day (Sawa *et al*., 2007). However, the *FKF1-GI* complex requires *CDF1* in order to regulate *CO* expression (Fornara *et al*., 2009; Imaizumi *et al*., 2005). Three homologs of *CDF1* play a redundant role in repressing *CO* transcript levels by directly binding to the *CO* promoter (Fornara *et al*., 2009).

Analysis of the abundance of the three proteins showed that *CDF1* and its homologs peak first, followed by *GI*, and then finally *FKF1* peaks in the afternoon under long day conditions (Imaizumi *et al*., 2005; Sawa *et al*., 2007). In the morning, *CDF1* and its homologs bind to the *CO* promoter (Fornara *et al*., 2009). Next, *GI* accumulates and forms a *CDF-GI* complex to repress *CO* (Fornara *et al*., 2009). Once *FKF1* protein is abundant, it interacts with the *CDF-GI* complex and targets *CDFs* for degradation (Imaizumi *et al*., 2005; Sawa *et al*., 2007), resulting in the transcription of *CO* (Fornara *et al*., 2009; Imaizumi *et al*., 2005; Sawa *et al*., 2007). Furthermore, plants that carry mutations in *CDF1* and *CDF* homologs suppressed the late flowering phenotype caused by *gi* mutation, confirming the redundant function of the *CDF1* homologs (Fornara *et al*., 2009).

2.7.4. Floral integrators and floral meristem identity

All of the flowering pathways converge to floral integrators including *FLOWERING LOCUS T (FT)* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1),* and induce the expression of Floral Meristem Identity (FMI)

genes, including *APETALA 1 (API)*, *APETALA 3 (AP3)*, and *AGAMOUS (AC)*, that control floral organ development (Blazquez, 2000; Lee *et al*., 2000). *LEAFY (LFY)* gene also plays a critical role in the convergence of all these flowering pathways (Borner *et al*., 2000).

FT" is known as a key flowering induction signal called florigen. *FT*" expression is gradually induced with maximum expression at floral initiation (Turck *et al.*, 2008). In Arabidopsis, *FT* is induced by the *CO* gene in LD (Samach *et al*., 2000). FT" also acts downstream of the autonomous and vernalization pathway, and, therefore, is a key flower integrator. Similarly, the expression of *SOC1* is regulated by the light pathway, but also by the autonomous, temperature, and gibberellin pathways (Borner *et al.*, 2000; Hepworth *et al.*, 2002). *LFY* functions in part downstream of *SOC1* (Lee *et al.*, 2000).

2.8. Genomics of shoot branching

Auxin is the first plant hormone shown to be involved in shoot branching, and it has been established that it controls the shoot tip apical dominance and, consequently, inhibits axillary bud outgrowth. Additionally, the replacement of the shoot apex with exogenous auxin maintains the inhibition of axillary buds (Cline 1996). Cytokinins show the opposite physiological role to auxin, since they act directly to promote axillary bud outgrowth. Studies have demonstrated that either exogenous cytokinin application or overexpression of genes encoding enzymes involved in cytokinin biosynthesis often induce bud outgrowth (King and Staden 1988; Medford *et al*., 1989; Miguel *et al.*, 1998). In addition, some of the mutants with a greater level of cytokinin show more shoot branching (Dun *et al.*, 2006).

Shoot branches arise from axillary meristems that form in the axils of leaves on the primary shoot axis. The axillary meristems themselves the shoot apex, and later identified as auxin (indole-3-acetic acid), could inhibit the initiate leaves to form a bud. Removal of the primary shoot apex results in activation of arrested axillary buds. The ability of the shoot apex to repress axillary bud growth is termed apical dominance. Thimann and Skoog (1933) reported that a compound, derived from the shoot apex, and later identified as auxin (indole-3-acetic acid), could inhibit the growth of lateral buds when applied to the stump of a decapitated plant. Subsequent work has provided multiple lines of evidence in

support of auxin-mediated bud inhibition in planta. However, a second messenger must relay the auxin signal into the bud because apically derived auxin is not transported into buds (Morris 1977) and exogenous auxin applied directly to buds does not inhibit their growth (Cline 1996).

MAX4 (MORE AXILLARY GROWTH 4) a gene of polyene dioxygenase family can act in the shoot to inhibit branching, expression in the root is sufficient for WT shoot branching levels (Sorefan *et al.*, 2003).

Sorefan *et al.* (2003) suggest that shoot branching is inhibited by auxin transported down the stem from the shoot apex. Auxin does not accumulate in inhibited buds and so must act indirectly. Mutations in the *MAX4* gene of Arabidopsis result in increased and auxin-resistant bud growth. Increased branching in *max4* shoots is restored to wild type by grafting to wild-type rootstocks, suggesting that MAX4 is required to produce a mobile branch inhibiting signal, acting downstream of auxin. A similar role has been proposed for the pea gene, *RMS1* (*RAMOSUS1*). Accordingly, *MAX4* and *RMS1*were found to encode orthologous, auxin-inducible members of the polyene dioxygenase family.

2.9. Key candidate genes for modifications in plant architecture

2.9.1. *GIGANTEA (GI)*

Induction of flowering in response to day length synchronizes flowering to the changing seasons and is believed to be important in adaptation of plants to growth at different latitudes (Ray and Alexander, 1966). Physiological experiments implicated the circadian clock as the time keeping mechanism that enables the measurement of day length (Samach and Coupland, 2000; Yanovsky and Kay, 2003). Forward genetics in *Arabidopsis thaliana* identified a genetic pathway that promotes flowering specifically on exposure to long days (LDs) (Searle and Coupland, 2004), and the role of the circadian clock in photoperiodic time measurement was confirmed by demonstrating that transcription of the genes that act in this pathway is circadian clock controlled. Mutations in one of these genes, *GIGANTEA (GI),* both impair circadian rhythms and delay flowering.

Flowering of Arabidopsis is promoted by long days and delayed by short days. Mutation in the *GIGANTEA* gene delay flowering under long days but have little or no effect under short days (Flowler *et al.*, 1999) showed that *GI* expression is regulated by the circadian clock with a peak in transcript levels 8-10h after dawn. The timing, height and duration of this peak are influenced by day length. Thus results were consistent with the idea that *GI* plays an important role in regulating the expression of flowering time genes during the promotion of flowering by photoperiod.

2.9.2. *CONSTANS (CO)*

CO is central in all plants analysed because it coordinates light and clock inputs in leaves to trigger the expression of *FLOWERING LOCUS T (FT)* whose protein, and possibly also its mRNA, can move from the phloem to the meristem (Corbesier *et al*., 2007; Tamaki *et al*., 2007). The *CO–FT* module is conserved in all known plants, but the final outputs of the signal diverge: whereas in *Arabidopsis thaliana*, a facultative long day (LD) plant, *CO* promotes the expression of *FT* under inducing long days (Lopez *et al*., 2001), in rice, a short day (SD) plant, the signals are different and *CO* is a repressor in non-inductive long days (Hayama *et al*., 2003).

Another important aspect of *CO* regulation involves the spatial coordination of the photoperiodic flowering signals due to the fact that light and photoperiod sensing occurs in leaves and probably in other actively photosynthetic tissues, whereas the developmental switch takes place in the non-photosynthetic apical meristem (Knott, 1934; Zeevart, 2008). The movement of a developmental signal from the leaves to the meristem was proposed early last century (Chailakhyan, 1936), but was only recently attributed to the movement of *FT* factors from the companion cells of the phloem to the apical meristem.

CONSTANS encodes a typical transcription factor with three characteristic domains which makes it a unique kind of transcriptional regulator present only in the plant kingdom (Putterill *et al*., 1995). It was soon found that a family of proteins closely similar to *CO* was present in the Arabidopsis (Robson *et al*., 2001) and rice genomes (Shin *et al*., 2004) and that representatives of this family could be identified in several expressed sequence tag (EST) databases from many phylogenetically diverse plants

(Griffiths *et al*., 2003). These *CO* like or *COL* proteins include homologues closely related to *CO* such as *COL1*, which is encoded in a gene next to *CO* in the genome and seems to be the result of recent tandem duplication (Putterill *et al*., 1995) and with which it shares an amino acid identity >80%.

2.9.3. *LEAFY (LFY)*

The *LEAFY* gene is an important element of the transition from the vegetative to the reproductive phase, as *LEAFY* is both necessary and sufficient for the initiation of individual flowers (Blázquez *et al*., 1997).

The flowering response to these environmental factors involves several signalling pathways that converge towards the regulation of floral meristem identity genes (Mouradov *et al*., 2002). Two of these genes *LEAFY (LFY*) and *APETALA 1(AP1)* were identified first because their mutation clearly perturbs the fate of the SAM productions. Other genes, called 'integrators', act upstream of *LFY/ AP1*and their mutation severely delays flowering in different growing conditions. These genes include *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*)

Although *AP1* and *LFY* are necessary to specify floral meristem identity, they do not function independently of one another. Instead, AP1 functions largely downstream of *LFY*. The best evidence for this comes from analysis of combinations of gain of-function and loss-of-function alleles of *LFY* and *AP1*. The floral promotion effects of 35S: *LFY* are blocked in an *ap1* mutant (Weigel and Nilsson, 1995), but the floral promotion effects of 35S:*AP1* are not blocked in a *lfy* mutant (Mandel and Yanofsky, 1995). However, in 35S:*AP1 lfy*, floral organ identity is not properly specified, demonstrating that *LFY* is necessary for the proper expression of floral organ identity genes, and this activity of *LFY* is independent of *AP1*.

It has been proposed that the indeterminate architecture of the *Arabidopsis* inflorescence is the result of the interplay between genes that confer floral identity, including *LFY*, and genes that promote shoot identity, including *TERMINAL FLOWER 1* (*TFL1*), such that *TFL1* represses *LFY* expression in shoots and *LFY* represses *TFL1* expression in flowers (Shannon and Meeks-Wagner, 1991; Alvarez *et al*., 1992; Weigel *et al*., 1992; Bradley *et al*., 1997). Similarly to *LFY*, *TFL1* is expressed not only

in the reproductive phase, but also during the vegetative phase, and *tfl1* mutants flower early (Shannon and Meeks-Wagner, 1991; Bradley *et al*., 1997).These observations are in agreement with a role of *TFL1* in the regulation of *LFY* expression during the vegetative phase.

2.9.4. *TERMINAL FLOWERING (TFL)*

The meristem produces phytomers, which are shoot units consisting of a leaf, axillary bud and a stem segment. Arabidopsis mutant plants of unfunctional *TERMINAL FLOWER1 (TFL1)* transcription factor (Shannon and Meeks-Wagner 1991) have a determinant meristem. *TFL1* controls the growth of the phytomer by delaying the expression of floral-related genes, such as *LEAFY (LFY)* and *APETALA1 (AP1),* and hence gives more time for the vegetative axillary buds to initiate and grow (Ratcliffe *et al.*,1999). Determination of the mechanism by which an axillary bud initiates and develops is important to genetically design plants with a desired branching habit.

TFL1 belongs to the *CETS (CENTRORADIALIS/TERMINAL FLOWER 1/SELF-PRUNING)* family of genes that encode PEBPs (phosphatidylethanolamine binding proteins) (Pnueli *et al*., 2001). PEBPs are evolutionally conserved among eukaryotes and play important roles in diverse organisms *TFL1* is homologous to phosphatidy lethanolamine binding proteins that play diverse roles related to signaling pathways controlling growth and differentiation (Benlloch *et al*., 2007). *TFL1* belongs to a small gene family, one of whose members, *FLOWERING LOCUS T (FT),* is also a regulator of flowering time. However, *FT* acts in an opposite manner to *TFL1; FT* promotes flowering and conversion of the SAM to a flower (Kardailsky *et al*., 1999; Kobayashi *et al*., 1999). The structures of the *TFL1* and *FT* proteins are very similar; the predicted polypeptides encoded by the Arabidopsis *FT* and *TFL1* genes are 175 and 177 amino acids long, respectively, with differences in only 39 residues from non-conservative changes, including substitutions and insertions/deletions (Ahn *et al*., 2006). Based on the results of swapping experiments of single amino acids and discrete domains between Arabidopsis *TFL1* and *FT*, the amino acid substitution from His to Tyr at residue 88 in *TFL1* (residue 85 in *FT*) in the ligand-binding pocket and different segments in exon 4 of both genes were found to be responsible for their differential bindings to interactors, resulting in opposite activities for *TFL1*and *FT* (Hanzawa *et al*., 2005; Ahn *et al*., 2006).

Different Regulation between *TFL1* homologs has been discovered in pea and Arabidopsis. In pea, two *TFL1*homologs have two distinct functions: *LF (LATE FLOWERING*) is involved in the control of the vegetative phase by delaying floral initiation, the transition from the vegetative to the inflorescence meristem and *DET* is involved in the control of the floral phase by preventing the transition from the inflorescence meristem to the flower. This regulation is different from that in Arabidopsis, in which only one *TFL1* gene controls the length of both the vegetative and floral phases (Foucher *et al.*, 2003)

2.9.5. *RAMOSUS (RMS)*

A number of genes involved in the control of shoot branching have been identified in pea (*Pisum sativum*). These include five *Ramosus (RMS)* genes, *RMS1*through *RMS5* (Beveridge, 2006). Grafting studies have demonstrated a role for these genes in shoot and rootstock tissues. The inability of exogenous auxin applications to rescue the increased branching phenotypes of the *rms* mutants (Beveridge *et al*., 2000), and the auxin inducible *RMS1*and *RMS5* gene expression (Foo *et al*., 2005; Johnson *et al*., 2006), suggests these genes are involved in the synthesis of auxin's secondary messenger. *Rmsl* is one of the series of five ramosus loci in pea in which recessive mutant alleles confer increased branching at basal and aerial vegetative nodes. *Rmsl* gene activity was more effective at inhibiting branching when expressed in the shoot because branching was slightly greater in *rmsl* / WT plants than in WT/*rmsl* plants.

Sorefan *et al*. (2003) used RT–PCR to investigate the expression of the *RMS1*gene in pea using the classical apical dominance test involving decapitation and replacement of the apex by exogenous auxin. Decapitation caused a substantial drop in *RMS1*expression within 6 h after treatment. This reduction was not only prevented by replacement of the apex with 500 and 3000 mg/L exogenous IAA, but *RMS1*expression was up regulated compared with intact controls. Mutation at the *Rms1*or *Rms5* locus produced plants with a highly branched phenotype, particularly at basal nodes in dwarf backgrounds, but also at aerial nodes in tall backgrounds; (Arumingtyas *et al*., 1992; Beveridge *et al*., 1997; Murfet and Symons, 2000). Other effects on plant growth and development were relatively minor, but included a decrease in main stem length of mutant plants compared with WT plants (Murfet and Symons, 2000).

Grafting studies with *rms1, rms2, rms5,* and WT seedlings provided further evidence for similarities in action of *Rms1*and *Rms5,*as both mutants responded similarly when grafted with *rms2* (Beveridge *et al*., 1997). This indicates that in *rms2* scions grafted to *rms1* or *rms5* rootstocks, the *rms2* mutation may block the action of *Rms1* and *Rms5,* resulting in a branching phenotype. In a reciprocal manner, branching is inhibited in *rms1* or *rms5* scions grafted to *rms2* rootstocks because *Rms1*and *Rms5* genes present in the *rms2* rootstock are able to facilitate synthesis or transport of the graft-transmissible signal.

2.10. Genetic manipulation of flowering time and branching habit

Nucleotide polymorphisms are major components of heritable phenotypic variation and thus drivers of evolution and domestication. Where natural nucleotide differences are limited or difficult to recover, induced mutations can be created in the genomes of living organisms in order to generate novel genetic diversity (Muller, 1927; Stadler, 1928). Mutations have been a powerful tool for the discovery and characterization of major biological processes and gene functions. Their utility for practical applications may be best exemplified in plant breeding (Jain, 2010a; Jain. 2010b).Currently over 3000 mutant crop varieties are registered in a database curated by the International Atomic Energy Agency (http://mvgs.iaea.org/). Traits improved via mutagenesis include enhanced yield and resistance to biotic and abiotic stresses (Jain, 2010b). The global economic impact of crops improved through mutation techniques is estimated in billions of US dollars annually (Ahloowalia *et al*., 2004).

2.10.1. Forward genetics Vs Reverse genetics

Kurowska *et al.* (2011) suggest one of the most direct ways of establishing gene function is to identify a mutation in the specific gene and to link this mutation to the phenotypic change in the mutated organism. In the forward genetics approach (“from mutation through phenotype to the gene”), large mutated populations have been created and screened for alterations in the trait or biological process of interest. Over the decades, large mutant collections have been developed for many model organisms. These isolated mutants have then served for the identification of the genes underlying the change in phenotype. The sequence of the gene responsible for the altered phenotype can be isolated using the process of map-based cloning. Although this approach is both time-

consuming and labour-intensive, it has been successfully applied for cloning several genes, even in species with large genomes, such as barley and wheat (Keller *et al*., 2005; Komatsuda *et al*., 2007; Krattinger *et al*., 2009; Zhang *et al*., 2009). Recent advances in large-scale genome sequencing projects have opened up new possibilities for the application of mutation techniques in basic studies and in the improvement of crops. The reverse genetics strategy ("from gene sequence to phenotype") has widely replaced the forward approach in studies involved in detecting gene function. This strategy is based on the alteration of a gene structure or its activity, followed by an analysis of the associated change in plant phenotype.

With the availability of large amounts of DNA sequences from model organisms and the incentives to determine the functions of genes discovered from DNA sequence, reverse genetic approaches are becoming increasingly important. Among these are genome wide mutagenesis methods followed by screening within individual gene segments, which is made possible by using PCR (Henikoff and Comai 2003). Although PCR based detection of insertions and deletions is straightforward, detection of point mutations, such as those introduced by chemicals, is challening, because the amplified fragment does not change in size. Nevertheless, detection of single base changes has improved rapidly with advances in single nucleotide polymorphism (SNP) detection technologies (Kwok, 2001), and this has fueled the application of new technologies to reverse genetic mutational screening.

One example of SNP detection technology being applied to reverse genetics is TILLING (Targeted Induced Local Lesions IN Genome), in which chemical mutagenesis is followed by screening for point mutations (McCallum *et al*., 2000a). The rapid increase in the acquisition of genomic sequence information in the past decade has enabled reverse genetic approaches to directly probe the function of specific genes by testing the *in vivo* on sequence of disruption or over expression of a gene on the phenotype of an organism (Tierney and Lamour, 2005). This is the "reverse" of traditional genetic analysis where phenotypes are observed and afterwards the gene causative for the phenotypic difference is cloned and validated. TILLING (Targeting Induced Local Lesions in Genomes) is a general reverse genetics strategy that combines traditional mutagenesis with high throughput mutation discovery methods (McCallum *et al*., 2000b).

A TILLING library consists of stored germplasm typically in the form of seed, and genomic DNA extracted from mutagenized material. Both DNA and germplasm can be stable for many years, allowing for screening for a variety of different traits and sharing of the library with a network of plant researchers as a public service (Till *et al.,* 2003). This represents an efficiency gain over traditional forward genetics or mutation breeding strategies where only a small subset of mutated alleles are recovered and maintained, while the majority of induced changes are discarded.

Additionally, reverse genetic strategies allow the selection of only potentially useful allelesfor careful phenotypic evaluation. For example, screening a diploid population of 6000 chemically mutagenized Arabidopsis plants will yield < 30 potentially deleterious mutations (Greene *et al*., 2003; Till *et al*., 2003).

The effort in phenotypic characterization and field evaluation can be reduced by two orders of magnitude when compared to forward genetics and mutation breeding. A disadvantage is that such estimations hold for monogenic traits where a single gene is controlling a phenotype, and mutation-based modification of polygenic traits can be more cumbersome (Jankowicz-Cieslak *et al*., 2011). However, reverse genetics strategies such as TILLING allow the recovery of potentially useful alleles that do not produce phenotypic differences alone, but do so when combined with others. Examples include genetic redundancy where disruption of multiple genes is required to reveal a phenotype, and in polyploid species where alteration of homeologous sequences is a requisite for the recovery of recessive trait (Enns *et al.,* 2005; Slade *et al.,* 2005). The probability of co disruption of two or three target genes in a single plant, or the random combination of such alleles in cross breeding experiments is low, making TILLING an efficient way to recover phenotypes that are either difficult or impossible by forward genetic approaches (Jankowicz-Cieslak *et al*., 2011).

2.10.2. Application of TILLING

The application of TILLING makes the functional analysis of large genomes as well as small genes, which are difficult targets for insertional mutagenesis, possible. Another great advantage of TILLING technology relies on the ability of chemical mutagens to create a spectrum of mutations, including missense changes, truncation and

mutations in splice junction sequences. In contrast to insertional mutagenesis that generates mostly gene knockouts, using TILLING, it is possible to induce a series of alleles in a targeted locus. In addition to loss-of-function alleles, chemical mutagens generate gain-of-function and hypomorphic alleles that can provide a range of phenotypes (Alonso and Ecker 2006).

The mutations are stable, which is not always the case for alternative methods of reverse genetics utilising RNAi silencing or transposon, e.g. Ac/Ds tagging. In addition, RNAi technology and insertional mutagenesis through T-DNA or transposon tagging relies on genetic transformation. TILLING does not require transformation and, thus, is the only reverse genetic strategy applicable for species that are not transformable or recalcitrant. It is recommended as non-GMO technology, so when using TILLING, GMO procedures and controversies are avoided. Moreover, TILLING is not technically demanding and can be performed at a relatively low cost. The TILLING strategy was initially developed as a discovery platform for functional genomics, but it soon became a valuable tool in crop breeding as an alternative to the transgenic approach (Kurowska *et al.*, 2011).

Till *et al.* (2003) revealed that chemical mutagenesis causes both point mutations, which are irreversible and produced in relatively high densities, and also chromosome breaks that cause various chromosomal rearrangements, which can reduce fertility and affect lethality. Unlike insertional mutagenesis, the high density of chemically induced point mutations makes TILLING suitable for targeting small genes or single protein domains that are encoded by large genes.In contrast to insertional mutagenesis, the TILLING strategy is general, because chemical mutagenesis has been successfully applied to most major taxa (Henikoff and Comai, 2003).

2.10.3. TILLING- a high-throughput harvest for functional genomics

TILLING (Targeted Induced Local Lesions IN Genome) was developed a decade ago as an alternative to insertional mutagenesis in *Arabidopsis thaliana* (McCallum *et al.*, 2000a). TILLING takes advantage of classical mutagenesis, sequence availability and high-throughput screening for nucleotide polymorphisms in a targeted sequence. It combines the high frequency of mutations induced by traditional mutagenesis with sensitive techniques for discovering single nucleotide mutations. The main advantage of

TILLING as a reverse genetics strategy is that it can be applied to any plant species, regardless of its genome size, ploidy level or method of propagation (Kurowska *et al.,* 2011).

Chemical mutagens, which are usually used in TILLING protocols, provide a high frequency of point mutations distributed randomly in the genome. An analysis of mutations induced by ethyl methane sulphonate (EMS) in192 Arabidopsis genes revealed about ten mutations per gene among the 3,000 M_2 plants examined (Greene *et al*., 2003). It was estimated that each M_2 plant carried, on average, 720 mutations (Till *et al*., 2003), while only 1.5 T-DNA insertions per mutant line were detected in the Arabidopsis insertion populations (Alonso *et al*. 2003). Thus, much smaller populations are required to reach saturation mutagenesis using TILLING 5,000 M_1 plants in Arabidopsis (Ostergaard and Yanofsky 2004) as compared to 360,000 lines in T-DNA mutagenesis (Alonso and Ecker 2006).

2.10.4. TILLING strategy

The first strategy that was described for the TILLING procedure (McCallum *et al*., 2000b) included the EMS treatment of Arabidopsis seeds, DNA isolation and pooling, PCR reaction of the fragment of interest, heteroduplex formation and the identification of heteroduplexes using DHPLC. Since then, TILLING has been used with many different organisms and many modifications to the procedure have been introduced that help to automate the screening of mutations and reduce its cost. Over time, the DHPLC method for detecting mutations in the TILLING approach was, in most cases, replaced by the digestion of heteroduplexes using specific endonucleases followed by polyacrylamide electrophoresis and visualisation in the very sensitive LI-COR gel analyser system (LI-COR Biosciences), which is less expensive and faster than DHPLC (Colbert *et al*., 2001; Till *et al.,* 2006).

2.10.5. Developing a mutagenized population

The ideal mutagen for TILLING is one that randomly induces single nucleotide substitutions, or small insertions/deletions (<30 nucleotides) at a high rates in the genome. The chemical mutagen ethyl methane sulfonate (EMS) generates mostly SNPs, and can be controlled to produce a high density of point mutations, causing a variety of lesions

including nonsense and missense mutations (Greene *et al*., 2003, Koornneef *et al.*, 1982). The effect of treatment with EMS is highly predictable; G: C->A: T transition changes represent the majority of induced mutations in most organisms. This is especially striking in *Arabidopsis* and wheat where > 99% of mutations identified by TILLING are G: C->A: T transitions (Greene *et al*., 2003, Slade *et al.*, 2005).

Seeds are soaked in a dilute solution of chemical mutagen for approximately 10–24 hrs. Due to the multicellular nature of the embryo that is mutagenized, different tissues in the resulting adult plant (termed the "M_1" generation), will contain different genotypes (Henikoff and Comai, 2003). Thus, mutations present in the somatic tissue will not match those in the germinal tissues, and this generation is not suitable for TILLING screens. Mutations in the M1 germline will be heterozygous, and therefore M_2 progeny from a self-cross of the M_1 should segregate mutations in a typical 1:2:1 Mendelian ratio. For *Arabidopsis*, only one M_2 sibling was chosen at random from the progeny of a single M_1 self-cross (Colbert *et al*., 2001). The single seed descent approach provides a predictable ratio of mutant to wild-type alleles in a single individual (either 1:1 for heterozygotes or 2:0 for homozygous alleles) and allows for straightforward segregation analysis of the M_3 generation.

The density of induced mutations is a major factor in the efficiency and cost of mutation discovery. For example, to discover 10 mutations in maize TILLING populations, with a density of 1 mutation per 500 kb, will take approximately twice as long and cost twice as much as to discover the same number of mutations as in *Arabidopsis* TILLING populations with a density of 1 mutation per 250 kb. Differences in mutation density between organisms may result from differences in the uptake of, cytotoxic response to, and repair of lesions induced by treatment with the mutagen. Following established mutagenesis protocols would therefore seem a good route to success, however, an approximately 1.5-fold range in mutation density was observed when following a standard protocol for *Arabidopsis* seed mutagenesis (Till *et al.*, 2003). To control for variability in mutagenesis, phenotypic markers can be used to predict the level of induced mutations before investing in the resources required for a large population. For *Arabidopsis*, a correlation between embryo lethality in the M_2 seed and density of mutations was found (Till *et al*., 2003).

EMS has been used as the mutagen in TILLING experiments performed on *Brassica napus, Brassica oleracea, Glycine max, Hordeum vulgare, Lotus japonicus, Medicago truncatula, Oryza sativa, Sorghum bicolor, Triticum aestivum, Triticum durum* and *Zea mays* by Gilchrist and Haughn (2005); Martín *et al*. (2009). EMS induced mutations are randomly distributed in the genome and a high degree of mutational saturation can be achieved without excessive DNA damage studied by Gilchrist and Haughn (2005). In Arabidopsis, 5% of the mutations induced by this mutagen in coding regions result in the premature termination of the gene product (nonsense mutations) or mutations in splice sites, whereas 50% lead to missense mutations said by Greene *et al.* (2003); Martín *et al*. (2009).

Henikoff and Comai (2003) revealed the EMS alkylates guanine bases and leads to mispairing: alkylated guanine pairs with thymine, which results mainly in G/C to A/T transitions. This type of transition makes up more than 99% of all EMS induced mutations in Arabidopsis, maize and wheat. However, when the frequencies of various types of EMS induced mutations were analysed in other species (tomato, rice and barley) G/C to A/T transitions constituted not more than 70% studied by Minoia *et al*. (2010).

2.10.6. Bioinformatic tools in the TILLING strategy

Kurowska *et al.* (2011) reported that the bioinformatics tools are used in the TILLING strategy from the beginning, when the candidate gene is the newly identified homologue and the amplicon is determined, till the end, when the analysis of obtained alleles are performed in terms of their impact on protein function. It is important to state that there is no need to know the full genome of the studied species in order to analyse it with the TILLING strategy. The sequence of the gene of interest can be retrieved for many species from databases, such as GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and then a proper homologue can be identified.

In the case of barley, whose genome is not fully sequenced, cloning homologues enables their functional analysis. Candidate genes for analysis, when not available for particular species, as e.g. in barley, can be retrieved from GenBank database of Arabidopsis or rice. Afterwards, in the case of Arabidopsis genes, a BLAST search is performed against the rice genome to identify the rice homologue.The determination of

an amplicon is a crucial step for TILLING analysis. The selection of a suitable amplicon provides a higher probability to identify changes in the DNA sequence with an impact on the protein function during TILLING screenings. It is worthwhile to choose a fragment as much as possible within the coding region. The second condition is to identify the region with the most potential to generate deleterious changes. This can be achieved with software such as CODDLE (Codons Optimized to Discover Deleterious LEsions; (http://blocks.fhcrc.org). CODDLE performs BLAST alignment in order to identify a conserved region, uses SIFT (Sort Intolerant From Tolerant) and PSSM (Position-Specific Scoring Matrix). In addition to CODDLE analysis, the alignment of genomic and amino acid sequences from closely related species with the use of BLASTN and ClustalW2 (http://www.ebi.ac.uk/Tools/ msa/clustalw2/), respectively, can be performed (Kurowska *et al*., 2011).

Each of the identified alleles is then analysed in terms of influence protein. A bioinformatic tool designed for displaying and analysing nucleotide polymorphisms is PARSESNP (Project Aligned elated Sequences and Evaluate SNPs; http://www. proweb.org/parsesnp/parsesnp_help.html; Taylor and Greene 2003). PARSESNP determines the effect of single nucleotide polymorphisms (SNPs) on protein, based on the alignment of related proteins with the use of PSSM and SIFT. Sequence alignments are converted to the Blocks format and then to PSSM. PSSM is aligned to the gene with the use of the Multiple Alignment Search Tool (MAST). It determines mapping of PSSM onto a sequence. Large positive PSSM (>10) means that missense change in the analysed sequence of the DNA can be dramatic for protein function. SIFT allows to predict the severity of change; a SIFT score lower than 0.05 could have the same effect as a PSSM higher than 10. Another feature of PARSESNP is the ability to predict changes in the restriction enzyme recognition sites (Kurowska *et al.*, 2011)

2.10.7. Next Generation Sequencing

At the beginning, the most time-consuming step of this method was the cleavage of heteroduplex using a purified enzyme which was, and still is, an expensive reagent. A few years later, it was discovered that crude celery juice extract could also be used for mismatch cleavage and that its endonuclease activity is the same or even higher than in

the case of the purified enzyme. The process of preparing it from celery is simple and very cheap, and takes less than two days. The protocol for crude celery juice extract isolation is based on a series of dialyses of the extract directly after juicing the celery. Once isolated, celery juice extract can be used for up to a few years when stored at –80°C (Till *et al*., 2004b). However, it should be remembered that techniques employing mismatch-specific cleavage enzymes are often limited, as some enzymes do not recognise all mismatch types, have a low sensitivity in detecting one allele in a pool of DNA or lead to a high gel background caused by nonspecific cleavage (Triques *et al*., 2007).

Ultra-high throughput sequencing is becoming available at low cost and it is a very attractive approach for TILLING. A new and advantageous model for a TILLING service is now possible. Academic laboratories, public and private breeding programs add their favorite genes to the TILLING service list. The advances in DNA sequencing technologies have been used in two ways in the TILLING strategy. Firstly, the growing number of whole genome sequencing projects (Mochida and Shinozaki, 2010) in plants for both crop and model species has led to an increase in the possible choices of target genes in TILLING. Secondly, new strategies for sequencing, so called next generation sequencing methods (NGS), which has been applied to a limited number of sequenced individuals using Sanger's method, can be used for direct mutation determination without any prescreening. The advent of NGS platforms has dramatically increased the speed at which a DNA sequence can be acquired and has also reduced the cost of sequencing by more than two orders of magnitude (Deschamps and Campbell, 2010). The NGS platforms, which are available today, include the 454 Genome Sequencer FLX and its smaller version, the GS Junior System, both of which are pyrosequencing based instruments (Roche Applied Science; http://454.com/products/ index.asp), the Solexa 1 G Genome Analyzer (Illumina; http://www.illumina.com), the SOLiD instrument (Applied Biosystems; https://products.appliedbiosystems.com) and the HeliScope Single Molecule Sequencer (Helicos; http:// www.helicosbio.com).

Rigola *et al*. (2009) used the 454 Roche technology for mutation detection in a tomato population obtained after EMS treatment and identified two mutations in the *eIF4E* gene based on the screening of more than 3,000 M_2 families in a single GS FLX sequencing run.

2.11. TILLING in crops

Since the inception of TILLING, this method has been widely used for the study of functional genomics in plants, especially for the model plant *Arabidopsis thaliana*. Greene *et al*. (2003) reported that the *Arabidopsis* TILLING Project (ATP), which was set up and introduced as a public service for the *Arabidopsis* community (Till *et al.,* 2003), had detected 1,890 mutations in 192 target gene fragments. Heterozygote mutations were detected at twice the rate of homozygote mutations. Therefore, the mutational density for treatment of *Arabidopsis* with EMS was approximately 1 mutation / 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.

A Maize TILLING Project established in 2005 at Purdue University has already identified 319 mutations in 62 genes, which has greatly assisted functional genomic studies in maize(Weil and Monde 2007).

Barley, which is also an important cereal crop with a fairly large genome size of ~5,300 Mb, was evaluated for the ability of induced mutations to be detected by TILLING (Caldwell *et al.,* 2004). Two genes (*Hin a* and *HvFor1*) were examined and 10 variants were identified, six of which were missense mutations. Phenotyping the M_3 individuals demonstrated that 20 per cent had visible phenotypes.

Wheat is an extremely important agronomic staple crop with an estimated production level of 600 million tons per year (Bagge *et al.,* 2007). A polyploid plant investigation to locate variants in the *waxy* locus (*GRANULE BOUND STARCH SYNTHASE I, GBSSI)* in wheat was implemented. Partial *waxy* wheat cultivars are desirable because production of amylose starch is reduced, which leads to the production of superior flour and noodle products for human consumption. Wheat genetics can be complicated because its genome is complex, it is an allohexaploid, and the total genome size is quite large (17,000 Mb). A total of 246 alleles were uncovered in three *waxy* gene homoeologues (*Wx*-A1, *Wx*-B1, and *Wx*-D1) from allohexaploid and allotetraploid wheat *via* TILLING. This comprehensive allelic series provided 84 missense, three nonsense

and five splice site mutations. Phenotyping of M_3 progeny demonstrated reduction of amylose production. Detecting genetic variants *via* phenotyping in wheat can be difficult because redundant copies of loci in the genome can mask expression. This study identified more extensive allelic variation in *GBSSI* than was identified in any report produced in the last 25 years (Slade *et al.*, 2005).

Rice, which is also a staple and important economic crop around the world, currently estimated to provide 80 per cent of the caloric intake for three billion people (Storozhenko *et al.*, 2007), has been the focus of a few TILLING studies. The rice genome has been predicted to contain ~50,000 genes, of which gene function needs to be determined empirically. In 2005, a report was published on the generation of a large mutation population (60,000) using multiple chemical mutagens on IR64, a widely grown Indica rice (Wu *et al.*, 2005). This study demonstrated that TILLING was suitable for reverse genetic studies with mutations detected in two genes; albeit, the mutational density in the population was fairly low. In addition, extensive phenotypic variation was assessed for the various chemical mutagens used to develop the mutant population and albinism was a common phenotype no matter which mutagen was applied. In a separate study, EMS and Az-MNU were used to induce an elevated mutational density in rice, with 57 polymorphisms identified from 10 target genes by TILLING (Till *et al.*, 2007). Another report on rice TILLING published in 2007, demonstrated the efficacy of TILLING to detect mutations by separation of products on agarose gels (Raghavan *et al.*, 2007). Results were analogous to pooling DNA and detecting mutations on a LI-COR DNA Analyzer.

Another investigated trait was spike morphology (Gottwald *et al*., 2009). A TILLING population in barley was created using the two rowed malting cultivar 'Barke'. Thirty one mutations were identified by screening a 1,270bp fragment of the homeodomain leucine zipper (HD-ZIP) gene *HvHox1* in 7,348 M_2 lines. Three of the newly identified mutants exhibited either a six rowed or an inter medium spike phenotype, and these mutations constituted a direct link between the gene and the phenotype. Reverse genetic screening of mutagenised populations could be also used as a molecular tool for crop improvement. Enhanced shelf life was the goal for research in *Cucumis melo* (Dahmani- Mardas *et al*., 2010).

TILLING screening in melons was performed for 4,032 M_2 plants and 11 genes related to fruit quality were chosen. In total, they identified and confirmed by sequencing 134 induced mutations in an 18.3kb total length of tilled amplicons. A detailed investigation was performed for CmACO1-ACC oxidase 1, the enzyme that catalyses the last step of ethylene biosynthesis and is connected with the shelf life of fruit. One mutation out of the seven detected in this gene, G194D, occurred in a highly conserved amino acid position and an assumption was made using crystallographic analysis that it affects the enzymatic activity. A phenotypic analysis confirmed this assumption that the mutant showed a significant delay in ripening and yellowing, with improved shelf life. Among the broad range of genes that encode traits that are of great interest to breeders, key genes in the lignin and β-glucan biosynthetic pathways were chosen in *Avena sativa* (Chawade *et al*., 2010).

2.11.1. TILLING in legumes

Another model plant, *Lotus japonicus*, has also been the focus of elucidating gene function through TILLING. *Lotus japonicus* is a perennial temperate legume that is a model plant for genomic studies because it has a short life cycle, is a diploid ($2n = 2x = 12$), has a relatively small genome (472 Mb), and is self-fertilized (Sato and Tabata 2006). TILLING was used to investigate induced mutations occurring in the protein kinase domain of the SYMRK gene, which is necessary for root symbiosis (Perry *et al.*, 2003).

Perry *et al*. (2009) identified a large allelic series for 12 genes known to be essential for nodule development in *Lotus japonicus*. A total population of 4,904 M_2 plants was screened and 97 mutant alleles were detected. All possible types of mutation were identified: silent alleles that caused no change in amino acid, changes in splice sites and missense to nonsense types. This unique data set, which combines genotypic and phenotypic information, is an excellent tool for structure function studies. Among the mutants identified, 19 alleles did not have an effect on gene function and 78 influenced the phenotype, including lines where nodulation deficiency was observed.

Six missense mutations were identified along with a mutation in the splice acceptor site. Nitrogen fixation and the functional role of sucrose synthase was the target of another *Lotus japonicus* TILLING study (Horst *et al.*, 2007). Six isoforms of sucrose

synthase were identified and several mutations including missense and nonsense were located in four of the six isoforms. Quantitative RT-PCR was performed to examine expression levels in *Lotus japonicus*, which were determined to have differential expression in various plant organs. Furthermore, 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 (Triques *et al.*, 2007). Some of the mutations discovered in the *LE* gene, which encodes 3β-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.

Soybean (*Glycine max*) contains approximately 35-50 per cent protein and has been shown to be beneficial for human health. It is an important economic crop that can improve soil quality by fixing nitrogen. Four mutant populations from two genetic backgrounds (Forrest and Williams 82) were created for soybean by treatment with EMS or NMU and evaluated for induced mutations (Cooper *et al.*, 2008). Several of the target genes initially tested amplified more than one target. Further work was carried out to produce a single product to employ TILLING so that mutation detection functioned optimally. A total of 116 mutations were identified *via* TILLING from seven target genes. The majority of the mutations uncovered by TILLING were determined to be the expected G/C to A/T transitions. This study demonstrated that soybean is suitable for TILLING studies.

Materials & Methods

CHAPTER III

MATERIALS AND METHODS

The present investigation was carried out at the Department of Plant Genetic Resources and Department of Pulses, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore during 2012-2014. Workflow of this experiment is depicted in Fig.1.The materials used in the course of experimentation and experimental methods adopted are described in this chapter.

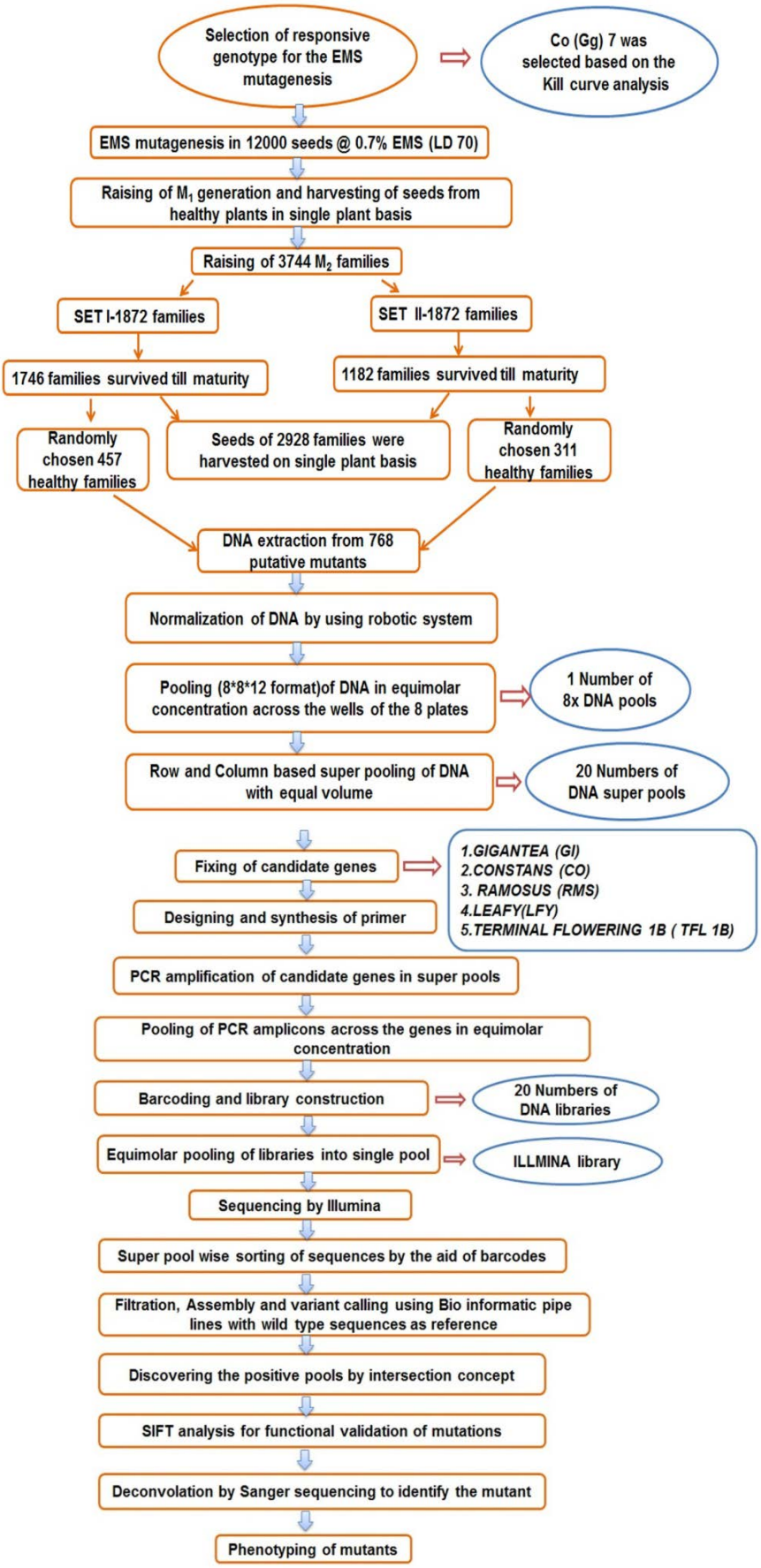
3.1. Generation of TILLING resources

Four genotypes of mungbean (Table 1) *viz*., Co (Gg) 6, Co (Gg) 7, VRM (Gg) 1, VBN (Gg) 1 was chosen for the Kill Curve analysis (Hohmann *et al*., 2005) for fixing a genotype/EMS dose combination that will lead to the production of a TILLING population with optimal density of point mutations (G>A transitions). Seeds of the genotypes were obtained from the Ramiah Gene Bank, Department of Plant Genetic Resources, Tamil Nadu Agricultural University, Coimbatore.

Table 1. Salient features of parents used in the present study

Characters	Parents			
	Co (Gg) 6	Co (Gg) 7	VRM (Gg) 1	VBN(Gg)1
Parentage	WGG 37X CO 5	MGG 336XCoGG 902	Pure line selection from K 851	Hybrid derivative of S.8 X PIMS 3
Year of release	1999	2006	2001	1989
Plant height(cm)	35-55	30-45	45-55	55-60
Days to 50 per cent flowering (days)	26-30	35-40	31-35	30-35
Maturity duration (days)	62-67	60-65	56-67	65-70
Seed yield (kg/ha)	900	978	1100	770
100 seed weight (g)	3.4	3.5-4.0	3.5	3.6

Figure 1. Work flow of the experiments



3.2. Optimization of mutagen concentration/ genotype combination

In order to optimize the mutagen concentration, initially well filled seeds of uniform size for the four genotypes of mungbean were sorted out for mutagenic treatments. To perform a 'Kill Curve analysis' a random sample of 100 seeds of each genotype was presoaked separately for 6 hours in water. Then the seeds were immersed for six hours in phosphate buffer along with the requisite concentration (0.10, 0.20, 0.30, 0.40, 0.50, 0.60, 0.70, 0.80, 0.90 and 0.100 ml/l) of EMS and 2% DMSO (v/v) with intermittent shaking. Immediately after the completion of treatment duration, the treated seeds were thoroughly washed with 1 % sodium thio-sulphate then washed in running tap water for half an hour to eliminate the residual effect of the chemical and the excess moisture in the seed coat was removed by using folds of blotting paper (Anon,1977). Then seeds of the genotypes were sown separately in the mud filled pot for recording survival percentage on 15^{th} after day after sowing.

3.3. Generation of M_1 population

Based on the results of the Kill Curve analysis (Fig 7) 0.7% EMS and genotype Co (Gg) 7 were chosen for mutagenizing a batch of 12000 seeds. Treated seeds of Co (Gg) 7 genotype were raised during summer 2012 (D.S: 29/02/2012) with spacing of 30x10cm 1W of Millet Breeding Station (MBS) (Plate 1). Lethality, injuries and sterility were recorded in M_1 generation (Plate 2). The seeds of each M_1 plant that survived till maturity were harvested on single plant basis.

The following observations were made in ten randomly selected plants in mutant population and control

3.3.1. Seed germination

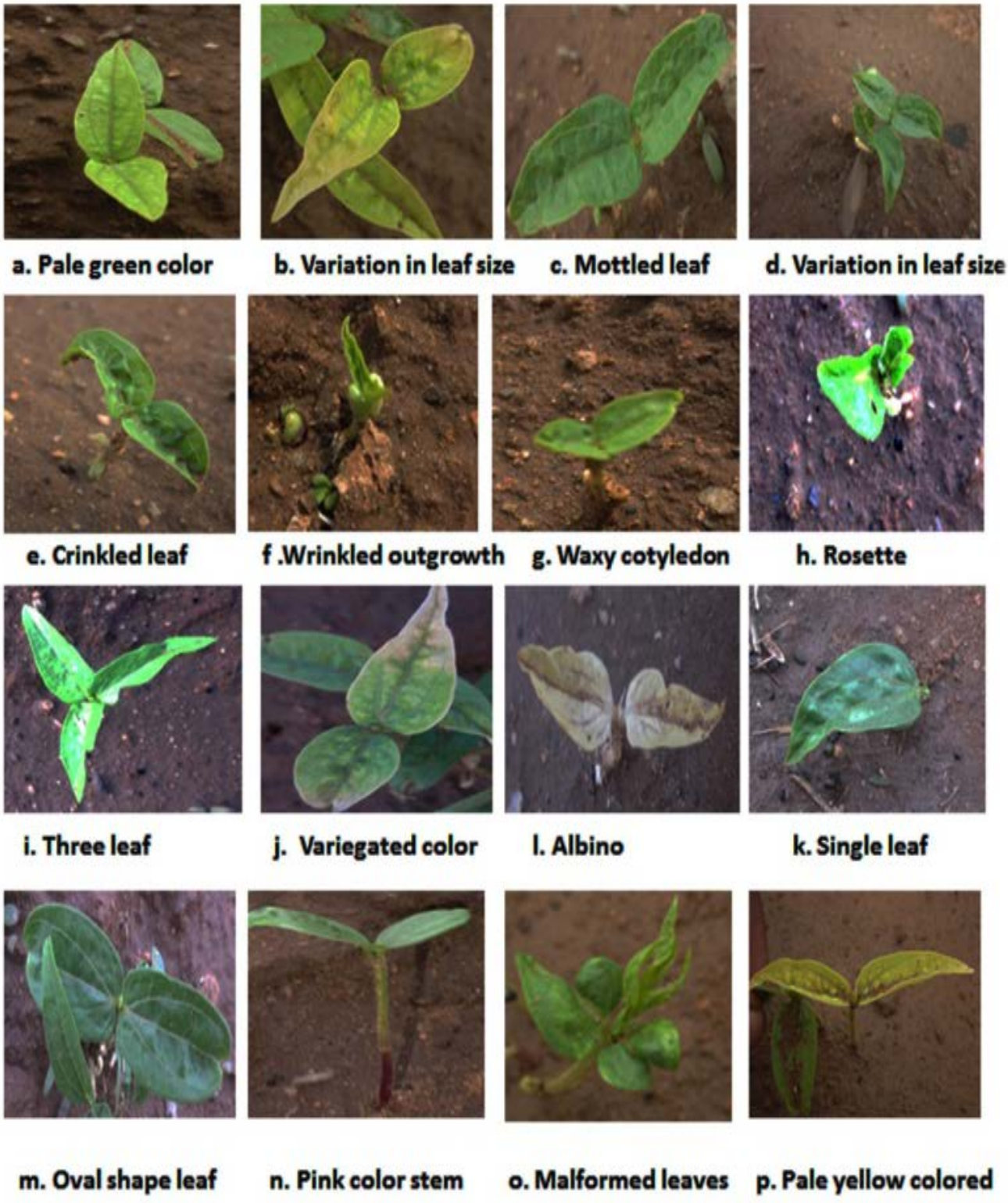
Germinated seeds were observed from third to seventh day. Emergence of leaf was taken as indication of germination. Germination percentage was worked out.

3.3.2. Shoot length

The length of the shoot from the collar region to the tip of the shoot was measured on ten randomly selected seedlings on the seventh day and expressed in cm.

Plate 1. Field view of M_1 generation mungbean TILLING population

Plate 2. Morphological variation observed in M_1 generation



3.3.3. Root length

The root length was also measured from collar region to the tip of the primary root on ten randomly selected seedlings and expressed in cm.

3.3.4. Plant height

The height of the plant from the base to the tip of the plant at 30^{th} day and at maturity was measured and expressed in centimeters.

3.3.5. Pollen fertility

Pollen grains were collected on clean glass slides by dusting anthers of single flower that were about to dehisce and stained with acetocaramine + glycerine (1:1) mixture. Well filled and fully stained pollens were counted as fertile, while the unstained and shrunken ones as sterile.

3.4. Generation of M_2 population (Set I)

The seeds of M_1 population were sorted out batches of 1872 families. About 1872 families were raised during *Kharif* 2012 (D.S: 13.08.2012) on a plant to progeny row basis at field 1W of Millet Breeding Station (MBS) (Plate 3a). Individual M_2 plants were harvested on single plant basis.

3.5. Generation of M_2 population (Set II)

The second set of remaining 1872 families was raised during *Rabi* 2012 (D.S: 13.12.2012) on a plant to progeny row basis at field 1D of Millet Breeding Station (MBS) (Plate 3b). Individual M_2 plants were harvested on single plant basis.

3.6. Seed banking of TILLING population

Generated 2928 MTP (Mungbean TILLING Population) in two batches (Table 2 and Fig 2). Banking seeds of MTP was done through packing seeds in aluminum pouches under vacuum pressure and stored in medium term cold room storage condition @ 5°c is found to more effective for extending viability without bruchid infestation. The process involved in banking TILLING population are presented in

Plate 3a. Field view of M2 generation Set I

mungbean TILLING population

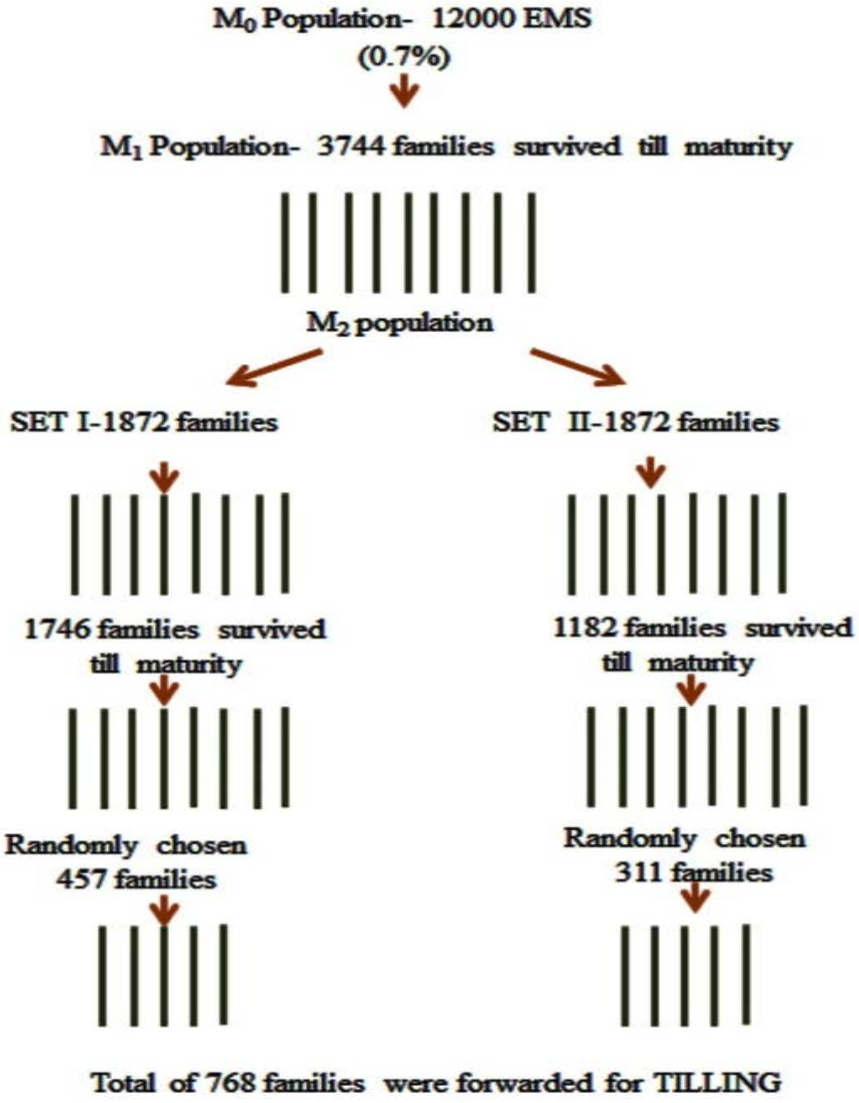
Plate 3b. Field view of M2 generation Set II

mungbean TILLING population

Table 2. Details on generation of mungbean TILLING populations

No of seeds treated in M_1	No. of M_1 plants harvested	Batch No	No. of M_2 families grown	No of M_2 plants tagged for Tilling
12000	3744	I	1872	1746
		II	1872	1182
			Total	**2928**

Figure 2. Schematic representation for the generation of mungbean TILLING population



(Fig 3) and the information was uploaded in Tamilnadu Genetic Resources integrated Database (TNGRID) software maintained at Department of Plant Genetic Resources, Centre for Plant Breeding and Genetics,TNAU,Coimbatore-641003.

3.7. Optimization of DNA Isolation protocol for TILLING in mungbean

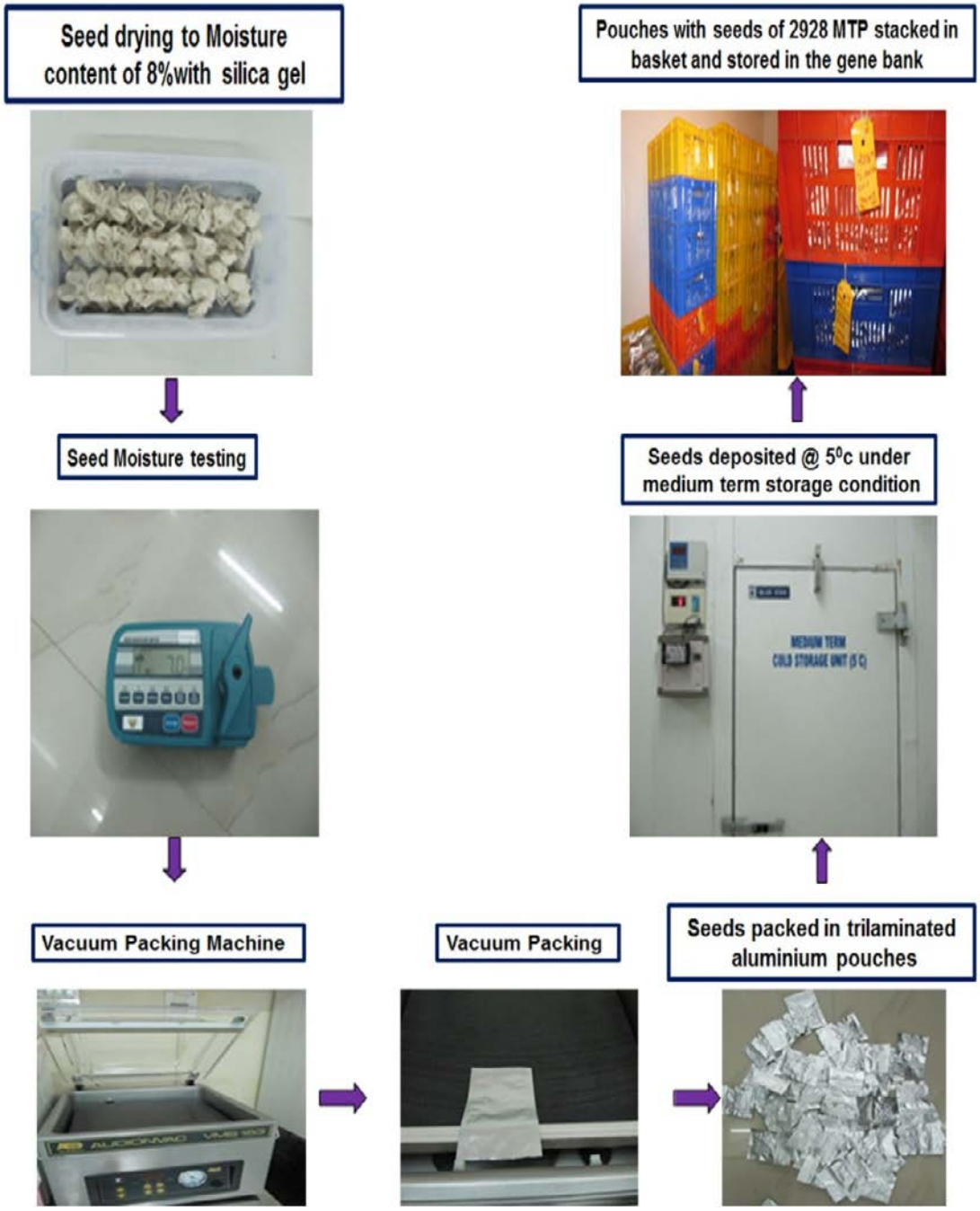
The quality and quantity of DNA used for amplification by PCR is the key to reproducible results and success of TILLING (Targeting Induced Local Lesions IN Genomes). The quantity and quality of DNA from each line must be consistent from sample to sample to allow equal pooling of DNA from several individuals. Many high throughput methods to isolate DNA from plant tissues are available; however, these methods produce either insufficient amounts or inconsistent quality of DNA for TILLING. Several commercial kits are also available to extract genomic DNA from plant tissues with sufficient quality but the yield of DNA produced from commercial kits is often low. Moreover, the cost can be prohibitive for small laboratories. Therefore, a part of the present study was under taken to extract of large quantity and high quality DNA which is often a limiting factor in genetic analysis of plant traits important to agriculture. Also standardized a low-cost, high yield, high quality, and high throughput method to prepare genomic DNA from mature leaves of *Vigna radiata* because TILLING programmes demands extraction of DNA from leaves of viable mutants after pod setting.

3.7.1. DNA isolation

Three protocols (enlisted below) were followed for standardizing of DNA isolation from mature leaves of mungbean. For this optimization protocol we had collected randomly ten leaves samples for extraction. The reagents used for extraction and protocols are given in **Annexure I**.

1. CTAB method of DNA extraction by (Doyle and Doyle 1987)
2. The Modified method of Murray and Thompson (1980)
3. Spin column based extraction with silica loaded binding buffer (B. Hofinger and B.Till, 2013)

Figure 3. Process involved in banking mungbean TILLING population at Ramiah Gene Bank



By adopting the best method we extracted DNA from 768 families to suit the wet lab 96 well formats of 8 rows and 12 columns. The extracted DNA from the families were distributed across 8 numbers of 96 well microplates.

3.8. High-throughput assessment of DNA concentration

Isolated DNA samples were transferred to 96 well plates. The DNA concentration was measured with Tecan Nano Quant Infinite M200 pro (Tecan, Switzerland) multimode reader using a nano quant plate designed for DNA quantification. The software Tecan i-control provides the DNA concentration in ng/ µl along with A_{260nm}/A_{280nm} purity ratio.

3.9. DNA normalization

After assessment of the concentration, DNA samples were normalized with different volume of water addition computed by using the formula

$V_1\ C_1/C_2 = V_2$.

Where,

V_1 = Final volume (µl) of normalized DNA required,

C_1 = Required final concentration of DNA,

C_2 = Actual concentration of the stock DNA,

V_2 = Volume of stock to be added.

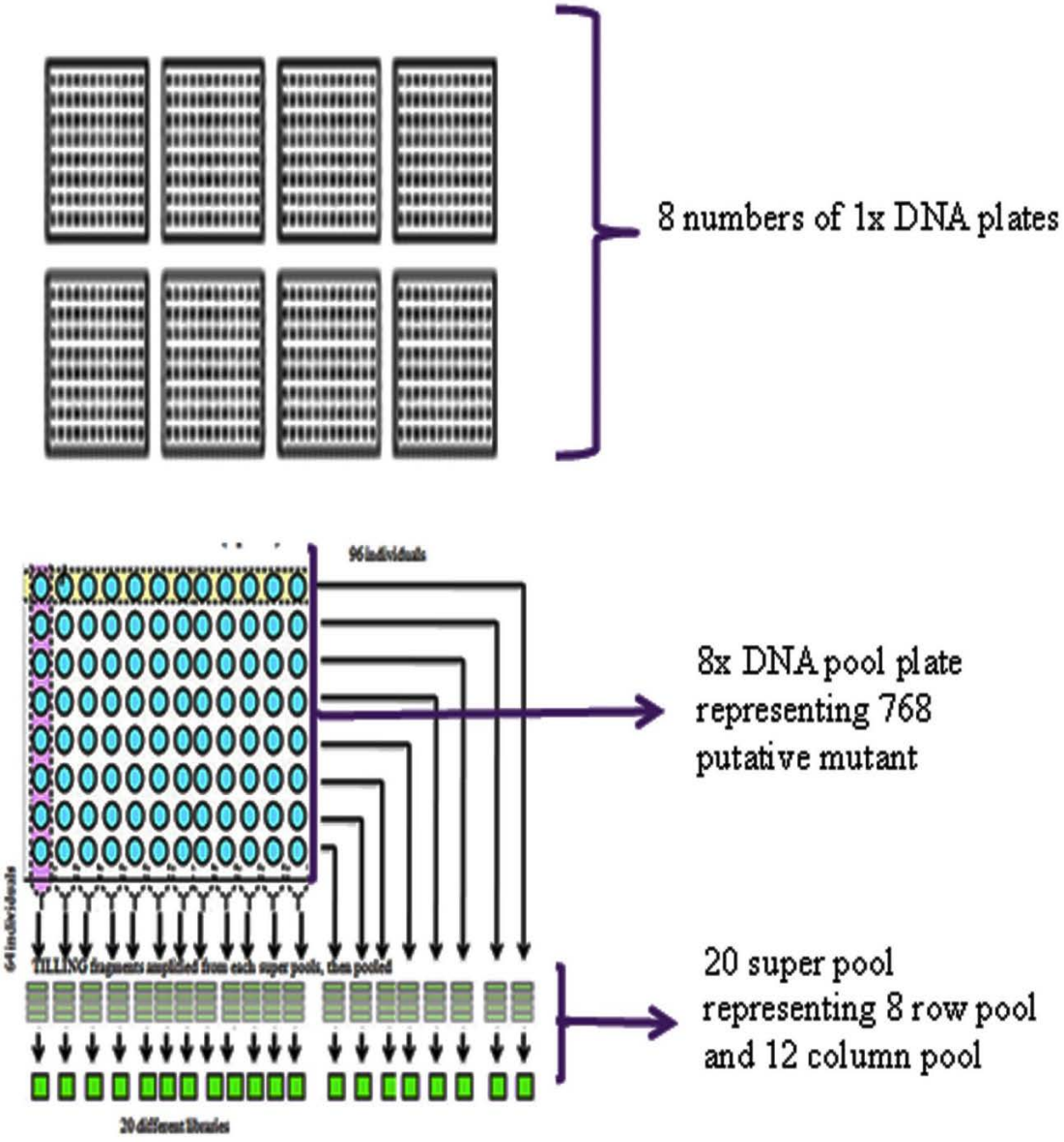
For dispensing different volumes of water in 768 samples in the deep well plates, a Tecan Freedom Evo75 (Tecan, Switzerland) robotic liquid handling system was employed (Plate 5).

3.10. Establishing a DNA bank of TILLING populations (DNA pools)

In order to increase the throughput during allele mining, pooling of normalized genomic DNA from a set of 768 M_2 families were accomplished. The strategy of DNA pooling is as depicted in the Figure 4.

Plate 5. High- throughput assessment of DNA normalization through robatic pipetting method

Figure 4. Schematic representation of pooling of genomic DNA of M_2 population



3.10.1. Pooling

Pooling strategy permits to decrease the number of reactions needed to screen an entire genomic library by PCR. So the extracted genomic DNA from 768 putative mutants were transferred to eight numbers of 2ml deep well plates in 8 rows X 12 column format representing 8 (rows) * 12 (columns) * 8 (plates) format . From the 8 numbers of 1x DNA plates, one 8x pooled **(Annexure II)** plate was prepared by pooling DNA in equimolar proportions in new well maintaining the row and column format. The 8x pooled plate was further pooled across the columns and rows (12+8) to prepare a super pool (pool of pool) plate which resulted in 20 super pools. DNA from the 20 wells of the super pool plates were used for PCR amplification of TILLING fragments.

3.11. Creation of reference Sample

Since whole genome sequence of mungbean is not yet available, we extracted the DNA from the control (Co (Gg) 7) sample using the above standardized protocol and kept it has a reference sample for further variant analysis.

3.12. Search for candidate gene sequences

As the sequence support for greengram (*Vigna radiata*) is very limited, hence resorted to use the sequences of the candidate genes (CGs) from other model legumes like Medicago, Soybean and Cowpea (Table.3). The gDNA and cDNA sequences of aforementioned genes were searched using BLAST tools of NCBI Genbank (http://www.ncbi. nlm.nih.gov/Genbank). These sequences were utilized for building up gene models and designing primers.

3.13. Designing primers to capture TILLING fragments of target key genes

EST sequences of *Medicago truncatula* will be ideally suitable for designing cross species primers for amplifying the *Gigantea* genes in mungbean, due to more query coverage and maximum identity it has recorded with all known sequences from crops like soybean and peas. Similarly for the *Leafy* and *Tfl* genes from *Vigna ungiculata* and *Constans, Ramosus* from *Glycine max.* The primers were designed using the software Primer3 (Primer3 http://frodo.wi.mit.edu/primer3/) and primer pairs are furnished in the Table 4.

Table 3. Key candidate genes and their function identified for TILLING by sequencing

S.No	Gene name	Sources	Accession ID	Gene length (bp)	Function	Consequence of disruption
1	*GIGANTEA(GI)*	*Medicago truncatula*	XM_003591999.1	7923	Regulating the expression of flowering time genes during the promotion of flowering by photoperiod	Late flowering
2	*RAMOSUS(RMS)*	*Glycine max*	XM_003522665.2	1765	Inhibit branching habit	Increased branching at basal vegetative nodes
3	*CONSTANS(CO)*	*Glycine max*	EU882819.1	2560	It is a central regulator of photoperiod pathway, triggering the production of the mobile florigen hormone *FT (FLOWERING LOCUS T)* that induces flower differentiation	Delayed flowering
4	*LEAFY(LFY)*	*Vigna unguiculata*	AB588745.1	2851	It promotes the transition from inflorescence to floral meristem	Delays floral primordial initiation
5	*TERMINAL FLOWERING1b (TFL1b)*	*Vigna unguiculata*	AB588742.1	1534	It maintains the indeterminate growth of the SAM by inhibiting the expression of the floral meristem identity genes *LFY* and *AP1*	Promotes terminal flowering

Table 4. Details of gene specific TILLING fragments discovered through CODDLe analysis

Gene	PSSM Score difference	Gene area of highest PSSM score difference	Primers (5' to 3')	Amplicon size (bp)
GIGANTEA(GI)	-51 to 169	548 to 1846	F-GCCCATGGTCCAGAAGTTG	1300
			R-AATTGGCGAGATCAGATGCC	
RAMOSUS(RAM)	-4 to 175	2271 to 3569	F- CGAGTTTTTGACGTTGTTGCC	780
			R- TCATCTTCCTCTGTTGCTCCTG	
CONSTANS (CO)	-1 to 641	60 to 1358	F- ACCAGCATTTTCAGCTTGG	1456
			R- ACCTGAATGGGAGGTCCAG	
LEAFY(LFY)	0 to 1364	586 to 1198	F- CCACCAAGGTATTCTTGTCA	652
			R- GGGACAATGTTATACCAGCA	
TERMINAL FLOWERING 1b (TFL1b)	0 to 787	586 to 1304	F- TGTGGGAAGAGTGATAGGAG	519
			R- AACACTTTGAGATTGAAACG	

The five TILLING fragments (1.3 to 2.8 kb) covering five key candidate genes with maximum mutation probability for PCR amplification were fixed using the bioinformatic pipeline CODDLE (Codons Optimized to Detect Deleterious Lesions; http://bioinfo.ut.ee/primer3/).The Primers for PCR amplification of five TILLING fragments were designed with PRIMER 3 software embedded with CODDLE. For high robustness in PCR amplification, the primers were developed to meet the following criteria: (1) the amplified fragments were verified for genome-specificity (2) 3' end complementarity and self-complementarity value of the primers verified to be below 3 and 8 respectively to reduce the mispriming .The gene models were built for all the five candidate genes based on blasting protein and EST sequences with the genomic sequences using different bioinformatics pipelines *viz.,* Sim4, CODDLe, Genewise and Spidey and a consensus models build up by all the pipelines were considered as valid as depicted in Figure 5.

3.14. PCR Amplification of TILLING fragments with pooled DNA

Following primer design, to determine functionality of the primers, PCR amplification was carried out using *TaKaRa LA Taq polymerase* (Code No.RR02AG) obtained from TAKARA BIO INC.

3.14.1. Optimization PCR protocol for amplifying TILLING protocol

An integrated long range (LR) PCR approach was applied to amplify all the five TILLING fragments in 20 super pools along with the control Co(Gg) 7. Multiple LR-PCR available to amplify long genomic fragments and some of them are advertised as being able to amplify up to 15 kb or even longer genomic DNA which can work well for specific genomic regions. TaKaRa LA *Taq® DNA Polymerase* Hot-Start Version (Cat#RR042A) obtained from Takara Clonotech Laboratories, USA was used for PCR amplification, in 50 µl reactions with pooled genomic DNA. TaKaRa *LA Taq® DNA Polymerase* which gives two fold higher fidelity than other Taq DNA Polymerase.

The default protocol provided by manufacture was not successful in amplifying the TILLING fragment of all the candidate genes. So further modification in the reaction mix and conditions (Table 5 and 6) such as a step down PCR cycle was followed to avoid non-specific amplification. By adopting the optimized protocol we could successfully

Figure 5. Gene models for candidate genes

a. Gene model for *GIGANTEA (GI)*

b. Gene model for *RAMOSUS (RMS)*

c. Gene model for *CONSTANS(CO)*

d. Gene model for *LEAFY (LFY)*

e. Gene model for *TERMINAL FLOWERING 1b(TFL1b)*

Table 5. PCR reaction condition followed for the amplification of TILLING fragments

S.No	Reaction Step	Temperature (°C)	Time	Cycles
1.	Initial denaturation	94	1 min	1
2.	Denaturation	94	30 seconds	10
3.	Annealing with 1°C decrement per cycle	63*/60**	45seconds	
4.	Extension	72	1min/kb (Fixed based on TILL fragment size)	
5.	Denaturation	94	30 sec	30
6.	Annealing	55	1minutes	
7.	Extension	72	1min/kb	
8.	Final extension	72	10 minutes	
9.	Hold at	10	Till end	

*PCR cycle I **PCR cycle II

Table 6. PCR master mix followed for the amplification of TILLLNG fragments

S.No	Reagent	Concentration	Volume(µl)
1.	TaKaRa GC Buffer I	2x	25
2.	TaKaRa LA Taq	5 units	0.5*/1.0**
3.	dNTPs	2.5mM(each)	8
4.	DMSO	99%	1
5.	$Mgcl_2$	50mM	1*/2**
6.	Template DNA	25 ng/µl	6*/7**
7.	Forward TILL primer	10 mM	3
8.	Reverse TILL primer	10 mM	3
9.	H_2O	-	2.5
10.	Total reaction volume	50.00	

*PCR master mix I **PCR master mix II

amplify the TILLING fragment for all the five candidate genes in 20 super pools. A total of 100 PCR products and 5 products from control sample were amplified using ABI 2720 (Applied Bio systems, Foster City, CA) thermal cycler.

3.15. Equimolar pooling of PCR products

The concentration of the PCR products were quantified using the Qubit dsDNA BR assays system (Invitrogen, Carlsbad, CA) to avoid errors from free nucleotides, excess enzymes present in the PCR. The amplified products from five TILLING fragments of super pooled samples were normalized to 25 ng /µl and equimolarly pooled across the genes on the predicted length of the individual TILLING fragments. Due weightage for amplicon size and of copy numbers for the computation of equimolarity was adopted. The final PCR pool for individual amplified superpools was prepared with the objective of having the final concentration of 2.0 µg of long amplicons (Plate 7).

3.16. Next-generation sequencing of pooled TILLING libraries

The NGS was outsourced to Genotypic Technology Pvt. Ltd., Bangalore-560094.

3.16.1. Materials

Sure Select QXT Library Prep Kit (Cat #5500-0120)

Agencourt AMPURE XP beads (Beckman Coulter, #A63881)

High Sensitivity Bioanalyzer Chips (Agilent, #5067-4626)

Nuclease free water (Ambion, #AM9939)

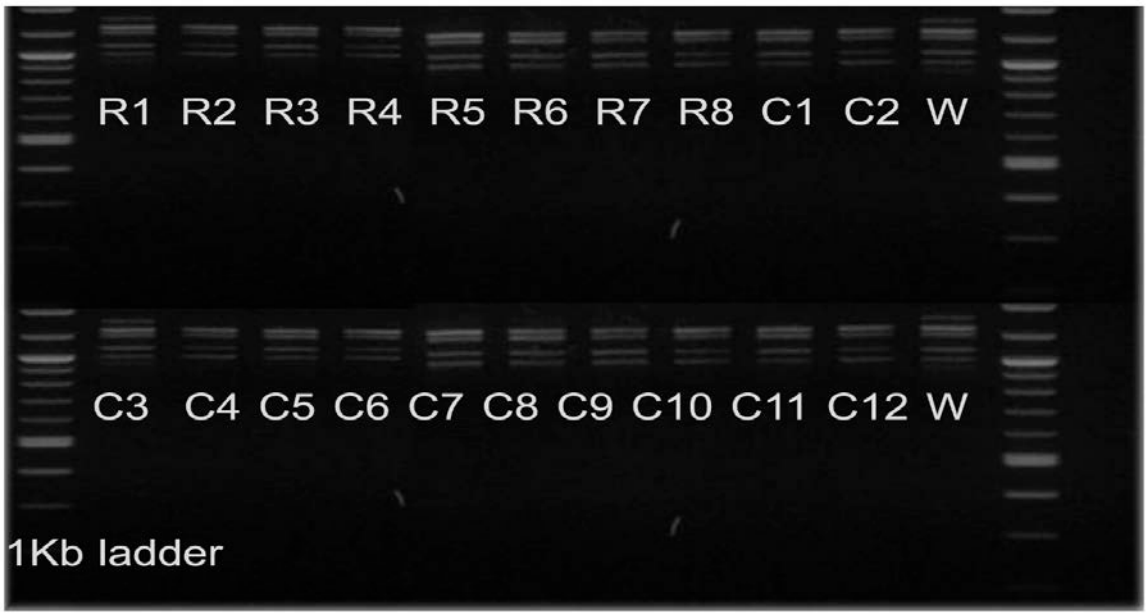
Qubit® dsDNA HS Assay Kit (Invitrogen, Cat # Q32854)

Absolute Ethanol (Millipore, Cat#K45259083 406)

3.16.2. Methods

Library preparation was performed at Genotypic Technology's Genomics facility following Sure Select QXT Library Preparation protocol outlined in Sure Select QXT Whole genome Library Prep for Illumina Multiplexed sequencing Protocol (Cat # 5500-0120).

Plate 7. PCR amplified products of all candidate genes in 20 super pools and in wild types



R1,R2,R3,R4,R5,R6,R7,R8- Row pools
C1,C2,C3,C4,C5,C6,C7,C8,C9,C10,C11,C12- Column pools
W- Wild type as Reference set

20ng of Qubit quantified genomic DNA was taken for Library preparation. Genomic DNA was fragmented and adapter-tag was added using Sure Select QXT Enzyme. Fragmented DNA was cleaned using Ampure XP beads. Cleaned adapter tagged fragments were amplified and indexed according to the protocol. Sample cleanup was done using Ampure XP beads. The prepared libraries were quantified using Qubit flourometer and validated for quality by running an aliquot on high sensitivity bioanalyzer chip (Agilent).

3.16.3. Analysis methodology

3.16.3.1. Sequencing

The samples were sequenced with Illumina Miseq Sequencer. Once the base calling is done, the raw reads were processed using inhouse **PERL** programme to process of trimming and filtering to get only the high quality reads.

3.16.3.2. Assembly

The sequence of five TILLING fragments was used to create a template assembly of the sequence from library 21st (Control) using **SPAdes** and **SSPACE**. The above template of five gene fragments was used to assemble sequences from rest of the samples.

3.16.3.3. Trimming and filtering the reads

Trimming is done to remove any undesired base calls at the 3' end of the read, to remove the adapter sequence, to remove the lower quality 3'ends. Filtering at the next step removes reads estimated to contain the low quality base calls. This two steps ensures the reads which taken further are of high quality.

3.16.3.4. Reads summary

The total number of reads obtained per sample is included.

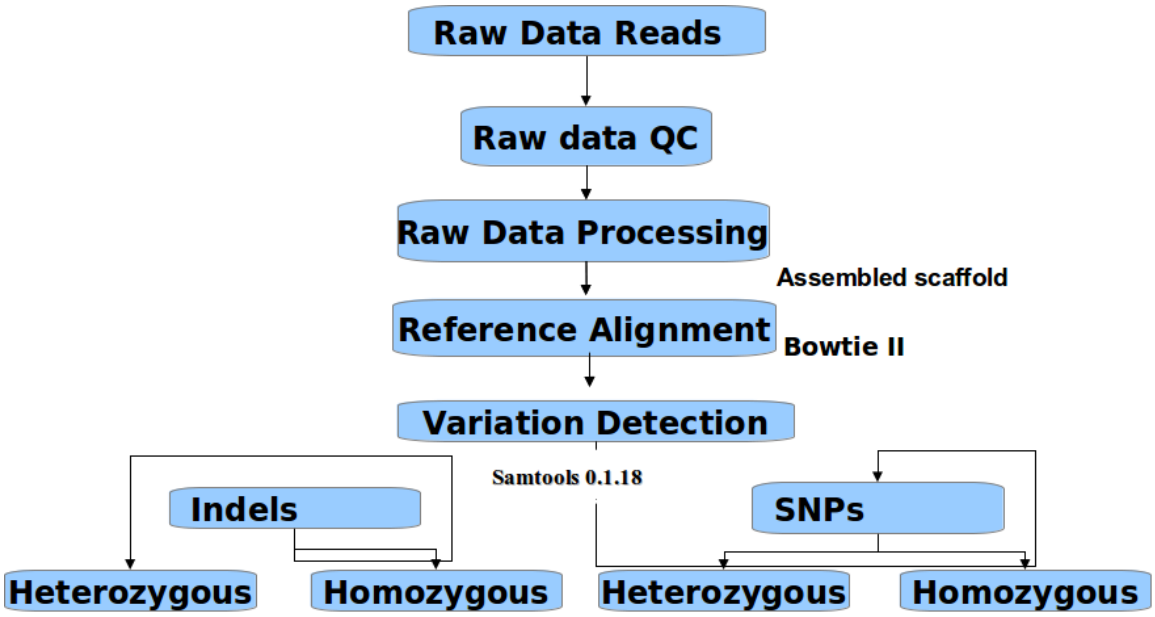
3.16.3.5. Alignment

The trimmed reads obtained are aligned to the reference scaffold with **Bowtie II** (Langmead and Salzberg. 2012). The percentage of coverage for all the samples were found.

3.16.3.6. Variant identification

The variants were detected using the **samtools 0.1.18** (Li *et al*., 2009). (Fig 6)

Figure 6. Overview of Variant analysis



3.17. Functional validation of SNPs through Sorting Tolerant from Intolerant (SIFT) analysis

Discovered sequence variants were analysed by the PARSESNP program (http://www.proweb.org/parsesnp/), which provides information on the location along with the details about amino acid changes and severity of mutations. This pipeline also provides information on the gain or loss of restriction sites caused by the induced polymorphisms.

Addition, deletion and substitution of the each amino acid have the potential effect on protein function (Sim *et al.*, 2012). SIFT (http://sift.jcvi.org/www/ SIFT_seq_ submit2.html.) a web based tool which predicts whether amino acid substitution affects protein function and structure based on sequence homology and the physical properties of amino acids (Ng and Henikoff, 2002) was employed to discover functional mutations. The predicted SIFT score ranges from 0 to 1. The amino acid substitution is predicted damaging is the score is < 0.05, and tolerated if the score is > 0.05.

3.18. Deconvolution of pooled samples

Deconvolution is the process of identifying the individual genotype carrying the mutation for the gene of interest. The discovered mutations were deconvolved using the concept of intersection (presence of same mutation in one of the row as well as a column super pool) and located the well (8X) carrying the mutant. The genotypes present in the pools were identified by using aforementioned concept (Table 7). The individual genotypes were amplified for discovered mutations and the same were detected through Sanger sequencing.

3.18.1. Sanger sequencing

For the discovery of individual mutation located in the genotypes, primers covering mutant of interest were designed by using web based tool PRIMER 3 (http://www.ncbi.nlm.nih.gov/tools/primers-blast/. The details of primers used for deconvolution of mutations through Sanger sequencing is given in Table 8. The Sanger sequencing was performed using BigDye® Terminator version 3.1 cycle sequencing kit (Applied Biosystems) on an ABI3730L (96 well) sequencer (Applied Biosystems) according to the manufacturer's instructions. By comparing the gene sequences of the individual PCR products from the pool with reference sequence were carried out to know the genotypes with positive mutation.

3.19. Biometrical characterization of the discovered mutants suitable for flowering time and branching habit

M_3 families were raised for MTP-399, MTP-580 and MTP 134 of the discovered mutants for candidate genes *GIGANTEA, RAMOSUS* and *TERMINAL FLOWERING (TFL 1b)* respectively. About 25 seeds of each family of discovered mutants were raised along with control Co (Gg) 7 during *kharif* 2014 (Plate 10) at 1W field of Millet Breeding station (MBS) with spacing of 30x10cm control for the following biometrical characterization to identify altered phenotypes.

3.19.1. Days to 50 per cent flowering (FPF)

The number of days taken from the date of sowing to the date when 50 per cent of the flowering in population was recorded.

Table 7. List of mutants selected in 8x super pool through interception concept

S.No	Well Position	Gene	Mutants
1	R2,C3	*GI*	MTP-15,MTP-111, MTP-207,MTP-303 MTP-399,MTP-495, MTP-591,MTP-687
2	R1,C4	*RMS*	MTP-4,MTP-100, MTP-196,MTP-292, MTP-388,MTP-484, MTP-580,MTP-676
3	R4,C2	*TFL1b*	MTP-38,MTP-134, MTP-230,MTP-326 MTP-422,MTP-518, MTP-614,MTP-710

Table 8. Details of primers used for deconvolution of variants through Sanger sequencing

Gene	Mutation	Primers (5'to3')	Amplicon Size (bp)
GI	G605A	F- GCTGTGGCAGAGCTTCGTA	119
		R- TGGGCTTCATGACTGACAC	
RMS	T1511A	F- CCTGATGCTAGGGTTGGTCG	251
		R- ACAGCACCTTCTTCATGCCA	
TFL1b	G165A	F- ATGGAACCACTTTCTGTGGGAA	330
		R- TCCTTGCAGCAGTCTCTCTCT	

Plate 10. Field view of M_3 generation for evaluating mutants

Plate 11. Wooden structure designed to access pods for mechanical harvesting

3.19.2. Plant height (PLH)

The height of the plant from the ground level to the tip of the main stem at the time of physiological maturity was measured and expressed in centimeters.

3.19.3. Number of branches per plant (NOB)

When first pod changes colour, count only number of primary branches whose origin is in the leaf axils of the main stem.

3.19.4. Length of longest branch (LLB)

Length of longest branch for each plant measured at physiological maturity and expressed in centimeters.

3.19.5. Height of first branching from the ground (HFA)

Height of the first branch from base measured from each mutant at pod maturity stage and expressed in centimeters.

3.19.6. Peduncle length (PEL)

Length of the longest peduncle measured when first pod changes colour and expressed in centimeters.

3.19.7. Days to first mature pod (DMT)

From sowing to stage when 50% of plants have mature pods.

3.19.8. Height of the first branch (HFB)

It is obtained by subtracting the plant height with the height of the first branching and expressed in centimeters.

3.19.9. Pod spread (POS)

Distribution of pods within the plant is observed in such a way that, the distance in centimeter is measured between left side extreme pod to right side extreme pod of the plant at physiological maturity.

3.19.10. Height of first pod (HFP)

Height of the first pod from base measured at physiological maturity and expressed in centimeters.

3.19.11. Internodal distance (INL)

The internodal distance measured for each mutant and recorded and expressed in centimeters. .

3.19.12. Number of clusters per plant (NCP)

The number of clusters per plant was counted and recorded at the time of maturity.

3.19.13. Number of pods per cluster (NPC)

The number of pods per cluster was counted and recorded at the time of maturity.

3.19.14. Number of pods per plant (NPP)

The number of fully matured pods per plant was counted at the time of maturity and recorded.

3.19.15. Pod length (POL)

Pods from each of the selected plant were taken and the pod length was measured from base to tip and expressed in centimeters.

3.19.16. Number of seeds per pod (NSP)

The number of seeds in each of the five randomly selected pods in a plant was counted and their average was recorded as number of seeds per pod.

3.19.17. Accessible pods per plant (APP)

A wooden structure was designed used to fix the height and width of the plant with in which pods were harvested .The structure (Plate 11) consisted of a flat plank as a platform and two vertical poles of height 50 cm with 4 rulers at a height of 19 cm, 25 cm, 30 cm and 35 cm. The spacing between the rulers is used tie the thread, placed at a distance of 30 cm that were marked as fixed grooves which represents the fixed width

of the plant. Using this device, the pods which were above the height of the ruler and within the width of 30 cm were harvested. Height of 25 cm for dwarf plants, 30 cm for moderate plants and 35 cm for tall plants were fixed. The number of harvested pods was counted.

3.19.18. Harvestable pod percentage (HPP)

It is the percentage of accessible pods to the total pods.

3.19.19. 100 seed weight (HSW)

Weight of 100 randomly selected seeds expressed in grams.

3.19.20. Yield per plant (SPY)

The weight of total seeds collected from each plant was recorded in gram.

3.20. Statistical analysis

The mean data recorded on five plants for different characters over the genotypes and replications were subjected to statistical analysis.

3.20.1. Unit analysis

The estimate of mean, variance and standard error were worked out by adopting standard methods (Panse and Sukhatme, 1967).

1. General mean (G.M) = Total of all values /n

Where, n = number of observations

2. Variance = SS-CF/ DF

Where,

SS = Sum of squares of all observations of a variate

Correction Factor = $(\text{Grand total})^2$/ N

CF = Correction factor

DF = Degree of freedom

3. Standard deviation (SD) = $(\text{Variance})^{1/2}$

4. Standard Error (SE) = SD/ n

5. Coefficient of variation (CV%) = _SD X 100/ mean

3.21. Mutation frequency

The mutation frequency for each amplicon is calculated as follows

$$\text{Mutation frequency} = \frac{(\text{size of amplicon} - 100\text{ bp} \times \text{total number of screened samples})}{\text{Total number of identified mutations}}$$

100 bp were subtracted because of the diminished ability to detect mutations in the upper and lower 50 bp; Gottwald *et al*., 2009.

Experimental Results

CHAPTER IV

EXPERIMENTAL RESULTS

Present investigation included the following research components *viz*., 1.Optimizing mutagenesis in mungbean for the saturational mutagen and generation of TILLING population. 2. Discovery of putative mutants from branching and flowering for robust branching type and delayed flowering type through TILLING by sequencing. 3. Biometrical characterization of the discovered mutants suitable for branching and flowering habit. The results obtained from these experiments are presented here under.

4.1. Optimizing mutagenesis in mungbean for the saturational mutagen and generation of TILLING population

In this study TILLING was performed by sequencing in 768 families of M_2 population with their DNA represented as 20 super pools.

4.1.1. Generation of TILLING resources

The results of the Kill Curve analysis (Table 9 and Fig 7) revealed that among EMS concentration tried 70mM was found to be the optimal for achieving 70 per cent kill for the genotype Co (Gg) 7 with 29.2 per cent survival as compared to the other three genotypes *viz*., Co (Gg) 6, VRM (Gg) 1 and VBN (Gg) 3 are 21 per cent, 19.4 per cent and 22.1 per cent respectively. The genotype Co (Gg) 7 showed relatively higher tolerance (high survival %) to EMS doses and was this suitable for creating TILLING populations as these genotype can harbour more mutations with in a manageable population size than the genotypes that exhibit high mortality due to cytotoxicity. Hence genotype /EMS combination of Co (Gg) 7 /70mM was fixed for generating Mungbean TILLING Populations (MTP).

4.1.2. M_1 population

Table 10 revealed that at 70mM concentration of EMS, the mean germination percentage in M_1 population was 30.14 per cent whereas in control 93.37 per cent was observed. Germination percentage was ranged from 28.75 per cent to 33.54 per cent with a 67.72 per cent reduction was recorded. The average shoot length in M_1 population was

Table 9. Data on survival (%) observed after 15 days of treatment of seeds in four mungbean genotypes with different doses of EMS

EMS doses in mM										
Genotypes	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**
Co 6	89.4	68.5	54.1	42.7	32.5	27.6	21.0	18.2	8.9	4.1
Co (Gg) 7	93.4	77.1	66.8	55.2	47.4	34.5	29.2	15.6	11.4	9.8
VRM (Gg) 1	78.3	65.7	53.3	48.5	37.7	26.7	19.4	13.3	7.3	2.1
VBN (Gg) 3	84.1	72.3	65.3	51.0	43.1	30.6	22.1	13.4	7.8	4.2

Figure 7. Graphical representation of results of Kill curve analysis

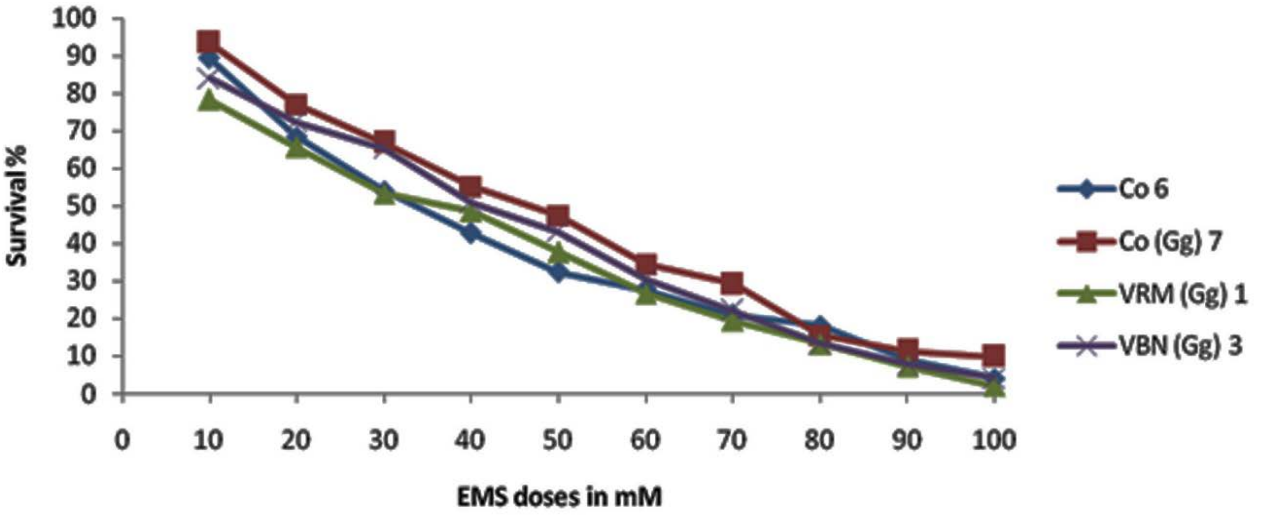


Table 10. Effect of mutagen on various characters in M_1 generation (EMS 70mM)

S.No	Characters	Mean	Min	max	SD	CV	Control	Percent over control	Percent reduction
1	Shoot length (cm)	7.53	6.98	8.30	0.50	6.64	11.65	64.63	35.37
2	Root length (cm)	3.88	3.17	4.21	0.33	8.50	5.50	61.45	38.55
3	Plant height (cm)	25.93	24.98	27.01	0.78	3.00	34.95	74.10	25.90
4	Pollen fertility (%)	77.04	72.05	85.36	3.69	4.78	87.49	88.05	11.95
5	Plant height at maturity	43.77	37.98	48.65	4.09	9.34	48.95	89.41	10.59
6	Germination%	30.14	28.75	33.54	1.69	5.60	93.37	32.28	67.72

7.53cm, whereas in control it was 11.65cm. Shoot length was ranged from 6.98cm to 8.30cm with 35.37 per cent reduction was recorded. The average root length in M_1 population was 3.88cm, whereas in control it was 5.50cm. It was ranged from 3.17cm to 4.21cm with 38.55 per cent reduction also recorded. The mean pollen fertility percentage in M_1 population was 77.04 per cent, whereas in control it was 87.49 per cent. It was ranged from 72.05 per cent to 85.36 per cent with 11.95 per cent reduction also recorded. Similarly the mean plant height at maturity in M_1 population was 43.77cm, whereas in control it was 48.95cm. It was ranged from 37.98cm to 48.65cm with 10.59 per cent reduction also recorded. The seeds of each M_1 plant which survived till maturity were harvested on single plant basis. 3744 plants survived till maturity.

4.1.3. M_2 population Set I

The seeds of M_1 population were sorted out in batches of 1872 families. About 1872 families were raised during *Kharif* 2012, of which 1746 families survived till maturity. Among them 457 healthy families were randomly chosen (Fig 2). Leaves were collected from these families and their pods were harvested on single plant basis. Pods were threshed to obtain a seed which was stored in medium term storage at Ramiah Gene Bank.

4.1.4. M_2 population Set II

The second set of remaining 1872 families was raised during *Rabi* 2012, out of which 1182 families survived till maturity.311 families were randomly chosen (Fig 2) among them. Leaves were collected from these families and their pods were harvested on single plant basis. Pods were threshed to obtain a seed which was stored in medium term storage cold room at Ramiah Gene Bank.

4.2. Discovery of putative mutants from branching and flowering for robust branching type and delayed flowering type through TILLING by sequencing

4.2.1. DNA isolation for TILLING by sequencing

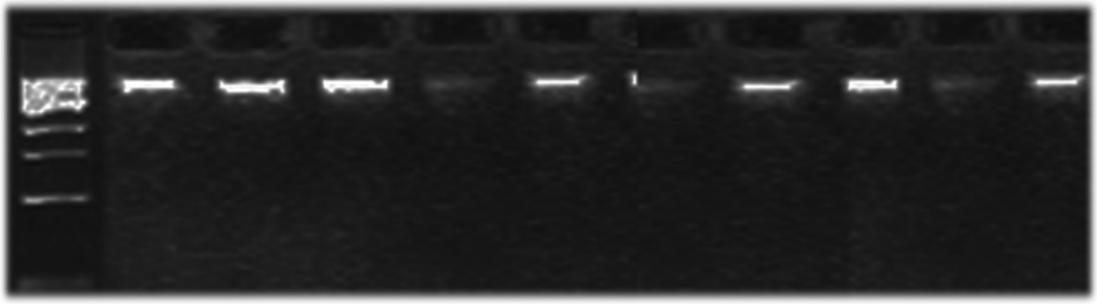
Table 11 shows the differences in DNA yield and quality with respect to different methods of extraction. In CTAB method (Plate 4a), mean DNA yield in ten test samples was 266.47 ng/µl and it varied from 182.2 to 382.1 ng/µl. The mean quality score of 1.54

Table 11. Optimization of DNA isolation protocol for TILLING in mungbean

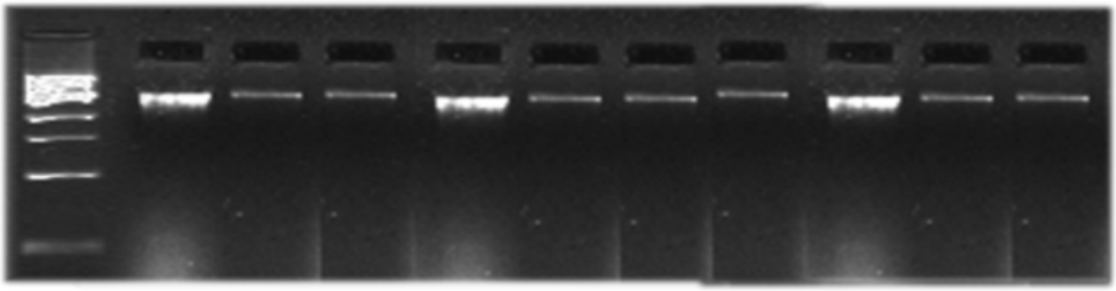
Samples	Extraction Methods					
	CTAB		Murray and Thompson (1980) with few modifications		Spin coloumn based extraction with silica loaded binding buffer	
	Quantity of DNA (ng/µl) obtained from 250 mg leaf tissue	Absorbance $_{260/280\ nm}$	Quantity of DNA (ng/µl) obtained from 250 mg leaf tissue	Absorbance $_{260/280\ nm}$	Quantity of DNA (ng/µl) obtained from 250 mg leaf tissue	Absorbance $_{260/280\ nm}$
1	362.2	1.56	425.3	1.84	762.5	2.01
2	225.8	1.35	396.1	1.78	845.2	2.01
3	186.5	1.64	284.2	1.89	912.3	2.02
4	282.2	1.66	421.2	1.78	689.5	1.98
5	320.7	1.75	440.1	1.95	659.7	2.11
6	382.1	1.52	417.6	1.84	885.4	2.07
7	245.6	1.44	369.7	1.82	789.6	2.01
8	266.5	1.46	312.5	1.77	909.2	2.03
9	182.2	1.58	298.2	1.68	885.7	1.99
10	210.9	1.47	315.4	1.89	874.3	1.98
Mean	266.47	1.54	368.03	1.82	811.34	2.02
Range	182.2-382.1	1.35-1.75	284.2-440.1	1.68-1.95	659.7-912.3	1.98-2.11
SD	66.63	0.11	56.83	0.07	81.48	0.03

Plate 4. Optimization of DNA isolation protocol for TILLING in mungbean

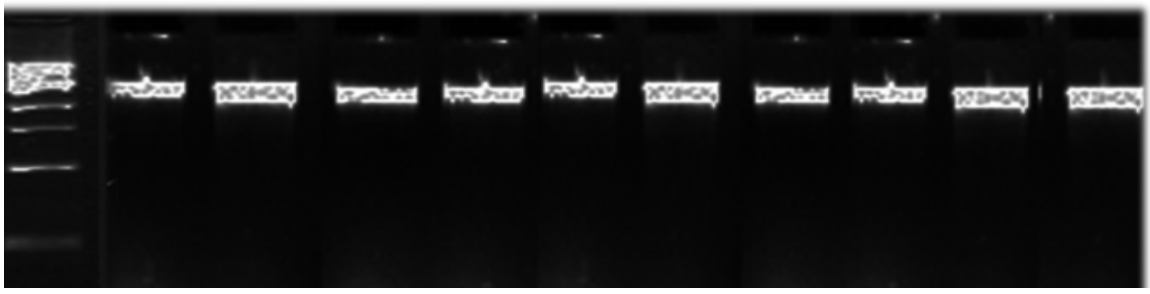
4a.DNA isolated by the CTAB method by J.J.Doyle and J.L.Doyle (1987)



4b. DNA isolated from the modified method of Murray and Thompson (1980)



4c. DNA isolated from spin column based extraction with silica loaded binding buffer (Bernhard Hofinger and Bradley Till 2013)



which varied from 1.35 to 1.75. In the modified method of Murray and Thompson (1980) (Plate 4b) the average yield was recorded 368.03 ng/µl and ranged from 284.2 to 440.1 ng/µl with average quality score of 1.82 which varied from 1.68 to 1.95. In the spin column based extraction with silica loaded binding buffer (Plate 4c) showed the average recovery of 811.34 ng/µl with ranged from 659.7 to 912.3 ng/µl. An average quality score of 2.02 was recorded in this method with ranges from 1.98 to 2.11. Among the methods investigated, binding buffer loaded with silica showed consistent high recovery with desirable quality score.

By adopting the best method *viz.,* the spin column based extraction with silica loaded binding buffer DNA were extracted from 768 putative mutants to suit the wet lab 96 well format of 8 rows and 12 columns. The extracted DNA from the families were distributed across 8 numbers of 96 well microplates.

4.2.2. Normalization of DNA for TILLING

The values on the concentrations of DNA normalised to an expected concentration of 100 ng/µl along with its associated variation accomplished through manual and robotic pipetting are furnished in Table 12. In plate 1, the mean normalized DNA yield through manual pipetting for 96 samples was 97.61 ng/µl and varied from 95.60 to 99.20 ng/µl whereas in robatic pipetting, it ranged from 99.98 to 100.11 ng/µl with mean yield of 100.02 ng/µl. Average normalized DNA for 96 samples in plate 2 through manual pipetting was 97.82 ng/µl and varied from 95.80 to 99.20 ng/µl whereas through robatic pipetting gives a average yield of 100.02 ng/µl and ranged from 99.97 to 100.11 ng/µl. In plate 3 the normalized DNA yield using manual pipetting method was 97.33 ng/µl with ranged from 94.60 to 99.70 ng/µl whereas through robatic pipetting, it ranges from 99.89 to 101 ng/µl with average yield of 100.11 ng/µl. Average yield of normalized DNA in plate 4 through manual pipetting was 98.10 ng/µl with varied from 95.80 to 99.70 ng/µl but maximum yield obtained through robatic pipetting with mean of 100 and ranged from 99.99 to 100.03 ng/µl. In the Plate 5 average yield of normalized DNA through manual pipetting was 97.83 ng/µl with varied from 95.90 to 99.10 ng/µl but through robatic pipetting it was achieved mean yield of 99.87 ranged from 98.90 to 100.02 ng/µl. The mean yield of normalized DNA in plate 6 through manual pipetting was 98.04 ng/µl

Table 12. Comparison of efficiency of DNA normalization methods for TILLING worked out from 8 plates

S. No	Parameters	Manual pipetting								Robotic pipetting (RLHS)							
		1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1.	Expected concentra-tion (ng/µl)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
2.	Mean (ng/µl/)	97.6	97.8	97.3	98.1	97.8	98.0	97.8	98.6	100.0	100.0	100.1	100.0	99.87	99.65	99.89	100.0
3.	Minimum (ng/µl)	95.6	95.8	94.6	95.8	95.9	96.0	96.0	95.6	99.98	99.97	99.89	99.99	98.90	98.00	99.60	99.87
4.	Maximum (ng/µl)	99.2	99.2	99.7	99.7	99.1	99.8	99.2	99.6	100.1	100.1	101.0	100.0	100.0	100.0	100.0	100.9
5.	Standard error	0.16	0.17	0.23	0.15	0.13	0.16	0.14	0.15	0.01	0.01	0.10	0.00	0.10	0.21	0.05	0.10
6.	Standard deviation	1.15	1.23	1.68	1.11	0.94	1.16	1.02	1.12	0.04	0.04	0.31	0.01	0.34	0.66	0.16	0.31
7.	CV %	1.17	1.25	1.72	1.13	0.96	1.18	1.04	1.13	0.04	0.04	0.31	0.01	0.34	0.66	0.16	0.31

with varied from 96.00 to 99.80 ng/µl. whereas through robatic pipetting was ranged from 98.00 to 100.01 ng/µl. Similarly the average yield of normalized DNA in plate 7 and 8 through manual pipetting was 97.86 ng/µl and 98.63 ng/µl respectively and ranged from 96.00 to 99.20 ng/µl and 95.60 to 99.60 ng/µl respectively, whereas through robitic pipetting average yield for plate 7 and 8 was 99.89 ng/µl and 100.08 ng/µl respectively with ranged from 99.60 to 100.00 ng/µl and 99.87 to 100.98 ng/µl respectively.

4.2.3. Primer designing and synthesis for TILLING analysis

The details of gene specific TILLING fragments discovered through CODDLe analysis is presented in Table 4. TILLING fragment length varies from 519 to 1456bp where *CO* gene shows maximum amplicon size (1456bp) and *TFL1b* gene shows minimum amplicon size (519 bp). Subsequently primers were designed for TILLING fragments through Primer 3 software.

4.2.4. PCR optimization for the amplification of TILLING fragments

Various PCR cycling conditions and master mix compositions standardized for the amplification of different candidate gene/TILLING fragments are presented in Table 13. The genes *GI* (Plate 6a), *RMS* (Plate 6b), *CO* (Plate 6c) were amplified in combinations of the PCR cycle I and PCR master mix II. The genes *LFY* (Plate 6d), and *TFL1b* (Plate 6e), were amplified by adopting PCR cycle II and PCR master mix I

4.2.5. Sequencing efficiency of NGS

Table 14 shows super pool wise yield and depth of coverage statistics of the sequencing reads generated by Illumina MySeq sequencer. The average reads of libraries varied from 1.52 to 1.99 millions. Average length of reads varied from 74 to 81 bp. The percentage of bases with quality score ≥ 20 ranged from 89.48 (C7) to 99.51 (C11). The mean value of trimmed reads was 3.41million and ranged from 2.14 to 4.98millions**.** Percentage of reads contributing for > 20 X depth ranged from 60.12 to 68.07. The average read depths were ranged from 1950 to 7332 (Table 15). The maximum average read depth was recorded for the gene *RMS* (7332) followed by *LFY* (5784), *TFL Ib* (3808), *GI* (2077) and *CO* (1950).

Table 13. PCR cycle condition and compositions standardized for the amplification of different candidate gene/ TILLING fragments

S.No	Candidate gene/ TILL fragment	GC content (%)	PCR*	
			Cycle Condition	PCR master mix
1.	*GI*	54	PCR Cycle I	PCR master mix II
2.	*RAM*	48.8	PCR cycle I	PCR master mix II
3	*CON*	52.6	PCR cycle I	PCR master mix II
4	*LFY*	45	PCR cycle II	PCR master mix I
5	*TFL1b*	42.5	PCR cycle II	PCR master mix I

*details are given in table 5 and 6

Plate 6a. PCR amplified products of *GIGANTEA* gene in 20 super pools

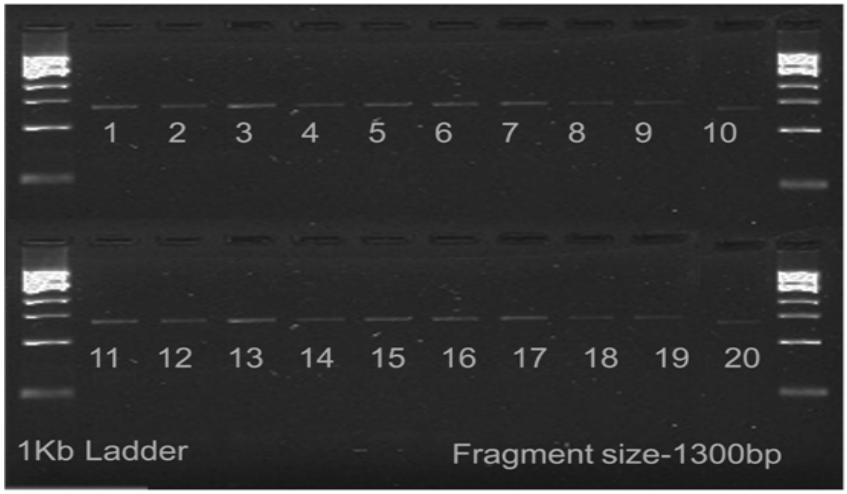


Plate 6b. PCR amplified products of *RAMOSUS* gene in 20 super pools

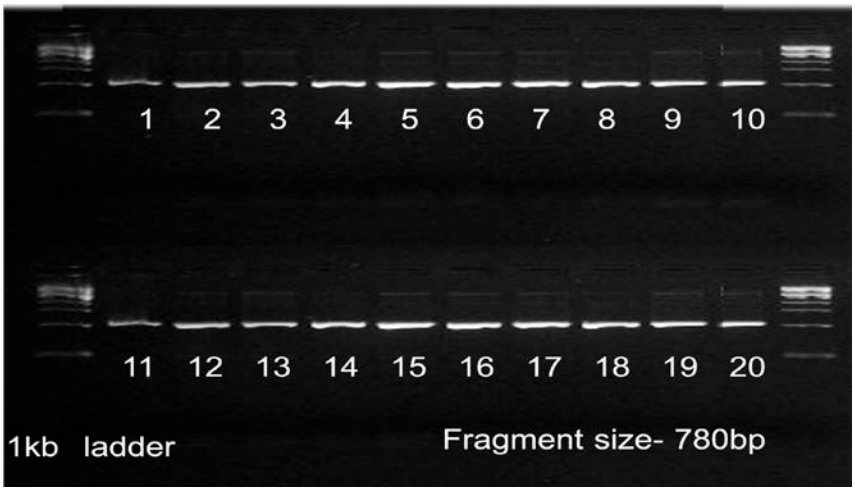


Plate 6c. PCR amplified products of *CONSTANS* gene in 20 super pools

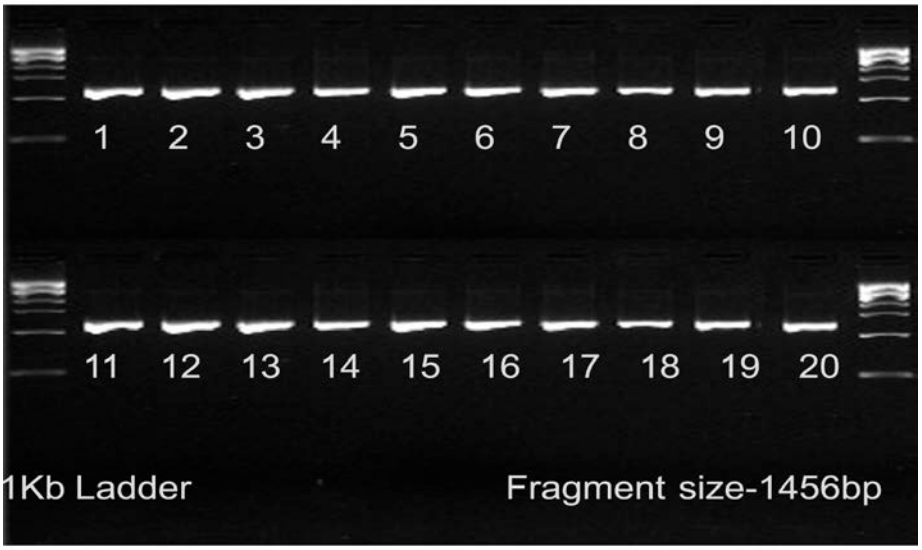


Plate 6d. PCR amplified products of *LEAFY* gene in 20 super pools

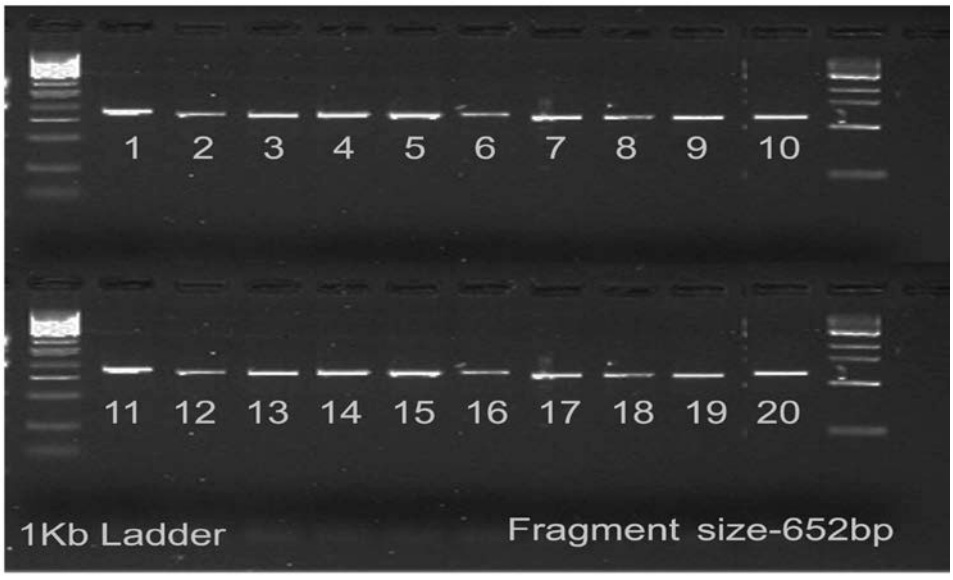


Plate 6e. PCR amplified products of *TERMINAL FLOWERING* gene in 20 super pools

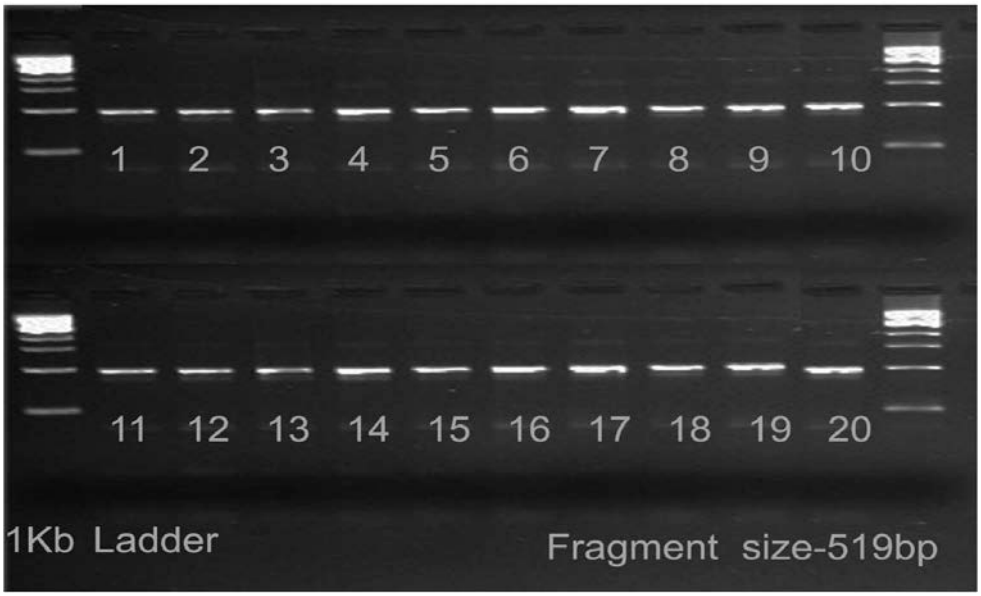


Table 14. Super pool wise statistics as generated by Illumina myseq sequencer

S.No	Super pool ID	No. of reads (in millions)	Percentage of bases with quality score ≥ 20	Average length of reads (bp)	Total bases (Millions)	No.of Trimmed reads in millions	Percentage of reads contributing for > 20 X depth
1.	R1	1.76	99.32	76	134.16	3.25	66.91
2.	R2	1.90	97.25	81	144.43	3.52	65.67
3.	R3	1.52	99.30	75	115.70	2.67	66.91
4.	R4	2.49	99.34	74	189.43	4.71	66.71
5.	R5	2.23	99.32	75	169.69	4.12	66.42
6	R6	1.73	99.32	76	131.24	3.22	65.78
7	R7	2.05	99.35	76	156.03	3.89	66.25
8	R8	1.80	99.32	78	136.74	3.36	65.64
9	C1	1.56	99.29	76	118.79	2.92	65.58
10	C2	1.93	99.28	77	149.28	3.72	63.12
11	C3	1.99	99.26	78	134.51	3.12	65.24
12	C4	1.82	98.34	79	114.25	2.14	63.21
13	C5	1.89	99.30	77	126.51	2.16	62.15
14	C6	1.91	98.88	75	132.15	3.89	65.15
15	C7	1.85	89.48	74	162.32	4.11	65.32
16	C8	1.89	96.30	77	158.21	3.69	62.21
17	C9	1.92	99.45	79	159.11	2.45	60.12
18	C10	1.78	99.50	78	114.21	2.78	65.42
19	C11	1.86	99.51	77	116.41	4.98	65.28
20	C12	1.95	98.85	76	212.68	3.14	65.31
21	Control	1.96	99.28	76	149.28	3.86	68.07
Mean		**1.89**	**98.53**	**76.67**	**144.05**	**3.41**	**65.07**
Range		**1.52-1.99**	**89.48-99.51**	**74-81**	**114.21-212.68**	**2.14-4.98**	**60.12-68.07**

Table 15. Average depth of coverage of various candidate genes through Illumina sequencing

S.No	Candidate gene	Average read depth
1	*GIGANTEA (GI)*	2077
2	*CONSTANS(CO)*	1950
3	*RAMOSUS(RMS)*	7332
4	*LEAFY(LFY)*	5784
5	*TERMINAL FLOWERING1B (TFL1b)*	3808

4.2.6. Variants discovered in 8x super pools

The number of well wise variants discovered in candidate genes from super pools through TILLING by sequencing is presented in Table 16. Among the candidate genes investigated, *CO* showed maximum variant of 3 followed by *GI*, *RMS*, and *LFY* with 2 variants each while *TFL1b* showed lowest number of variant 1 in the pooled library.

4.2.7. SNP/Indel discovery and detection

The nature and position of the sequence variants discovered is furnished in the Table 17. Out of 10 variants discovered 8 were found to be SNP and 2 of them were identified as Indels. Out of the 8 SNPs discovered 2 of them G→A transition and others were found to be transversion *viz.*, 2 of C→A, 2 of C→T, T→A and A→T.

4.2.8. Functional analysis of sequence mutants

The results of SIFT analysis of the variants depicting the functional effects on their protein are furnished in the Table 18.

4.2.8.1. *GI (GIGANTEA)*

GI gene registered two variants *viz*., G→A transition and C →T transversion at base positions 605 and 630 respectively. Both these variants were resided in the exon one of the gene. The SIFT analysis revealed that sequence variant G→A (605) transition resulted amino acid substitution of C→Y at the position of 202 in protein sequence (Plate 8a). Further the SIFT analysis also indicated that this was intolerant with high deleterious effect as indicated SIFT score of 0.00. Further the functional mutation G→A (605) was also found introduced a restriction sites variation for the enzymes *AvaIII* and *NdeI*. It also resulted the loss of the restriction site for the enzyme *NlaIII*. The sequence variant C→T (630) was found to be a silent as it did not cause any amino acid change as revealed by SIFT analysis.

4.2.8.2. *RMS (RAMOSUS)*

RMS gene registered two variants *viz*., T→A and C →T transversion at base positions 1511 and 1520 respectively. Both these variants resided in the exon four of the gene. The SIFT analysis revealed that sequence variant T→A (1511) transversion

Table 16. Number of position wise sequence variants discovered in each super pool through TILLING by sequencing

1. *GIGANTEA (GI)*

Column/Row	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	Total
R1	1	0	0	0	0	0	0	0	0	0	0	0	1
R2	0	0	1	0	0	0	0	0	0	0	0	0	1
R3	0	0	0	0	0	0	0	0	0	0	0	0	0
R4	0	0	0	0	0	0	0	0	0	0	0	0	0
R5	0	0	0	0	0	0	0	0	0	0	0	0	0
R6	0	0	0	0	0	0	0	0	0	0	0	0	0
R7	0	0	0	0	0	0	0	0	0	0	0	0	0
R8	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	1	0	1	0	0	0	0	0	0	0	0	0	2

2. RAMOSUS (RMS)

Column/Row	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	Total
R1	0	0	0	1	0	0	0	0	0	0	0	0	1
R2	0	0	0	0	0	0	0	0	0	0	0	0	0
R3	0	0	0	0	0	0	0	0	0	0	0	0	0
R4	0	0	0	0	0	0	0	0	0	0	0	0	0
R5	0	0	1	0	0	0	0	0	0	0	0	0	1
R6	0	0	0	0	0	0	0	0	0	0	0	0	0
R7	0	0	0	0	0	0	0	0	0	0	0	0	0
R8	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	0	0	1	1	0	0	0	0	0	0	0	0	2

3. CONSTANS(CO)

Column/Row	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	Total
R1	0	0	0	0	0	0	0	0	0	0	0	0	0
R2	1	0	0	0	0	0	0	0	0	0	0	0	1
R3	1	0	0	0	0	0	0	0	0	0	0	0	1
R4	0	0	0	0	0	0	0	0	0	0	0	0	0
R5	0	0	1	0	0	0	0	0	0	0	0	0	1
R6	0	0	0	0	0	0	0	0	0	0	0	0	0
R7	0	0	0	0	0	0	0	0	0	0	0	0	0
R8	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	2	0	1	0	0	0	0	0	0	0	0	0	3

4. LEAFY (LFY)

Column/Row	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	Total
R1	0	0	0	0	0	0	0	0	0	0	0	0	0
R2	0	0	0	0	0	0	0	0	0	0	0	0	0
R3	0	1	0	0	0	0	0	0	0	0	0	0	1
R4	0	0	1	0	0	0	0	0	0	0	0	0	1
R5	0	0	0	0	0	0	0	0	0	0	0	0	0
R6	0	0	0	0	0	0	0	0	0	0	0	0	0
R7	0	0	0	0	0	0	0	0	0	0	0	0	0
R8	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	0	1	1	0	0	0	0	0	0	0	0	0	2

5. TERMINAL FLOWERING (TFL1b)

Column/Row	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**	**Total**
R1	0	0	0	0	0	0	0	0	0	0	0	0	0
R2	0	0	0	1	0	0	0	0	0	0	0	0	1
R3	0	0	0	0	0	0	0	0	0	0	0	0	0
R4	0	0	0	0	0	0	0	0	0	0	0	0	0
R5	0	0	0	0	0	0	0	0	0	0	0	0	0
R6	0	0	0	0	0	0	0	0	0	0	0	0	0
R7	0	0	0	0	0	0	0	0	0	0	0	0	0
R8	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	0	0	0	1	0	0	0	0	0	0	0	0	1

Table 17. Gene wise list of sequence variants discovered through TILLING by sequencing

S. No	Gene Name	Variant ID	Nucleotide position change*	Reference Base	Called Base	Type of variant	Position of Variant
1.	*GI*	MTP-M1	630	C	A/T	SNP	Exon
2	*GI*	MTP-M2	605	G	A	SNP	Exon
3.	*RMS*	MTP-M3	1511	T	A	SNP	Exon
4.	*RMS*	MTP-M4	1520	C	A/T	SNP	Exon
5	*CO*	MTP-M5	732	C	T	SNP	Intron
6	*CO*	MTP-M6	1351	C	T	SNP	Intron
7	*CO*	MTP-M7	734	A	T	SNP	Intron
8	*LFY*	MTP-M8	512	TTCTTC	T	INDEL	Intron
9	*LFY*	MTP-M9	464	GTT	GT	INDEL	Intron
10	*TFL1b*	MTP-M10	165	G	A	SNP	Exon

***** Variant position based on TILLING fragment

Table 18. Functional mutations discovered in candidate genes through TILLING by sequencing

S.No	Candidate gene	Variant discovered Nucleotide change		Effect*	Amino acid change	Well position	Restriction sites		SIFT Score
		At Gene level	At Fragment level				Gained in variant	Lost from reference	
1	*Gigantea*	G605A	G380A	C202Y	Cysteine-Tyrosine	R2,C3	*AvaIII, NdeI*	*NlaIII*	0.00
2.	*Gigantea*	C630T	C405T	R210=	-	R1,C1	*AccI, HgaI, MjaIV*	*HhaI*	1.00
3	*Ramosus*	T1511A	T661A	F202L	Phenylalanine - Leucine	R1,C4			0.00
4	*Ramosus*	C1520T	C670T	T481=		R5,C3			1.00
5	*Terminal flowering*	G165A	G165A	G56S	Glycine - Serine	R4,C2			0.00

*=indicates silent mutation

Plate 8a. Gene model and position of mutations in *GI* gene

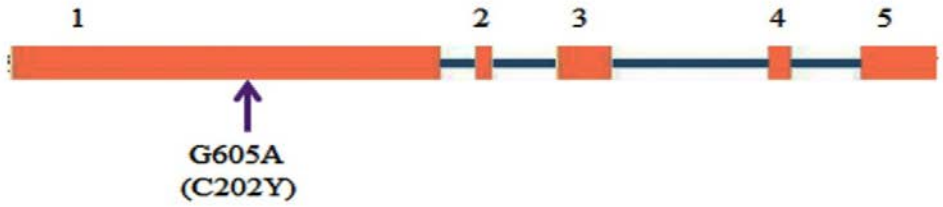


Plate 8b. Gene model and position of mutations in *RMS* gene

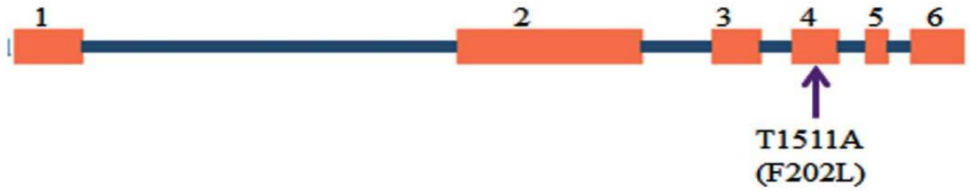


Plate 8c. Gene model and position of mutations in *TFL1b* gene

resulted amino acid substitution of F→L at the position of 202 in protein sequence (Plate 8b). Further the SIFT analysis also indicated that this was intolerant with high deleterious effect as indicated SIFT score of 0.00. The sequence variant C→T (1520) was found to be a silent variant as it did not cause any amino acid change as revealed by SIFT analysis.

4.2.8.3. *TFL1B (TEMINAL FLOWERING 1b)*

TFL 1b gene registered variant G→A transition at base positions 165 located in the exon one of the gene. The SIFT analysis revealed that sequence variant G→A (165) transition caused amino acid substitution of G→S at the position of 56 in protein sequence (Plate 8c). Further the SIFT analysis also indicated that this was intolerant with high deleterious effect as indicated SIFT score of 0.00.

4.2.9. Deconvolution of pooled samples through Sanger's sequencing

Sanger's sequencing of the PCR amplicons generated from DNA templates of individual putative mutants was carried out. The genotype discovered through Sanger sequencing and sequence alignment are presented in Table 19. Sanger's sequencing results revealed that genotypes; MTP-399 (Plate 9a) in super pools R2, C3 showed functional mutation (MTP-M2) for the gene *Gigantea.* The genotype MTP-580 (Plate 9b) in super pool R1, C4 showed functional mutation (MTP-M3) for the *Ramosus* gene and MTP-134 (Plate 9c) in super pool R4, C2 showed functional mutant (MTP-M10) for the *Terminal flowering* gene. The seeds from these mutants were recovered from Ramiah Gene bank for raising M_3 generation.

4.3. Biometrical characterization of the discovered mutants suitable for flowering time and branching habit

4.3.1. Characterization of M_3 families for identifying altered phenotypes

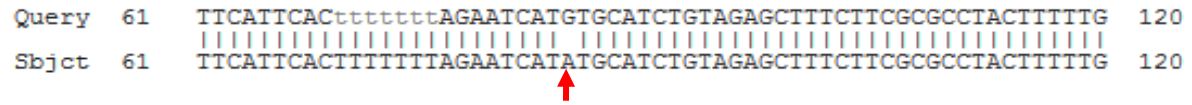
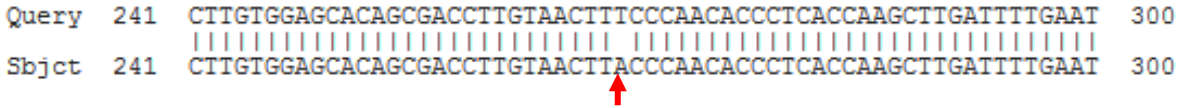
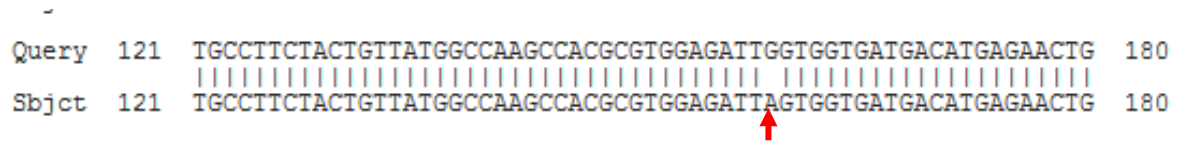
MTP-399, MTP-580 and MTP 134 are the discovered mutants for candidate genes *GIGANTEA, RAMOSUS* and *TERMINAL FLOWERING (TFL 1b)* respectively. About 25 seeds of each family of discovered mutants were raised along with control for characterization (Table 20)

Plate 9a. Decovolution of *GI* mutant by sanger sequencing

Plate 9b. Decovolution of *RMS* mutant by sanger sequencing

Plate 9c. Decovolution of *TFL1b* mutant by sanger sequencing

Table 19. List of mutants discovered through Sanger sequencing

S. No	Mutation Id	Genotype deconvolved through Sanger sequencing*	Sequence alignment
1	MTP-M2 *GI* (G605A)	MTP-399	
1	MTP-M3 *RMS* (T1511A)	MTP-580	
2	MTP-M10 *TFL1b* (G165A	MTP-134	

Indicates base change position

Table 20. Details of M_3 mutants selected for positive ideotype based on sanger sequencing

S.No	Gene	Mutant family identified	Total no of seeds sown per mutant family	No. of mutants survived still maturity	List of mutant shows positive ideotype
1	*GI*	MTP-399	25	19	MTP-399-04 MTP-399-08 MTP-399-11 MTP-399-16
2	*RMS*	MTP-580	25	21	MTP-580-07 MTP-580-19 MTP-580-21
3	*TFL 1 b*	MTP-134	25	17	MTP-134-03 MTP-134-15

4.3.2. Characterization of *GI* mutants

Mutation in candidate gene is expected to express delay flowering which is desirable. Among the 25 seeds of MTP 399 (Table 20) sown 19 survived till maturity. Four plants showed altered phenotype with late flowering habit in comparison with control (Plate 12). They were tagged and characterized for biometrical parameters and harvested individually for further evaluation in M_4 generation.

4.3.2.1. Fifty per cent flowering (FPF)

The maximum delayed flowering with FPF 47days was recorded for the mutant MTP-399-16 than the control (Co (Gg) 7) with 31.50 days, followed by 40 days for the mutant MTP-399-11. The mean FPF for *GI* was 40.50 days which ranged from 37 to 47 days. The other two mutants *viz.,* MTP-399-04 and MTP-399-08 recorded FPF of 37 days and 38 days respectively (Table 21).

4.3.2.2 Plant height (PHT)

Among the four mutants MTP-399-11 had highest PHT of 42.5cm than the control Co (Gg) 7 (31 cm), followed by MTP-399-04 and MTP-399-16 with 38.2cm. The mean PHT for *GI* mutants was 38.77 cm which ranged from 36.2 to 42.5cm. MTP-399-08 had PHT of 36.2 cm. (Table 21).

4.3.2.3 Days to maturity (DMT)

The mutant MTP-399-16 recorded the maximum DMT of 58 days than the control Co (Gg) 7 (42 days), followed by MTP-399-11 which had DMT of 57 days. The mean DMT for *GI* mutants was 53 days which ranged from 48 to 58 days. The other two mutants *viz.,* MTP-399-04 and MTP-399-08 recorded DMT of 49 days and 48 days respectively (Table 21).

4.3.2.4. Height to first branch (HFA)

HFA recorded a mean value of 7.35 cm for *GI* mutants which ranged from 5.5 to 9.5 cm. Among the four mutants, MTP-399-11 registered highest HFA of 9.5cm than the control Co (Gg) 7 (3.10 cm), followed by MTP-399-16 (8.7 cm). The other two mutants *viz.,* MTP-399-04 and MTP-399-08 recorded HFA of 5.5cm and 5.7 cm respectively (Table 21).

4.3.2.5. Number of branches (NOB)

The NOB for *GI* mutants recorded a mean value of 4 which ranged from 3 to 5 branches. Among them, maximum NOB was recorded for MTP-399-11 with 5 branches than the control Co (Gg) 7 with 1.85 branches, followed by two mutants (MTP-399-16 and MTP-399-04) with 4 branches. MTP-399-08 recorded NOB with 3 branches (Table 21).

4.3.2.6. Length of the longest branch (LLB)

Among the four mutants MTP-399-11 had highest length of 37.5 cm than the control Co (Gg) 7 10.25cm, followed by MTP-399-04(36.5 cm). The mean of LLB for *GI* mutants was 33.2cm which ranged from 27.5 to 37.5cm. MTP-399-08 and MTP-399-16 was observed with LLB of 27.5cm and 31.3cm respectively (Table 21).

4.3.2.7. Peduncle length (PEL)

The mutant MTP-399-08 recorded the maximum PEL of 10.7cm than the control Co(Gg)7 (4.6cm), followed by MTP-399-11 which had 9.3cm PEL. The average PEL for *GI* mutants was 9.32 cm which ranged from 8.6 to 10.7cm. PEL for the mutant MTP-399-04 and MTP-399-16 was recorded 8.7cm and 8.6cm respectively (Table 21).

4.3.2.8. Height of the first branch (HFB)

The mean value for the trait HFB was 31.4cm for *GI* mutants which ranged from 29.5cm to 33.0cm. Among the four mutants HFB was highest for MTP-399-11 (33.0cm) than the control Co (Gg) 7 (26.4), followed by MTP-399-04 (32.7cm).HFB for other two mutants v*iz*., MTP-399-08 and MTP-399-16 was 30.5 cm and 29.5 cm respectively (Table 21).

4.3.2.9. Pod spread (POS)

The mean value for the trait POS was 17.82 cm for *GI* mutants with a range of 14.2 to 22.1 cm was observed. Among the four mutants, lowest POS was observed in mutant MTP-399-16 with 14.2cm than the control Co (Gg) 7 (22.75cm), followed by MTP-399-11 (16.5 cm). POS for other two mutants *viz*., MTP-399-04 and MTP-399-08 was 22.1cm and 18.5 cm respectively (Table 21).

4.3.2.10. Height of the first pod (HFP)

The mean HFP for the *GI* was 19.75 cm which ranged from 17.2 to 23.5cm. Among them MTP-399-16 recorded the highest HFP of 23.5cm than the control Co (Gg) 7 (10cm), followed by MTP-399-11 with 20.5 cm. MTP-399-04 and MTP-399-08 had recorded 17.8cm and 17.2 cm respectively (Table 21).

4.3.2.11. Internodal length (INL)

The mean value for the trait INL was 2.07cm for *GI* mutants with ranged from 1.5 to 2.4cm. Among them lowest INL was observed in MTP-399-11 (1.5cm) than control Co (Gg) 7 (2.75cm), followed by MTP-399-04 (2.1cm). MTP-399-08 and MTP-399-16 had recorded INL of 2.3cm and 2.6cm respectively (Table 21).

4.3.2.12 Number of clusters per plant (NCP)

The mean NCP for the *GI* was 10.5 which ranged from 9 to 12. Among them mutant MTP-399-11 recorded maximum NCP with 12 than control Co (Gg) 7 (5.4), followed by MTP-399-16 produced 11 NCP. MTP-399-04 and MTP-399-08 recorded 9 and 10 NCP respectively (Table 21).

4.3.2.13. Number of pods per cluster (NPC)

The NPC for *GI* mutants recorded a mean value of 6.25 which ranged from 6 to 7. Among them maximum NPC recorded for MTP-399-08 (7) than the control Co (Gg) 7 (3.6), followed by MTP-399-04, MTP-399-11 and MTP-399-16 (6) (Table 21).

4.3.2.14. Accessible pods per plant (APP)

Regarding APP the mean value for *GI* mutants was 59 which ranged from 49 to 63. Mutant MTP-399-08 and MTP-399-11had maximum APP (63) than control Co(Gg) 7(11.6), followed by MTP-399-16 (61).MTP-399-04 had recorded 49 APP (Table 21).

4.3.2.15. Pod length (POL)

The POL for *GI* mutants recorded mean value of 5.97cm which ranged from 5.2cm to 6.7cm. Among them maximum POL recorded for MTP-399-04 (6.7cm) than the control Co (Gg) 7(5.1cm), followed by MTP-399-16 (6.2cm). MTP-399-11 and MTP-399-08 had recorded POL 5.2cm and 5.8cm respectively (Table 21).

Table 21. Biometrical observation recorded for *GI* mutants

Mutant lines	FPF	PLH	NOB	LLB	HFA	PEL	DMT	HFB	POS	HFP	INL	NCP	NPC	NPP	POL	NSP	APP	HPP	HSW	SPY
MTP-399-04	37	38.2	4	36.5	5.5	8.7	49	32.7	22.1	17.8	2.1	9	6	54	6.7	8	49	90.7	4.6	19.5
MTP-399-08	38	36.2	3	27.5	5.7	10.7	48	30.5	18.5	17.2	2.3	10	7	70	5.8	9	63	90.0	4.5	18.6
MTP-399-11	40	42.5	5	37.5	9.5	9.3	57	33.0	16.5	20.5	1.5	12	6	72	5.2	8	63	87.5	4.7	21.5
MTP-399-16	47	38.2	4	31.3	8.7	8.6	58	29.5	14.2	23.5	2.4	11	6	66	6.2	10	61	92.4	4.8	20.6
Mean	40.5	38.77	4	33.2*	7.35	9.32	53	31.42	17.82	19.75	2.07	10.5	6.25	65.5*	5.97	8.75	59*	90.15*	4.65	20.0
SD	4.5	2.65	0.81	4.67	2.04	0.96	5.22	1.69	3.34	2.88	0.4	1.29	0.5	8.06	0.63	0.95	6.73	2.03	0.12	1.26
Control	31.5	31	1.85	10.25	3.1	4.6	42	26.4	22.75	10	2.75	5.4	3.6	22.75	5.1	8.1	11.6	49.35	3.4	10.6

* -Significant at 1%

FPF- Days to 50 per cent flowering

PLH- Plant height

NOB- Number of branches per plant

LLB- Length of longest Branch

HFA- Height of first branching from the ground

PEL- Peduncle length

DMT- Days to first mature pod

HFB- Height of the first branch

POS- Pod spread

HFP- Height of first pod

INL- Internodal distance

NCP- Number of clusters per plant

NPC- Number of pods per cluster

NPP- Number of pods per plant

POL- Pod length

NSP- Number of seeds per pod

APP- Accessible pods per plant

HPP -Harvestable pod percentage

HSW-100 seed weight

SPY- Yield per plant

Plate 12. Evaluation of *GI* mutants in M_3 generation

CO (Gg)7-(Wild type) completed the flowering stage within 54th DAS

Late Flowering types and bushy growth observed in *GI* mutant MTP-399 at 54th DAS

4.3.2.16. Number of pods per plant (NPP)

NPP recorded a mean value of 65.5 for *GI* mutants which ranged from 54 to72. Among them MTP-399-11 had maximum NPP of 72 than control Co (Gg) (65.5), followed by MTP-399-08(70). The other two mutants *viz.*, MTP-399-04 and MTP-399-16 had recorded 54.0 and 66 respectively (Table 21).

4.3.2.17. Number of seeds per pod (NSP)

Among the *GI* mutants MTP-399-16 had recorded maximum of 10 NSP than the control Co (Gg) 7 (8.1), followed by MTP-399-08 (9). The mean of *GI* mutants was 8.75 which ranged from 8 to 10. MTP-399-04 and MTP-399-11 had recorded NSP of 8 (Table 21).

4.3.2.18. Harvestable pods percentage (HPP)

The mean value of HPP for the *GI* mutants was 90.15 per cent which ranged from 87.5 to 92.4 per cent was observed. Among them HPP was maximum for MTP-399-16 (92.4 per cent) than the control Co (Gg) 7 (49.35 per cent), followed by MTP-399-04 (90.7 per cent). The other two mutants *viz.*, MTP-399-11 and MTP-399-08 had recorded HPP of 87.5 per cent and 90 per respectively (Table 21).

4.3.2.19. Hundred seed weight (HSW)

The HSW for *GI* recorded a mean value of 4.6g which ranged from 4.5 to 4.8g. Among them MTP-399-16 had maximum HSW with 4.8g than the control Co (Gg) 7 (3.4g), followed by MTP-399-11 (4.7g). MTP-399-08 and MTP-399-04 had recorded HSW 4.5g and 4.6g respectively (Table 21).

4.3.2.20. Seed yield per plant (SPY)

The mean value of SPY for the *GI* mutants was recorded 20g which ranged from 18.6 to 21.5g. Among them SPY maximum for MTP-399-11(21.5g) than the control Co (Gg) 7 (10.6g), followed by MTP-399-16 (20.6g). MTP-399-08 and MTP-399-04 recorded SPY of 18.6g and 19.5 g respectively (Table 21).

4.3.3. Characterization of *RMS* mutants

Mutation in this candidate gene is expected to express profuse branching. Among the 25 seeds of MTP 580 (Table 20) 21 were survived till maturity. Three plants (MTP-580-07, MTP-580-19 and MTP-580-21) were showed altered phenotype with profuse branching (Plate 13). They were tagged and characterized for biometrical parameters and harvested individually for further evaluation in M_4 generation.

4.3.3.1 Fifty per cent flowering (FPF)

The mean FPF for *RMS* mutants was 37.67 days which ranged from 36 to 39 days. Among them MTP-580-21 recorded the maximum delayed flowering with FPF of 39 days than the control Co (Gg) 7(31.5 days), followed by MTP-580-07 with 38 days. MTP-580-19 recorded the FPF with 36days (Table 22).

4.3.3.2 Plant height (PHT)

Among the *RMS* mutants MTP-580-19 had the highest PHT of 42.2cm than the control Co (Gg) 7, followed by MTP-580-21(41.7cm). Average plant height for the *RMS* mutants was 41.76cm which ranged from 41.4 to 42.2cm. MTP-580-07 recorded PHT of 41.4cm (Table 22).

4.3.3.3 Days to maturity (DMT)

The mutant MTP-580-07 and MTP-580-19 recorded the maximum DMT of 49 days than the control Co (Gg) 7 (42days). Average DMT for *RMS* mutants was 48.33 days which ranged from 47 to 49 days. MTP-580-21 had recorded of DMT of 47 days (Table 22).

4.3.3.4. Height to first branch (HFA)

HFA recorded a mean value of 9.66 cm for *RMS* mutants which ranged from 8.2 to 11.3 cm. Among them MTP-580-19 registered highest HFA of 11.3cm than the control Co(Gg) 3.1cm, followed by MTP-580-21 (9.5 cm).The mutant MTP-580-07 recorded the HFA of 8.2cm (Table 22).

4.3.3.5. Number of branches (NOB)

The NOB for *RMS* mutants recorded a mean value of 6.33 which ranged from of 6 to 7. Among them maximum NOB was recorded for MTP-580-19, with 7 branches than the control Co (Gg) 7 (1.85), followed by MTP-580-07 and MTP-580-21 with 6 branches (Table 22).

4.3.3.6. Length of the longest branch (LLB)

Among three *RMS* mutants MTP-580-19 had the highest length of 29.7cm than the control Co (Gg) 7 (10.25), followed by MTP-580-07 (29.5 cm). Average LLB for *RMS* mutants was 28.9cm which ranged from 27.5 to 29.7cm. MTP-580-21 had LLB of 27.5cm (Table 22).

4.3.3.7. Peduncle length (PEL)

The mutant MTP-580-19 recorded the maximum PEL of 9.7cm than the control Co (Gg)7 (4.6cm), followed by MTP-580-21 which had 9.3cm PEL, which ranged from 8.1 to 9.7cm and lowest PEL was observed for the mutant MTP-580-07 (8.1cm) (Table 22).

4.3.3.8. Height of the first branch (HFB)

The mean value for the trait HFB was 32.1cm for *RMS* mutants which ranged from 30.9cm to 33.2cm. Among the *RMS* mutants HFB recorded maximum for MTP-580-07 (33.2cm) than the control Co (Gg) 7 (26.4cm), followed by MTP-580-21 (32.2cm). MTP-580-19 exhibited the HFB of 30.9 cm (Table 22).

4.3.3.9. Pod spread (POS)

The mean value for the trait POS was 18.66cm which ranged from 16.4 to 21.2 cm was recorded. Among them POS was minimum for MTP-580-19 (16.4cm) than the control Co (Gg) 7 (22.75cm), followed by MTP-580-21 (18.4cm). MTP-580-07 exhibited the POS with a value 21.2 cm (Table 22).

4.3.3.10. Height of the first pod (HFP)

The mean HFP for the *RMS* mutants was 22.63cm which ranged from 21.9 to 23.6cm. Among them MTP-580-07 recorded the maximum HFP of 23.6cm than the

Table 22. Biometrical observation recorded for *RMS* mutants

Mutant lines	FPF	PLH	NOB	LLB	HFA	PEL	DMT	HFB	POS	HFP	INL	NCP	NPC	NPP	POL	NSP	APP	HPP	HSW	SPY
MTP-580-07	38	41.4	6	29.5	8.2	8.1	49	33.2	21.2	23.6	2.1	11	7	77	5.3	7	69	92.20	4.9	21.1
MTP-580-19	36	42.2	7	29.7	11.3	9.7	49	30.9	16.4	22.4	2.3	14	5	70	5.2	6	61	91.42	5.2	22.3
MTP-580-21	39	41.7	6	27.5	9.5	9.3	47	32.2	18.4	21.9	2.2	13	6	78	5.7	8	69	91.02	4.7	20.7
Mean	37.7	41.8	6.3	28.9	9.7	9.0	48.3	32.1	24.7	22.6	4.5	12.7	6.0	75.0*	5.4	7.0	66.3*	91.5*	4.9	21.4
SD	1.52	0.4	0.57	1.21	1.55	0.83	1.15	1.15	2.41	0.87	0.1	1.52	1	4.35	0.26	1	4.61	0.6	0.25	0.83
control	31.5	31	1.85	10.25	3.1	4.6	42	26.4	22.75	10	2.75	5.4	3.6	22.75	5.1	8.1	11.6	49.35	3.4	10.6

* -Significant at 1%

FPF- Days to 50 per cent flowering

PLH- Plant height

NOB- Number of branches per plant

LLB- Length of longest Branch

HFA- Height of first branching from the ground

PEL- Peduncle length

DMT- Days to first mature pod

HFB- Height of the first branch

POS- Pod spread

HFP- Height of first pod

INL- Internodal distance

NCP- Number of clusters per plant

NPC- Number of pods per cluster

NPP- Number of pods per plant

POL- Pod length

NSP- Number of seeds per pod

APP- Accessible pods per plant

HPP -Harvestable pod percentage

HSW-100 seed weight

SPY- Yield per plant

Plate 13. Evaluation of *RMS* mutants in M_3 generation

Less number of branches in wild type Co(Gg) 7

Profuse branching in *Ramosus* mutant MTP-580

control Co (Gg) 7 (10cm), followed by MTP-580-19 with 22.4 cm. The least performance for this trait was recorded for MTP-580-21 with 21.9 cm (Table 22).

4.3.3.11. Internodal length (INL)

The mean value for the trait INL was 2.2cm for *RMS* mutants which ranged from 2.1 to 2.3cm. Among them lowest INL was observed for MTP-580-07 than the control Co (Gg) 7, followed by MTP-580-21 (2.2cm). MTP-580-19 had INL of 2.3cm was recorded (Table 22).

4.3.3.12. Number of clusters per plant (NCP)

The mean value of NCP for *RMS* mutants was 12.66 which ranged from 11 to 14. Among them MTP-580-19 recorded maximum NCP of 14 than control Co (Gg) 7 (5.4), followed by MTP-580-21 produced 13 NCP. MTP-580-07 recorded NCP of 11.0 (Table 22).

4.3.3.13. Number of pods per cluster (NPC)

The NPC for *RMS* mutants recorded a mean value of 6.0 which ranged from 5 to 7. Among them maximum NPC recorded for MTP-580-07 (7) than the control Co (Gg) 7 with 3.6 NPC, followed by MTP-580-21(6). MTP-580-19 recorded NPC of 5 (Table 22).

4.3.3.14. Accessible pods per plant (APP)

Regarding APP the mean value for *RMS* mutants were 66.33 which ranged from 61 to 69. Mutants MTP-580-07 and MTP-580-21 had maximum APP of 69 than the control Co (Gg) 7 (11.6).MTP-580-19 recorded APP of 61(Table 22).

4.3.3.15. Pod length (POL)

The POL for *RMS* mutants recorded mean value of 5.4cm which ranged from 5.2cm to 5.7cm. Among them maximum POL recorded for MTP-580-21 (5.7cm) than the control Co (Gg) 7, followed by MTP-580-07 (5.3). MTP-580-19 had recorded lowest POL with a value (5.2cm) (Table 22).

4.3.3.16. Number of pods per plant (NPP)

NPP recorded a mean value of 75.0 for *RMS* mutants which ranged from 70 to 78. Among them MTP-580-21 had more NPP of 78 than the control Co (Gg) 7 (22.75), followed by MTP-580-07(77). MTP-580-19 had recorded NPP of 70 (Table 22).

4.3.3.17. Number of seeds per pod (NSP)

Among the *RMS* mutants MTP-580-23 had maximum NSP of 8 than the control Co (Gg) 7 (7.5).MTP-580-07 and MTP-580-19 recorded NSP of 7 and 6 respectively. Average NSP for *RMS* mutants was7 which ranged from 6 to 8 (Table 22).

4.3.3.18. Harvestable pods percentage (HPP)

The mean value of HPP for the *RMS* mutants was 91.54% which ranged from 91.02 per cent to 92.20 per cent. Among them HPP was maximum for MTP-580-07 (92.20 per cent) than the control Co (Gg) 7 (49.35 per cent), followed by MTP-580-19 (91.42 per cent). MTP-580-21 exhibited HPP of 91.02 per cent (Table 22).

4.3.3.19. Hundred seed weight (HSW)

The HSW for *RMS* mutants recorded a mean value of 4.93g which ranged from 4.7 to 5.2g. Among them MTP-580-19 had maximum HSW of 5.2g than control Co (Gg) 7 (3.4g), followed by MTP-580-07 (4.9g).MTP-580-21 recorded HSW of 4.7g (Table 22).

4.3.3.20. Seed yield per plant (SPY)

The mean value of SPY for the given *RMS* mutants was 21.36g which ranged from 20.7 to 22.3g was recorded. Among them MTP-580-19 recorded maximum SPY of 22.3g than the control Co (Gg) 7 (10.6g), followed by MTP-580-07 (21.1g). MTP-580-21 recorded SPY of 20.7g (Table 22).

4.3.4. Characterization of *TFL1b* mutants

Mutation in this candidate gene is top pod bearing is desirable**.** Among 25 seeds of MTP 134 (Table 20) 17 were survived till maturity. Two plants (MTP-134-03, and MTP-134-15) were showed altered phenotype with top pod bearing (Plate 14). They were tagged and characterized for biometrical parameters and harvested individually for further evaluation in M_4 generation.

4.3.4.1 Fifty per cent flowering (FPF)

The mean FPF for *TFL1b* mutants was 39 days which ranged from 37 to 41 days. Among them MTP-134-15 recorded the maximum FPF of 41 days than the control Co (Gg) 7 (31.5 days), followed by MTP-134-03 with 37 days (Table 23).

4.3.4.2 Plant height (PHT)

Among the two mutants MTP-134-15 had the highest PHT of 43.6cm than the control Co (Gg) 7 (31cm), followed by MTP-134-03 with 42.5cm (Table 23).

4.3.4.3 Days to maturity (DMT)

Average DMT for *TFL1b* mutants was 51days. The mutant MTP-134-15 recorded the maximum DMT of 53 days than the control Co (Gg) 7 (42 days), followed by MTP-134-03 (49 days) (Table 23).

4.3.4.4. Height to first branch (HFA)

HFA recorded a mean value of 8.25cm for two mutants which ranged from 7.9 to 8.6 cm. Among the two mutants, MTP-134-03 registered the highest HFA of 8.6cm than the Co(Gg)7(3.1cm), followed by MTP-134-15 of (7.9cm) (Table 23)

4.3.4.5. Number of branches (NOB)

The NOB for two mutants recorded a mean value of 4.5 which ranged from of 4 to 5. Among them maximum NOB was recorded for MTP-134-15 with 5 branches than the control Co (Gg) 7(1.85), followed by MTP-134-03 with 4 branches (Table 23).

4.3.4.6. Length of the longest branch (LLB)

Among the two mutants MTP-134-15 had the highest length of 22.3cm than the control Co(Gg)7 (10.25cm), followed by MTP-134-03 (21.5 cm). Average LLB for *TFL1b* mutants was 21.9cm (Table 23).

4.3.4.7. Peduncle length (PEL)

The mutant MTP-134-03 recorded the maximum PEL of 8.9cm than the control Co(Gg)7 (4.6cm), followed by MTP-134-15 which had 7.9cm PEL. Average PEL for *TFL1b* mutants was 8.4cm (Table 23).

4.3.4.8. Height of the first branch (HFB)

The mean value for the trait HFB was 34.8cm for *TFL1b* mutants was observed. Among them HFB recorded maximum for MTP-134-15 (35.7cm) than the control Co (Gg) 7 (26.4cm), followed by MTP-134-03 (33.9cm) (Table 23).

4.3.4.9. Pod spread (POS)

The mean value for the trait POS was 20.8cm for *TFL1b* mutants was observed. Among them POS was maximum for MTP-134-03 (21.2cm) than control Co (Gg) 7 (22.75cm), followed by MTP-134-15 (20.3cm) (Table 23).

4.3.4.10. Height of the first pod (HFP)

The mean HFP for *TFL1b* mutants was 23 cm. Among them MTP-134-03 recorded the maximum HFP of 25.1cm than control Co (Gg) 7 (10cm), followed by MTP-134-15 with 20.9 cm. (Table 23).

4.3.4.11. Internodal length (INL)

The mean value for the trait INL was 2.15cm for *TFL1b* mutants was observed. Among them INL was minimum for MTP-134-03 (2.1 cm) than control Co (Gg) 7 (2.75cm) followed by MTP-134-15 (2.2cm) (Table 23).

4.3.4.12. Number of clusters per plant (NCP)

The mean value of number of clusters per pod for *TFL1b* mutants was 7.5. Among them maximum NCP was recorded for MTP-134-15(8) than the control Co (Gg) 7 (5.4), followed by MTP-134-03(7) (Table 23).

4.3.4.13. Number of pods per cluster (NPC)

The NPC for *TFL1b* mutants recorded a mean value of 8.5. Among them maximum NPC recorded for MTP-134-15 (8) than the control Co (Gg) 7 (3.6), followed by MTP-134-03 (7) (Table 23).

4.3.4.14. Accessible pods per plant (APP)

Regarding APP the mean value for *TFL1b* mutants were 58. Mutant MTP-134-15 had maximum APP with a value of 65 than the control Co Gg) 7 (11.6), followed by MTP-134-03 with 51(Table 23).

4.3.4.15. Pod length (POL)

The POL for *TFL1b* mutants recorded mean value of 6.45cm. Among them maximum POL recorded for MTP-134-15 (6.7cm) than control Co (Gg) 7 (5.1), followed by MTP-134-03 (6.2) (Table 23).

Table 23. Biometrical observation recorded *TFL1b* mutants

Mutant lines	FPF	PLH	NOB	LLB	HFA	PEL	DMT	HFB	POS	HFP	INL	NCP	NPC	NPP	POL	NSP	APP	HPP	HSW	SPY
MTP-134-03	37	42.5	4	21.5	8.6	8.9	49	33.9	17.2	25.1	2.1	7	8	56	6.2	7	51	91.07	4.8	21.3
MTP-134-15	41	43.6	5	22.3	7.9	7.9	53	35.7	16.5	20.9	2.2	8	9	72	6.7	8	65	90.27	4.7	20.5
Mean	39.0	43.1	4.5	21.9	8.3	8.4	51.0	34.8	20.8	23.0	4.8	7.5	8.5	64.0*	6.5	7.5	58.0*	90.7*	4.8	20.9
SD	2.82	0.77	0.7	0.56	0.49	0.7	2.82	1.27	0.49	2.96	0.07	0.7	0.7	11.31	0.35	0.7	9.89	0.56	0.07	0.56
Control	31.5	31	1.85	10.25	3.1	4.6	42	26.4	22.75	10	2.75	5.4	3.6	22.75	5.1	8.1	11.6	49.35	3.4	10.6

* -Significant at 1%

FPF- Days to 50 per cent flowering
PLH- Plant height
NOB- Number of branches per plant
LLB- Length of longest Branch
HFA- Height of first branching from the ground
PEL- Peduncle length
DMT- Days to first mature pod
HFB- Height of the first branch
POS- Pod spread
HFP- Height of first pod
INL- Internodal distance
NCP- Number of clusters per plant
NPC- Number of pods per cluster
NPP- Number of pods per plant
POL- Pod length
NSP- Number of seeds per pod
APP- Accessible pods per plant
HPP -Harvestable pod percentage
HSW-100 seed weight
SPY- Yield per plant

Plate 14. Evaluation of *TFL1b* mutants in M_3 generation

Axillary bearing in wild type Co (Gg)7

Terminal bearing in *TFL* 1b mutant MTP-134

4.3.4.16. Number of pods per plant (NPP)

NPP recorded a mean value of 64 for *TFL1b* mutants. Among them MTP-134-15 had maximum NPP of 72 than control Co (Gg) 7 (22.75), followed by MTP-134-03 (56) (Table 23).

4.3.4.17. Number of seeds per pod (NSP)

Among the *TFL1b* mutants MTP-134-15 had the highest value of 8 than the control Co (Gg) 7 (7) followed by MTP-134-03 which had value of 7. The mean NSP for *TFL1b* mutants was 7.5 (Table 23).

4.3.4.18. Harvestable pods percentage (HPP)

The mean value of HPP for *TFL1b* mutants was 90.67per cent was observed. Among them HPP was maximum for MTP-134-03 (91.07per cent) than the control Co (Gg) 7 (49.35 per cent), followed by MTP-134-15 (90.27per cent) (Table 23).

4.3.4.19. Hundred seed weight (HSW)

The HSW for *TFL1b* mutants recorded a mean value of 4.75g was observed. Among them MTP-134-03 had maximum HSW with 4.8g than control Co (Gg) 7 (3.4g), followed by MTP-134-15(4.7g) (Table 23).

4.3.4.20. Seed yield per plant (SPY)

The mean value of SPY for *TFL1b* mutants was 20.9g was recorded. Among them two SPY maximum for MTP-134-03(21.3g) than the control Co (Gg) 7 (10.6g), followed by MTP-134-15 as value of 20.5g (Table 23).

Discussion

CHAPTER V

DISCUSSION

Plant breeders are attempting to enhance yield by selecting for individual traits since the beginning of plant breeding era. This approach has been broadened to encompass the breeding of model plants or ideotypes. An implicit assumption in the ideotype approach is that yield enhancement can be achieved through genetic manipulation of agronomic traits favorably into a single genotype. With that assumption in mind, some ideotypes have been proposed presenting a package of traits that may be very difficult to obtain or to combine in a single plant. A good example of that can be analyzed in the wheat ideotype presented by Donald (1968).

The ideotype approach tries to integrate principles of three important areas in crop production *viz.*, crop physiology, crop ecology and plant breeding which very often have not followed the same direction. To design and breed a plant from the material available, which is theoretically capable of greater production than the genotype it is to replace, in any defined environmental situation, the availability of following three resources is required: sufficient knowledge on the traits concerned, adequate genetic diversity and suitable techniques for introgression and testing.

Morphological traits, such as plant height, internode number and length, hypocotyl diameter and length, number of pods on top, middle and bottom third of the stem, first pod insertion height, and number of pods on the main stem are the traits of the major importance identified by various studies in legumes (Izquierdo and Hosfield, 1983; Nienhuis and Singh, 1986; Acquaah *et al.,* 1991, 1992; Kornegay *et al.*, 1992; Brothers and Kelly, 1993: Sulaiman *et al.,* 2007) aimed at plant architecture suitable for mechanical harvesting. This endeavor will demand the development of cultivars with a combination of traits, including determinate growth habit, more erect plants associated with more compact (bush-type) canopy, short internodes and short secondary branches as well as lodging resistance as observed by Ehlers and Hall, 1997 in cowpea. Lather (2000) also developed a chickpea brachytic mutant, as donor for erectness. The progenies were erect and compact in growth habit with strong and reasonably tall

stems and few but erect secondary and later order branches (for better light interception and air exchange of canopy). These chickpea ideotypes are also suitable for mechanical harvesting as the fruiting zone started at about 20 cm from base.

As to construe in modification of plant architecture suitable for mechanical harvesting in mungbean, there are two components that needs suitable manipulation to achieve an ideal plant type. The placement of majority of the pods at the accessibility zone of a mechanical harvester is the first requirement (Top podding character). Second component involves manipulation required for a compensatory enhancement of yield component due to the detrimental effects of reduced vegetative growth as a result of terminal flowering (top podding).

The first component can be easily achieved by deploying a few mutations which are highly characterized and commonly available in legumes named as determinant plant type (DT). Second component is the most challenging one which requires the deployment of more number of mutations especially to modify the flowering time thereby enhanced vegetative growth along with optimized plant architecture such as reduced internodal length, relatively longer main stem and optimal branch length for top podding at uniform level being the requirements.

5.1. TILLING as a method of choice for generating mutants for traits with complex inheritance

Two main approaches utilized to link genotype to phenotype are known as forward and reverse genetics. Both of these processes aim to determine the function of gene/genes through screening the phenotype or genotype of individual mutants to ultimately determine how it is controlled. Traditionally utilized, forward genetics (phenotype to genotype), in which one starts with a particular identified phenotype or biological process and the gene sequence is ultimately deduced through screening large numbers of mutagenized individuals for phenotypic variations is a useful approach. However, forward genetic approaches are not practical for genome wide analysis primarily due to the effort and time involved to identify each gene coding for a particular phenotype (Alonso *et al.,* 2006). In reverse genetics (from genotype to phenotype), the gene sequence is known and mutants are screened to identify individuals with structural

alterations in the gene of interest (Nagy *et al.*, 2003). This approach is generally less time demanding than forward genetics. Reverse genetic strategies have been successfully used for functional genomics in many animal and plant species. The wide spread availability of sequence data allows researchers to rapidly design reverse genetic strategies to determine gene function. TILLING, a high throughput mutation detection method, takes advantage of chemical mutagenesis to generate induced mutations in a population, which results in a high mutational density with very low levels of aneuploidy and dominant lethality (Greene *et al.*, 2003). Among the methodologies available currently to extend the benefits of genomics from models up to the level of functional genomics in crops TILLING seems to be the most viable option especially for grain legumes, as it does not require the transgenic procedures to create functional knockouts. When combined with chemical mutagenesis, the screening for mutant individuals in a large population is achievable only by TILLING. In the present investigation, it is very essential to use the functional genomic tools like TILLING to create targeted mutations and perform functional analysis of the putative genes involved in the branching and flowering control.

The family Leguminosae is one of the largest families of flowering plants and includes important grain legumes such as mungbean, chickpea, pigoenpea and lentil. Legumes vary in habit from annual to perennial and in their genomes from simple diploids to large and complex polyploids. Two legume species, *Medicago truncatula* and *Lotus japonicus*, are being used as models to study legume genetics and genomics. These two species were chosen as models because of their being diploid with relatively small genomes (450–550 Mb), self-fertility, relative ease of genetic transformation, and short generation time. The success of any species as a molecular genetic model depends on the availability of versatile genetic and genomic resources.

5.2. Creation of saturation mutagenesis

In TILLING, seeds are exposed to a strong mutagenic compound, which introduces random mutations across the entire genome. However, extra care is taken to achieve mutation saturation in the target genome. Before creating the TILLING population, most researchers therefore start by establishing a “kill curve” using their mutagen of choice where concentration is plotted against seed survivability. A general rule of

thumb is to aim for a 30–40% survival rate (Wang *et al*., 2010, Chawade *et al.,* 2010). After mutagenesis, the seeds (M_1) are planted and allowed to self-fertilize and produce a new generation of seeds (M_2). Typically, one seed from each line is sown to produce the M_2 population and, DNA is isolated from every single M_2 plant.

In the majority of TILLING experiments, especially those conducted on *Arabidopsis*, EMS has been applied as a mutagen. In addition to this species, EMS has been used as the mutagen in TILLING experiments performed on *Brassica napus*, *Brassica oleracea*, *Glycine max*, *Hordeum vulgare*, *Lotus japonicus*, *Medicago truncatula*, *Oryza sativa*, *Sorghum bicolor*, *Triticum aestivum*, *Triticum durum* and *Zea mays* (Gilchrist and Haughn 2005; Martín *et al*., 2009). EMS induced mutations are randomly distributed in the genome and a high degree of mutational saturation can be achieved without excessive DNA damage (Gilchrist and Haughn 2005). In Arabidopsis, 5% of the mutations induced by this mutagen in coding regions result in the premature termination of the gene product (nonsense mutations) or mutations in splice sites, whereas 50% lead to missense mutations (Greene *et al.,* 2003; Martín *et al.,* 2009). EMS alkylates guanine bases and leads to mispairing: alkylated guanine pairs with thymine, which results mainly in G/C to A/T transitions (Henikoff and Comai, 2003). This type of transition makes up more than 99% of all EMS-induced mutations in Arabidopsis, maize and wheat. However, when the frequencies of various types of EMS induced mutations were analysed in other species (tomato, rice and barley) G/C to A/T transitions constituted no more than 70% (Minoia *et al*., 2010).

In this investigation, EMS concentrations on the survival of four varieties of mungbean (Co7, Co6, VRM (Gg) 1 and VBN (Gg) 3) were studied. The variety which shows relatively higher tolerance (high survival %) to EMS doses is suitable for creating TILLING populations as these varieties can harbor more mutations within a manageable population size than the varieties that cannot survive high EMS doses. The results of kill curve analysis show that the variety Co (Gg) 7 showed relatively better tolerance to EMS treatment as compared to the other three varieties viz Co (Gg) 6, VRM (Gg) 1 and VBN (Gg) 3. Among the doses the concentration of 70mM was found to be the optimal EMS dosage for achieving 70% kills for the variety Co (Gg) 7. Hence, combination of Co (Gg) 7 with 70mM EMS was fixed for generating Mungbean TILLING Populations

(MTP). Similar study was conducted by Ndou *et al*., 2013 to determine the optimum EMS concentration for effective mutagenesis in selected wheat varieties. Seeds of four varieties (B936, B966, SST387 and SST875) were treated with four EMS concentrations (0, 0.3, 0.5and 0.7%) Percentage seedling emergence, germination and seedling height were recorded for the treatment combinations. The most effective treatment in variety B936 was 0.7% EMS. The study established varietal specific EMS dose and treatment conditions to be used in inducing large-scale mutagenesis in wheat.

About 12,000 M_0 seeds were treated with 70mM and grown in field conditions. About 3774 M_1 plants survived till maturity and they were sorted out into two batches of 1872 families each. The first set of 1872 families was raised during *Kharif* 2012. In that 1746 families survived till maturity. Among them 457 healthy families were randomly chosen. The second set of the remaining 1872 families was raised during *Rabi* 2012. Out of which 1182 families survived till maturity. Among them 311 families were randomly chosen. Leaves from them were collected and their pods were harvested on single plant basis. Pods were threshed to obtain a seed which was stored in medium term storage cold room at Ramiah Gene Bank.

Large numbers of mutants were also generated by Sabetta *et al*. 2011 in sunflower, where thirty thousand M_0 seeds of sunflower were mutagenized with 0.7% EMS for 6 h and grown in field conditions. About 13,000 M_1 plants were obtained, but only 50% of them reached the complete maturity and set seeds. Four to 10 M_2 seeds per family were sown in the field and, at the stage of 3-4 leaves; most of the emerged plants were manually thinned to leave only one M_2 plant per family. About 64% of the sown seeds were able to germinate: thereby, 4,211 M_2 plants were obtained and regularly monitored for the presence of phenotypic variations in comparison with the untreated wild-type. Finally, since M_3 seeds were harvested from 86.7% of fertile plants, an M_2 population of 3,651 lines was used for leaf DNA sampling and M_3 seed stocking.

5.3. DNA Isolation and pooling strategy

In any reverse genetic approach, pooling and super pooling of DNA isolated from an invariably large mutant resource is essential for limiting the sample numbers for PCR amplification and mutation discovery (Sreelakshmi *et al*., 2010; Missirian *et al*., 2011).

The quality of DNA from each line must be consistent from sample to sample to allow equal pooling of DNA from several individuals. Many high throughput methods to isolate DNA from plant tissues are available; however, these methods produce either insufficient amounts or inconsistent quality of DNA for TILLING (Dilworth *et al*., 2000; Xin *et al*., 2003; Geuna *et al*., 2000; Mace *et al*., 2003; Flagel *et al*., 2005). Several commercial kits are also available to extract genomic DNA from plant tissues with sufficient quality (Xin and Chen, 2006), but the yield of DNA produced from commercial kits is often low. Moreover, the cost can be prohibitive for small laboratories (Xin and Chen, 2012)

In this study, different methods of DNA isolation such as classical CTAB, modified method of Murray and Thompson (1980) and spin column loaded with silica methods were tried to assess a suitable recipe which could recover high and almost uniform good quality DNA from leaf tissues of mutants. Among the methods tried binding buffer loaded with silica gave consistent recovery and optimal quality of DNA good enough for pooling and super pooling (Table 11).

Till *et al*. 2007 and Uauy *et al*. 2009 expanded the mutation discovery by searching for new mutations in rice and wheat mutants populations respectively. The genomic DNA of 768 individuals was used to create two types of pools, eight “row pools” (96 individuals each) and 12 “column pools” (64 individuals each), for a total of 20 “pools”. Each individual was represented twice, once in the row pool and once in the column pool. A mutation found in both the row and column pools identified a pool of eight individuals where one of the eight individuals carries the mutation.

Similar pooling strategies were followed in this investigation, DNA was extracted from 768 M_2 families and normalized to expected concentration of 100ng/µl was used to create two types of pools, eight “row pools” (96 individuals each) and 12 “column pools” (64 individuals each), for a total of 20 “pools” depicted in Figure 4. So that a common mutation found in both the row and column pools on intersection can be designated to a pool of eight individuals where one of the eight individuals carries the mutation.

5.4. Primer designing and PCR optimization for the amplification of TILLING fragments

The sequence of the gene of interest can be retrieved for many species from databases, such as GenBank (http://www.ncbi.nlm.nih.gov/genbank) and then a proper homolog can be identified. In the case of barley, whose genome is not fully sequenced, cloning homologues enables their functional analysis. Candidate genes for analysis, when not available for particular species, as .in barley, can be retrieved from the GenBank data base of Arabidopsis or rice. Hence forth, in the case of Arabidopsis genes, a BLAST search is performed against the rice genome to identify the rice homologue. In order to amplify the coding sequence of a proper homologue, it is necessary to use mRNA and amino acid sequences as a query in the barley EST databases. EST sequences with the highest similarity are chosen as a template to design primers in order to amplify them (Kurowska *et al*., 2011). Primers for amplification were designed by entering genomic DNA sequence into the Codons Optimized to Deliver Deleterious Lesions (CODDLe) input form (http://blocks.fhcrc.org) to select the regions most likely to harbor deleterious changes induced by EMS and then using a modified version of Primer 3 (Rozen *et al*., 2000) to select primers.

In this investigation also, as the sequence support for greengram (*Vigna radiata*) is very limited, hence resorted to use the sequences of the candidate genes (CGs) listed in Table 3 from other model legumes like medicago, soybean and cowpea. The gDNA and cDNA sequences of aforementioned genes were searched from NCBI Genbank (http://www.ncbi.nlm.nih.gov/Genbank). These sequences were utilized for building up gene models and designing primers. EST sequences of *Medicago truncatula* was ideally suitable for designing cross species primers for amplifying the *Gigantea* genes in green gram, due to more query coverage and maximum identity it has recorded with all known sequences from crops like soybean and peas. Similarly for the *Leafy* and *Tfl* genes from EST sequences of *Vigna radiata* and *Constans, Ramosus* from ESTs of *Glycine max.* The primers were designed using the software Primer3 (Primer3 http://frodo.wi.mit. edu/primer3/) and primer pairs are furnished in the Table 4.The five TILLING fragments (1.3 to 2.8 kb) covering five key candidate genes with maximum mutation probability for

PCR amplification were fixed using the bioinformatic pipeline CODDLE (Codons Optimized to Detect Deleterious Lesions). The Primers for PCR amplification of five TILLING fragments were designed with PRIMER 3 software embedded with CODDLE.

GC content of the target template sequence, length of template amplicon, presence of PCR amplification inhibitors, self-complimentarily of primer etc., have been postulated to be the major determinants of success in PCR amplification (Housley *et al*., 2006; Jia *et al*., 2014). In this study, ultimate care were taken in the design of PCR primers in such a way that the length of target regions were >4 kb, the self-complementarity of primer sequence to be >3. Also adopted step down PCR cycle as recommended by Don *et al*. (1991) to minimize the off target amplification. Successfully amplified all the candidate genes/TILLING fragments with different PCR cycling conditions and master mix compositions (Table 13). To enhance the fidelity of PCR amplification Taq polymerase with proof read was used to minimize the introduction of non-target bases during extension. In this study, TaKaRa LA DNA polymerase was used.

5.5. TILLING by Next Generation Sequencing

The most commonly used method to identify mutations in a TILLING population is by using the Li-Cor system. However, there are a number of inherent drawbacks with the Li-Cor method that need to be considered. Parameters like fluorescent dye primer and DNA concentrations as well as the ratio between the cleavage enzyme and PCR product concentrations all affect the results and need to be optimized (Sikora *et al*., 2011).

Next-generation sequencing (NGS) has significantly accelerated the prospects of identifying mutations/variants at the whole-genome or gene levels. Decreasing sequencing costs due to improved technical accuracy, improved throughput, and increased capacity compared to only a few years ago has led to a great potential for NGS in TILLING. The two most commonly used NGS platforms are the 454 Genome Sequencer FLX Ti (Roche Applied Science) and the Illumina (Solexa) Genome Analyzer (Sikora *et al*., 2011). Experiments suggest that as many as 12,000 samples may be analyzed simultaneously on a single 454-picotiter plate (PTP) using Key Point technology, as successfully tested on a tomato TILLING population (Rigola *et al*., 2009).

As the amount of data generated from NGS is immense, knowledge of bioinformatics and access to computational resources are invaluable during analysis.

Considering the power of next generation sequencing (NGS), in this investigation the PCR amplicons of five different genes were pooled across the super pools to formulate 20 PCR amplicon libraries. Such pooling across the PCR amplicons was adopted as the gene sequences themselves are different from each other and serve as biological barcodes. Further with the aid of *in vitro* DNA bar coding addition technology the twenty libraries were pooled into one sequencing library under ILLUMINA myseq sequencer after adding variable four base length barcodes. Such barcoding strategy for using the power of next generation sequencing in pooled PCR amplicons has been reported by Knapp *et al*. (2010). Such tagged DNA libraries were sorted based on barcodes after assembling the contigs in the sequence assembler bioinformatics pipeline. The sequence of five TILLING fragments was used to create a template assembly of the sequence from library 21 (Control) using **SPAdes** and **SSPACE** under ILLUMINA myseq sequencer. The above template of five gene fragments was used to assemble sequences from rest of the samples since mungbean genome sequence is not completed yet.

5.6. Sequencing depth and variant calling efficiency

Illumina sequencing has also been adapted to high-throughput TILLING, and has been used to screen bread-wheat, durum-wheat, and rice populations (Tsai *et al*., 2011). A number of computational tools exist for finding SNPs in non-overlapping pools sequenced with next-generation technology, such as Var Scan (Koboldt *et al*., 2009), CRISP (Bansal, 2010; Bansal *et al*., 2010), SNP Seeker (Druley *et al*., 2009), SAM tools (Li *et al*., 2009), and MAQ (Li *et al.,* 2008).

Tsai *et al*. 2011 described the use of next-generation sequencing coupled with multidimensional pooling for the identification of rare alleles in populations using rice (*Oryza sativa*) and wheat (*Triticum durum* and *Triticum aestivum*) as examples. They concluded that sequencing of pooled samples is effective for TILLING with the combination of pooling strategies, PCR amplification, Illumina platform sequencing, and a bioinformatic pipeline that assigns probabilities to each candidate resulted in efficient detection with a low false-positive rate.

The average depth of coverage for the candidate gene under ILLUMINA myseq sequencer in this investigation was more than approximately 1000x which has surpassed the minimum of 50x required for unambiguous discrimination of error from the true variant(s) (Table 14 and 15). Hence, it is obvious that all the variants discovered in this investigation possess very high confidence limits. Liu *et al*., 2012 suggest that the highest integrity data and broader range of application, including amplicon sequencing, clone checking, ChIP-Seq, and small genome sequencing, are the outstanding parts of MiSeq. It is also flexible to perform single 36 bp reads (120 MB output) up to 2 × 150 paired-end reads (1–1.5 GB output) in MiSeq. Due to its significant improvement in read length, the resulting data performs better in contig assembly compared with HiSeq.

5.7. Candidate genes and sequence variants discovered

Various authors have reported the presence of conserved sequences in the central genes involved in the downstream of the photoperiod pathway in *Arabidopsis thaliana* are *GIGANTEA (G1), CONSTANS (CO),* and *FLOWERING LOCUS T (FT)* (Redei, 1962; Koorneef *et al.*, 1991, 1998). These genes are well conserved across many species of flowering plants (Hecht *et al.,* 2005). *CO* encodes a putative zinc finger transcription factor, and plays a central role in photoperiodic flowering control in Arabidopsis (Putterill *et al.,* 1995). The meristem produces phytomers, which are shoot units consisting of a leaf, axillary bud and a stem segment. Arabidopsis mutant plants of unfunctional *TERMINAL FLOWER1 (TFL1)* transcription factor (Shannon and Meeks-Wagner 1991) have a determinant meristem. *TFL1* controls the growth of the phytomer by delaying the expression of floral-related genes, such as *LEAFY (LFY)* and *APETALA1 (AP1),* and hence gives more time for the vegetative axillary buds to initiate and grow (Ratcliffe *et al.,* 1999). Determination of the mechanism by which an axillary bud initiates and develops is important to genetically design plants with a desired branching habit. *TFL1* belongs to the *CETS (CENTRORADIALIS/TERMINAL FLOWER 1/SELF-PRUNING)* family of genes that encode PEBPs (phosphatidylethanolamine binding proteins) (Pnueli *et al*., 2001). PEBPs are evolutionally conserved among eukaryotes and play important roles in diverse organisms *TFL1* is homologous to phosphatidylethanolamine binding proteins that play diverse roles related to signaling pathways controlling growth and differentiation (Benlloch *et al*., 2007).

A number of genes involved in the control of shoot branching have been identified in pea (*Pisum sativum*). These include five *Ramosus (RMS)* genes, *RMS1* through *RMS5* (Beveridge, 2006). Grafting studies have demonstrated a role for these genes in shoot and rootstock tissues. The inability of exogenous auxin applications to rescue the increased branching phenotypes of the *rms* mutants (Beveridge *et al*., 2000), and the auxin inducible *RMS1*and *RMS5* gene expression (Foo *et al*., 2005; Johnson *et al*., 2006), suggests these genes are involved in the synthesis of auxin's secondary messenger.Hence, in this investigation the genes *GI, CO* were chosen for alteration in flowering time, *TFL* for determinate growth habit and *RMS* for profuse branching. Detailed functional modifications that can be attained by interrupting those candidate genes are given in the Table 3.

The presence of very high proportions of conserved sequences in the candidate genes prompted us to use the bioinformatics pipeline Codon Optimisation for Discovery of Deleterious Lesions, (CODDLe) which helps in discovering gene regions which are less conserved and also with high probability of carrying functional sequence variants (McCallum *et al*., 2000b). Such selection is necessary to identify hotspot areas likely to be harbour intense variations.

By assembling the contigs across reference sequences, the variants in the form of SNPs and Indels were called for further validation of their putative functional effects. Based on the number of bases, nature and position of the variants a plethora of genetic information can be obtained by applying suitable computational algorithms. Even though gene targets for this study were chosen through CODDLe, some of the genes showed very less variant per kb of target which followed an order *RMS* (2.56)>*CO*(2.06) *TFL1b*(1.92)>*GI*(1.53)>*LFY*(0.00) However, functional variant per kb of target followed a different order; *TFL1b*(1.92)>*RMS* (1.28)>*GI*(0.76)>*CO*(0.00)>*LFY*(0.00) (Table 24). The average mutation frequency for the population was found to be one mutation per 0.22 Mb (Table 25). Therefore for the entire population of 2928 M_2 lines translates to an average of 13 mutations for any 1 kb DNA fragment. Similar studies were done for TILLING in the two-rowed barley cultivar 'Barke' for sites of functional diversity in the gene HvHox1 where the average mutation frequency for the population was found to be one mutation per 0.5 Mb. Therefore, the entire population of 10,279 M_2 lines translates to an average of 20 mutations for any 1 kb DNA fragment by Gottwald *et al*. 2009.

Table 24. SNP polymorphism in selected candidate genes responsible for alteration in flowering and growth habit

S.No	Candidate genes	Length of target (kb)	Proportion of variant /kb of target	Proportion of functional variant/kb of target
1.	*GI*	1.300	1.53	0.76
2.	*RMS*	0.780	2.56	1.28
3.	*CO*	1.456	2.06	0.00
4.	*LFY*	0.652	0.00	0.00
5.	*TFL Ib*	0.519	1.92	1.92

Table 25. Mutation frequency for the candidate genes

S.No	Candidate genes	Amplicon size (bp)	Silent mutant	Missense mutant	Total Mutants	Mutation frequency (Mb)
1.	*GI*	1300	1	1	2	0.46
2.	*RMS*	780	1	1	2	0.29
3.	*TFL Ib*	519	0	1	1	0.39
			Mean			**0.22**

5.8. Deconvolution of pooled samples

The concept of intersection (presence of same mutation in one of the row as well as a column super pool) helps to locate the 8x well carrying the variant. Further deconvolution of a genotype in the 8x well be achieved by adopting any of the three strategies; (i) analyzing *in silico* predicted restriction enzyme cleavage variations in the gene sequence arising due to the base change (mutation) in the eight putative lines as compared to the reference or (ii) by comparing the gene sequences of the PCR products of the eight putative mutants, for this Sanger's sequencing can be adopted and also (iii) through any SNP genotyping technology such as, allele specific PCR amplification. In our study through Sanger's sequencing and identified the variant carrying genotypes from the pooled samples (Table 19).

5.9. Gene wise variant discovery and its implications

5.9.1. *GIGANTEA* (*GI*)

In this investigation, the discovered SNP at coordinate 605 of this gene creates a C→Y substitution (Table 18 and Plate 8a). Cysteine has traditionally been considered to be a hydrophilic amino acid, based largely on the chemical parallel between its thiol group and the hydroxyl groups in the side-chains of other polar amino acids (Heitmann, 1968). However, the cysteine side chain has been shown to stabilize hydrophobic interactions in micelles to a greater degree than the side chain in the non-polar amino acid and the polar amino acid. In a statistical analysis of the frequency with which amino acids appear in different chemical environments in the structures of proteins, free cysteine residues were found to associate with hydrophobic regions of proteins. Their hydrophobic tendency was equivalent to that of known non-polar amino acids such as tyrosine (Nagano *et al*., 1999). Based on SIFT analysis this substitution was found to be deleterious and affecting the protein function.

Curtis *et al*. 2002 reported a late-flowering transgenic radish has been produced by the expression of an antisense *GIGANTEA (GI)* gene fragment using a floral-dip method. In the progenies of eleven T1 plants analysed (T2 generation), all lines showed a significant delay in both bolting and flowering times compared to wildtype and positive control plants, and that, the level of *GI* transcript was inversely proportional to the time

of bolting and flowering. At a maximum, bolting and flowering times were delayed by 17 and 18 days respectively, compared to wildtype plants (in positive control plants, the delay was 23 and 26 days, respectively). This study provides evidence that down-regulation of the *GI* gene by co-suppression could delay bolting in a cold-sensitive long-day (LD) plant.

Our deconvolution results also revealed that MTP-399 family was found to be the carrier of the *GI* gene (MTP-M2). Subsequent biometrical characterization showed maximum delayed flowering with fifty per cent flowering 47days for the mutant MTP-399-16 was recorded than control (Co (Gg) 7) 31.50 days, followed by 40 days for the mutant MTP-399-11. MTP-399-11 also had highest plant height of 42.5cm than control Co (Gg) 7 (31 cm), maximum number of branches was also recorded with 5 branches than control Co (Gg) 7 (1.85 branches), maximum accessible pods per plant (63) also observed than control Co (Gg) 7 (11.6).This result clearly indicates that the mutant MTP-399-11 carrier of *GI* mutation and it was further characterized molecular level and can be used for future breeding programmes to transfer high branching trait.

5.9.2. *RAMOSUS* (*RMS*)

In this investigation, the discovered SNP at coordinate 1511 of this gene creates a F→L substitution (Table 18 and Plate 8b). Phenylalanine can be substituted with other aromatic or hydrophobic amino acids. The phenylalanine side chain is fairly non-reactive, and is thus rarely directly involved in protein function, although it can play a role in substrate recognition. Being hydrophobic, leucine prefers to be buried in protein hydrophobic cores. It also shows a preference for being within alpha helices more so than in beta strand. It is very non-reactive and is thus rarely directly involved in protein functions like catalysis, although it can play a role in substrate recognition (Macias *et al.,* 2002). Based on SIFT analysis this substitution was found to be deleterious and affecting the protein function.

Foo *et al*. 2005 reported in *Pisum sativum*, the *RAMOSUS* genes *RMS1*, *RMS2*, and *RMS5* regulate shoot branching via physiologically defined mobile signals. Molecular, genetic, and physiological evidence that *RMS1* plays a central role in a shoot-to-root-to-shoot feedback system that regulates shoot branching in pea. Indole-3-

acetic acid (IAA) positively regulates *RMS1* transcript level, a potentially important mechanism for regulation of shoot branching by IAA.Our results also revealed that MTP-580 family was found to be the carriers of the *RMS* gene (MTP-M3). Subsequent biometrical characterization showed MTP-580-21 recorded the maximum delayed flowering with fifty per cent flowering of 39 days than control Co (Gg) 7(31.5 days), followed by MTP-580-07 with 38 days but fifty per cent flowering was shorter than MTP-399-16 and MTP-399-11 of *GI* mutant. But maximum number of branches was recorded for MTP-580-19, with 7 branches than control Co (Gg) 7 (1.85), followed by MTP-580-07 and MTP-580-21 with 6 branches which more than *GI* mutant MTP-399 (5 branches). Maximum accessible pods per plant in MTP-580-21 and MTP-580-07 of 69 was observed. This result clearly indicates that the mutant MTP-580-07and MTP-580-21 carrier of profuse branching of *RMS* mutant and this mutant after further functional and genetic characterization can be deployed in breeding programmes.

5.9.3. *TERMINAL FLOWERING 1b* (*TFL 1b*)

In this investigation, the discovered SNP at coordinate 165of this gene creates a G→S substitution (Table 18 and Plate 8c). The unique property of Glycine, can play a distinct functional role (use of backbone) to bind to phosphates (Gahmen *et al*., 1996). The loss of this amino acid residue (conserved glycine) could have a drastic impact on function. Serines are quite common in protein functional centres. The hydroxyl group is fairly reactive, being able to form hydrogen bonds with a variety of polar substrates. Extracellular serines can also be O-glycosylated where a carbohydrate is attached to the side chain hydroxyl group (Gupta *et al*., 1999). Based on SIFT analysis this substitution was found to be deleterious and affecting the protein function.

Alvarez *et al*. 1992 reported the growth of flowering stems in wild-type Arabidopsis is indeterminate. They isolated eight recessive mutants of a gene, terminal flower, in which inflorescences become determinate. Determination of the mechanism by which an axillary bud initiates and develops is important to genetically design plants with a desired branching habit reported by McSteen and Leyser 2005. Our deconvolution results also revealed that the MTP-134 family was found to be the carriers of the *TFL1b* gene (MTP-M10). Subsequent biometrical characterization showed MTP-134-15 had

highest plant height of 43.6cm than other two mutants *GI* (MTP-399-11) and *RMS* (MTP-580-19). Next to (MTP-399-16) *GI* mutant MTP-134-15 recorded the maximum fifty per cent flowering of 41 days. Next to *RMS* mutant MTP-580-21 (69) MTP-134-15 had maximum accessible pods per plant with a value of 65. This result clearly indicates that the mutant MTP-134-15 carrier of top pod bearing habit and which after further genetic and molecular characterization can be used in breeding programme.

5.10. Implications of the current investigation for attaining ideal plant type for Mechanical harvesting

Currently, the development of cultivars more suitable for mechanical harvesting is a major breeding objective aiming to reduce production costs as well as to address the problem of the labor force shortage. Manual harvesting comprises up to 30% of the total crop production cost, whereas in semi-mechanical harvesting systems this value might drop to 13% in cowpea (Mercantil, 2001). The conversion of mungbean to an entirely mechanized crop is a major breeding challenge. This endeavor will demand the development of cultivars with a combination of traits, including semi determinate growth habit, more erect plants associated with more compact (bush-type) canopy, short internodes and short secondary branches.

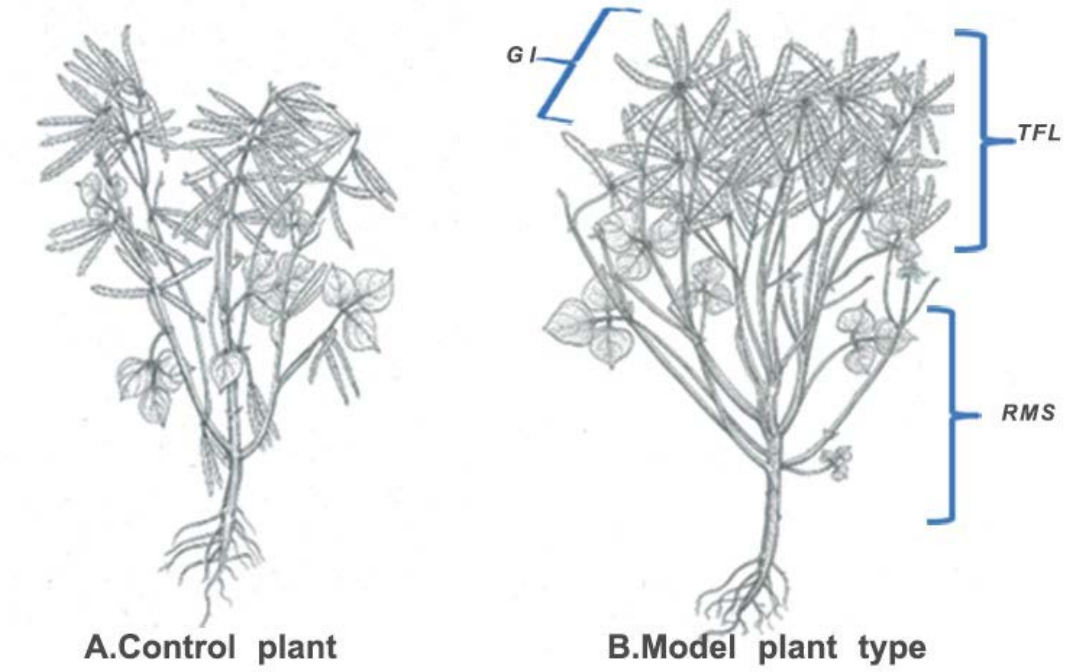
With this idea our investigation was carried out to bring the altered phenotype suitable for mechanical harvesting through isolation of mutation through TILLING approach in mungbean. Since mungbean sequence not yet completed, so targeted the candidate genes *viz*., *GI, CO, RMS, LFY* and *TFL1b* from other model legume crops such as *Medicago truncatula*, *Glycine max* and *Vigna ungiculata*. Those candidate genes were well characterized in this study for altering phenotypes such as delayed flowering, profuse branching, semi-determinate growth and terminal flowering.

In our investigation the functional mutations for *GI, RMS* and *TFL* were discovered from the mungbean TILLING population. The discovered mutants *viz*., MTP-399-16, MTP-134-15, MTP-134-03, MTP-134-15and MTP-399-11 could be deployed further for delayed flowering, semi determinate growth habit along with top pod bearing habit suitable for mechanical harvesting. The characterized mutations in MTP-580-19, MTP-134-15, MTP-580-07 and MTP-580-21 could be introgressed further for profuse branching, top pod

bearing along with maximum accessible pods per plant suitable for mechanical harvesting. Further marker assisted introgression of these mutations in to a common background could result in an ideal plant type for mechanical harvesting. In spite of an intensive mutant generation and mutant discovery process undertaken in this study, mutation in the gene *CONSTANS* which can ideally contribute for flowering time control could not be recovered.

Even though bushy phenotype is the implicated trait of *GI* mutants deployed, it also affects partial control on flowering time. Hence, the *GI* mutation in combination with *TFL* and *RMS* could result in desirable plant type with good scope for mechanical harvesting. The conceptual introgression programme for the phenotype modification which can be achieved by deploying the mutants discovered in this investigation is depicted in figure 8.

Figure 8. Comparison of control plant along with model plant type after introgression of all the discovered mutants in single plant suitable for mechanical harvesting



RAMOSUS (RMS)-for profuse branching, *TERMINAL FLOWERING (TFL)*-for top pod bearing *GIGANTEA(GI*)-for extending flowering time and bushy growth

Summary

CHAPTER VI

SUMMARY

The present investigation was carried out at the Department of Plant Genetic Resources, and Department of Pulses, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore during 2012-2014. This investigation aimed at optimizing mutagenesis in mungbean for saturational mutagen and generation of TILLING population, discovery of putative mutants from branching and flowering for robust branching type and delayed flowering type through TILLING by sequencing and biometrical characterization of the discovered mutants suitable for branching and flowering habit.

The results obtained from the present study are summarized below

- EMS doses /genotype combination for saturation mutagenesis was identified through kill curve analysis. Genotype Co (Gg) 7 and EMS doses of 0.7% (LD_{70}) were fixed among other mungbean genotypes *viz*., Co (Gg) 6, VRM (Gg) 1 and VBN (Gg) 1.
- A total of 12000 seeds was treated with 0.7% and raised during summer 2012. The seeds of each M_1 plant which survived till maturity of about 3744 families were harvested on single plant basis.
- The seeds of M_1 population were sorted out batches of 1872 families. In that 2928 M_2 families survived till maturity. Among them we randomly choose 768 healthy families and banking the seeds in medium term cold room storage at Ramiah Gene Bank.
- Different DNA isolation method were used, among the methods investigated, binding buffer loaded with silica showed consistent high recovery with desirable quality score. By adopting the best method *viz.*, the spin column based extraction with silica loaded binding buffer we extracted DNA from 768 families to suit the wet lab 96 well format of 8 rows and 12 columns. The extracted DNA from the families were distributed across 8 numbers of 96 well microplates.

- Pooling and super pooling of DNA were adopted as Till *et al*(2007) in 8*8*12 format
- EST sequences of *Medicago truncatula* will be ideally suitable for designing cross species primers for amplifying the *Gigantea* genes in Green gram, due to more query coverage and maximum identity it has recorded with all known sequences from crops like soybean and peas. Similarly for the *Leafy* and *Tfl* genes from *Vigna ungiculata* and *Constans, Ramosus* from *Glycine max.* The primers were designed using the software Primer3 (Primer3 http://frodo. wi.mit.edu/primer3/)
- The five TILLING fragments (1.3 to 2.8 kb) covering five key candidate genes with maximum mutation probability for PCR amplification were fixed using the bioinformatic pipeline CODDLe (Codons Optimized to Detect Deleterious Lesions)
- The genes *GI, RMS, CO* were amplified in combinations of the PCR cycle I and PCR master mix II. The genes *LFY* and *TFL1b* were amplified by adopting PCR cycle II and PCR master mix I.
- 20 amplicon products were outsourced for NGS under ILLUMINA myseq sequencer.
- Through variant calling and bioinformatics procedure 8 SNP and 2 Indels were discovered across five candidate genes
- By adapting SIFT analysis three mutations *viz.*, G→A, T→A and G→A residing on genes *GIGANTEA*, *RAMOSUS* and *TERMINAL FLOWERING 1b* respectively were found to be deleterious with amino acid substitution of C→Y for *GI* gene, F→L for *RMS* gene and G→S for *TFL1b* gene.
- The average mutation frequency for the population was found to be one mutation per 0.22 Mb. Therefore for the entire population of 2928 M_2 lines translates to an average of 13 mutations for any 1 kb DNA fragment
- The Putative mutants in the pools were deconvoluved by sanger sequencing and the M_2 mutant families MTP-399, MTP-580 and MTP-134 were identified as a

carrier for mutation residing in *GIGANTEA*, *RAMOSUS* and *TERMINAL FLOWERING 1b* respectively.

- Mutant families were biometrically characterized for 20 characters.
- The discovered mutants *viz*., MTP-399-16, MTP-134-15, MTP-134-03, MTP-134-15 and MTP-399-11 could be deployed further for delayed flowering, semi determinant type along with top pod bearing habit suitable for mechanical harvesting.
- MTP-580-19, MTP-134-15, MTP-580-07 and MTP-580-21 could be introgressed further for profused branching, top pod bearing along with maximum accessible pod per plant suitable for mechanical harvesting

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Annexures

ANNEXURE I

REAGENTS REQUIRED FOR DNA EXTRACTION

I. 2X CTAB buffer (1 litre)

20 g of CTAB dissolved in 860 ml of ddH_2O

81.82 g of NaCl

100 ml of 1 M Tris pH 8.0

40 ml of 0.5 M EDTA pH 8.0

Sterilize by autoclaving.

II. STOCK SOLUTIONS

1 M Tris pH 8.0 (1 litre)

121.1 g of Tris

700 ml of ddH_2o

Dissolve Tris and bring to 900 ml

Adjust pH to 8.0 using concentrated HCl (about 50 ml)

Make up the volume to 1000 ml using ddH_2O

Sterilize by autoclaving

0.5 M EDTA pH 8.0 (1 litre)

186.12 g of EDTA

750 ml of ddH_2O

Add about 20 g of NaOH pellets

Slowly add more NaOH until the pH 8.0

Make up the volume to 1000 ml using ddH_2O

Sterilize by autoclaving

TE buffer (1 litre)

10 ml of 1 M Tris pH 8.0 (10 mM)

2 ml of 0.5 M EDTA pH 8.0 (1 mM)

Make up the volume to 1000 ml using ddH_2O

Sterilize by autoclaving

III. Other chemicals

1. Ice cold isopropanol

It was stored at -20ºC.

2. Chloroform: Isoamyl alcohol (24:1 v/v)

About 24 parts of chloroform and 1 part of isoamyl alcohol was added and mixed properly.

3. Chloroform: Octanol (24:1 v/v)

About 24 parts of chloroform and 1 part of Octanol was added and mixed properly.

4. Ethanol (100 per cent and 70 per cent)

Absolute ethanol was used as such, 70 per cent ethanol was prepared by adding 30 parts of distilled water to 70 parts of absolute ethanol.

5. RNAase

About 10 mg of RNase was dissolved in 1 ml of solution containing 10 mM Tris (pH 7.5) and 15 mM NaCl. The prepared stock solution was stored at -20ºC, the working stock was stored at 4ºC.

6) Silica Powder (Celite 545 silica)

7) 2M Guanidine Hydrochloride

8) 5M NaCl

PREPARATION OF SILICA POWDER-DNA BINDING SOLUTION

1. Added silica powder (Celite 545 silica) into 15 mL Falcon-tube (approx. 100mg)
2. Added 3 mL dH2O in to the falcon tube
3. Shaked vigorously (vortex and invert)
4. Allowed slurry settle for approx. 15 min
5. Removed (pipette off) the liquid
6. Repeated it for 2 times (a total of 3 washes)
7. After last washing step: resuspended the silica powder in about the same amout of water (up to about 5 mL)
8. Added 8ml of DNA binding buffer (2M Guanidine Hydrochloride dissolved in absolute ethanol)
9. Added 2 ml of TE buffer (PH-8.00)
10. Finally suspended the silica powder in DNA binding buffer

PREPARATION OF WASH BUFFER

To 1mL of 5M NaCl 99mL of 95% EtOH

I. DNA EXTRACTION PROCEDURE - CTAB method

1. 0.5 g of leaf sample was taken in a pre-chilled sterile pestle and mortar.
2. The samples were grinded rapidly with 500 µl of pre-heated CTAB buffer.
3. The grinded samples were transferred to an eppendorf tube and kept in water bath having 65^{o}C temperature. The samples were maintained in the same temperature for 30 minutes.
4. The samples were allowed to cool. Equal volume of chloroform-isoamyl alcohol (24:1 V/V) was added and mixed by inversion.
5. The samples were centrifuged at 10000 rpm for 10 minutes at room temperature.
6. The clear aqueous phase was transferred to new sterile tube. Equal volume of ice-cold isopropanol was added and mixed gently by inversion and then kept in the freezer until DNA was precipitated. (4^{o}C for overnight).
7. A brief spin at 10000 rpm for 10 minutes was given and the supernatant was removed. To this, 500 µl of 70% ethanol was used to wash the DNA and then centrifuged for 3-5 minutes and the supernatant was discarded.
8. The residue was dried completely.
9. The DNA pellet was dissolved in 100 µl of TE buffer. Five µl of RNase was added and kept in water bath for 30 minutes at 37^{o}C
10. The DNA was thus extracted and stored at -20^{o}C.

II. DNA EXTRACTION PROCEDURE – The Modified method of Murray and Thompson 1980

1. 0.75 g of leaf sample was taken in a pre-chilled sterile pestle and mortar.
2. The samples were grinded rapidly with 500 µl of pre-heated CTAB buffer.
3. The grinded samples were transferred to an eppendorf tube and kept in water bath having 65^{o}C temperature. The samples were maintained in the same temperature for 30 minutes.
4. The samples were allowed to cool. Equal volume of chloroform-Octanol (24:1 V/V) was added and mixed by inversion.
5. The samples were centrifuged at 10000 rpm for 10 minutes at room temperature.
6. The clear aqueous phase was transferred to new sterile tube. 2/3 volume of ice-cold isopropanol was added and mixed gently by inversion and then kept in the freezer until DNA was precipitated. (4^{o}C for overnight).
7. A brief spin at 10000 rpm for 10 minutes was given and the supernatant was removed. To this, 500 µl of 70% ethanol was used to wash the DNA and then centrifuged for 3-5 minutes and the supernatant was discarded.
8. The residue was dried completely.
9. The DNA pellet was dissolved in 100 µl of TE buffer. Five µl of RNase was added and kept in water bath for 30 minutes at 37^{o}C
10. The DNA was thus extracted and stored at -20^{o}C.

III. DNA EXTRACTION PROCEDURE – Spin column based extraction with silica loaded binding buffer (Bernhard Hofinger and Bradley Till, 2013)

1. 0.50 g of leaf sample was taken in a pre-chilled sterile pestle and mortar.
2. The samples were grinded rapidly with 500 µl of pre-heated CTAB buffer.
3. The grinded samples were transferred to an eppendorf tube and kept in water bath having 65^{o}C temperature. The samples were maintained in the same temperature for 30 minutes.
4. The samples were centrifuged at 10000 rpm for 10 minutes at room temperature.
5. The clear aqueous phase was transferred to new sterile tube.
6. Equal volume of Silica based binding buffer (Preparation given above) was added to each centrifuge tube mixed well then transferred to spin column filter
7. Centrifuged the spin column filter at 11000rpm @2 min and then discard the substance collected in collection tube.
8. Added 500µl of Washing buffer (Preparation given above) to each filter then centrifuged for 11000rpm@ 2 min.
9. Repeated the washing procedure for twice. Dry spin was allowed to remove the excess ethanol in the filtrate.
10. Eluted the DNA with hot sterile water by adding 100 µl to each tube by centrifuge at 11000rpm for 2 min.

ANNEXURE II

LIST OF PUTATIVE MUTANTS PRESENT IN 8X POOLED PLATE

MTP-1	MTP-2	MTP-3	MTP-4	MTP-5	MTP-6	MTP-7	MTP-8	MTP-9	MTP-10	MTP-11	MTP-12
MTP-97	MTP-98	MTP-99	MTP-100	MTP-101	MTP-102	MTP-103	MTP-104	MTP-105	MTP-160	MTP-107	MTP-108
MTP-193	MTP-194	MTP-195	MTP-196	MTP-197	MTP-198	MTP-199	MTP-200	MTP-201	MTP-202	MTP-203	MTP-204
MTP-289	MTP-290	MTP-291	MTP-292	MTP-293	MTP-294	MTP-295	MTP-296	MTP-297	MTP-298	MTP-299	MTP-300
MTP-385	MTP-386	MTP-387	MTP-388	MTP-389	MTP-390	MTP-391	MTP-392	MTP-393	MTP-394	MTP-395	MTP-396
MTP-481	MTP-482	MTP-483	MTP-484	MTP-485	MTP-486	MTP-487	MTP-488	MTP-489	MTP-490	MTP-491	MTP-492
MTP-577	MTP-578	MTP-579	MTP-580	MTP-581	MTP-582	MTP-583	MTP-584	MTP-585	MTP-586	MTP-587	MTP-588
MTP-673	MTP-674	MTP-675	MTP-676	MTP-677	MTP-678	MTP-679	MTP-680	MTP-681	MTP-682	MTP-683	MTP-684
MTP-13	MTP-14	MTP-15	MTP-16	MTP-17	MTP-18	MTP-19	MTP-20	MTP-21	MTP-22	MTP-23	MTP-24
MTP-109	MTP-110	MTP-111	MTP-112	MTP-113	MTP-114	MTP-115	MTP-116	MTP-117	MTP-118	MTP-119	MTP-120
MTP-205	MTP-206	MTP-207	MTP-208	MTP-209	MTP-210	MTP-211	MTP-212	MTP-213	MTP-214	MTP-215	MTP-216
MTP-301	MTP-302	MTP-303	MTP-304	MTP-305	MTP-306	MTP-307	MTP-308	MTP-309	MTP-310	MTP-311	MTP-312
MTP-397	MTP-398	MTP-399	MTP-400	MTP-401	MTP-402	MTP-403	MTP-404	MTP-405	MTP-406	MTP-407	MTP-408
MTP-493	MTP-494	MTP-495	MTP-496	MTP-497	MTP-498	MTP-499	MTP-500	MTP-501	MTP-502	MTP-503	MTP-504
MTP-589	MTP-590	MTP-591	MTP-592	MTP-593	MTP-594	MTP-595	MTP-596	MTP-597	MTP-598	MTP-599	MTP-600
MTP-685	MTP-686	MTP-687	MTP-688	MTP-689	MTP-690	MTP-691	MTP-692	MTP-693	MTP-694	MTP-695	MTP-696
MTP-25	MTP-26	MTP-27	MTP-28	MTP-29	MTP-30	MTP-31	MTP-32	MTP-33	MTP-34	MTP-35	MTP-36
MTP-121	MTP-122	MTP-123	MTP-124	MTP-125	MTP-126	MTP-127	MTP-128	MTP-129	MTP-130	MTP-131	MTP-132
MTP-217	MTP-218	MTP-219	MTP-220	MTP-221	MTP-222	MTP-223	MTP-224	MTP-225	MTP-226	MTP-227	MTP-228
MTP-313	MTP-314	MTP-315	MTP-316	MTP-317	MTP-318	MTP-319	MTP-320	MTP-321	MTP-322	MTP-323	MTP-324
MTP-409	MTP-410	MTP-411	MTP-412	MTP-413	MTP-414	MTP-415	MTP-416	MTP-417	MTP-418	MTP-419	MTP-420
MTP-505	MTP-506	MTP-507	MTP-508	MTP-509	MTP-510	MTP-511	MTP-512	MTP-513	MTP-514	MTP-515	MTP-516
MTP-601	MTP-602	MTP-603	MTP-604	MTP-605	MTP-606	MTP-607	MTP-608	MTP-609	MTP-610	MTP-611	MTP-612
MTP-697	MTP-698	MTP-699	MTP-700	MTP-701	MTP-702	MTP-703	MTP-704	MTP-705	MTP-706	MTP-707	MTP-708
MTP-37	MTP-38	MTP-39	MTP-40	MTP-41	MTP-42	MTP-43	MTP-44	MTP-45	MTP-46	MTP-47	MTP-48
MTP-133	MTP-134	MTP-135	MTP-136	MTP-137	MTP-138	MTP-139	MTP-140	MTP-141	MTP-142	MTP-143	MTP-144
MTP-229	MTP-230	MTP-231	MTP-232	MTP-233	MTP-234	MTP-235	MTP-236	MTP-237	MTP-238	MTP-239	MTP-240
MTP-325	MTP-326	MTP-327	MTP-328	MTP-329	MTP-330	MTP-331	MTP-332	MTP-333	MTP-334	MTP-335	MTP-336
MTP-421	MTP-422	MTP-423	MTP-424	MTP-425	MTP-426	MTP-427	MTP-428	MTP-429	MTP-430	MTP-431	MTP-432
MTP-517	MTP-518	MTP-519	MTP-520	MTP-521	MTP-522	MTP-523	MTP-524	MTP-525	MTP-526	MTP-527	MTP-528
MTP-613	MTP-614	MTP-615	MTP-616	MTP-617	MTP-618	MTP-619	MTP-620	MTP-621	MTP-622	MTP-623	MTP-624
MTP-709	MTP-710	MTP-711	MTP-712	MTP-713	MTP-714	MTP-715	MTP-716	MTP-717	MTP-718	MTP-719	MTP-720
MTP-49	MTP-50	MTP-51	MTP-52	MTP-53	MTP-54	MTP-55	MTP-56	MTP-57	MTP-58	MTP-59	MTP-60
MTP-145	MTP-146	MTP-147	MTP-148	MTP-149	MTP-150	MTP-151	MTP-152	MTP-153	MTP-154	MTP-155	MTP-156
MTP-241	MTP-242	MTP-243	MTP-244	MTP-245	MTP-246	MTP-247	MTP-248	MTP-249	MTP-250	MTP-251	MTP-252
MTP-337	MTP-338	MTP-339	MTP-340	MTP-341	MTP-342	MTP-343	MTP-344	MTP-345	MTP-346	MTP-347	MTP-348
MTP-433	MTP-434	MTP-435	MTP-436	MTP-437	MTP-438	MTP-439	MTP-440	MTP-441	MTP-442	MTP-443	MTP-444
MTP-529	MTP-530	MTP-531	MTP-532	MTP-533	MTP-534	MTP-535	MTP-536	MTP-537	MTP-538	MTP-539	MTP-540
MTP-625	MTP-626	MTP-627	MTP-628	MTP-629	MTP-630	MTP-631	MTP-632	MTP-633	MTP-634	MTP-635	MTP-636
MTP-721	MTP-722	MTP-723	MTP-724	MTP-725	MTP-726	MTP-727	MTP-728	MTP-729	MTP-730	MTP-731	MTP-732
MTP-61	MTP-62	MTP-63	MTP-64	MTP-65	MTP-66	MTP-67	MTP-68	MTP-69	MTP-70	MTP-71	MTP-72
MTP-157	MTP-158	MTP-159	MTP-160	MTP-161	MTP-162	MTP-163	MTP-164	MTP-165	MTP-166	MTP-167	MTP-168
MTP-253	MTP-254	MTP-255	MTP-256	MTP-257	MTP-258	MTP-259	MTP-260	MTP-261	MTP-262	MTP-263	MTP-264
MTP-349	MTP-350	MTP-351	MTP-352	MTP-353	MTP-354	MTP-355	MTP-356	MTP-357	MTP-358	MTP-359	MTP-360
MTP-445	MTP-446	MTP-447	MTP-448	MTP-449	MTP-450	MTP-451	MTP-452	MTP-453	MTP-454	MTP-455	MTP-456
MTP-541	MTP-542	MTP-543	MTP-544	MTP-545	MTP-546	MTP-547	MTP-548	MTP-549	MTP-550	MTP-551	MTP-552
MTP-637	MTP-638	MTP-639	MTP-640	MTP-641	MTP-642	MTP-643	MTP-644	MTP-645	MTP-646	MTP-647	MTP-648
MTP-733	MTP-734	MTP-735	MTP-736	MTP-737	MTP-738	MTP-739	MTP-740	MTP-741	MTP-742	MTP-743	MTP-744
MTP-73	MTP-74	MTP-75	MTP-76	MTP-77	MTP-78	MTP-79	MTP-80	MTP-81	MTP-82	MTP-83	MTP-84
MTP-169	MTP-170	MTP-171	MTP-172	MTP-173	MTP-174	MTP-175	MTP-176	MTP-177	MTP-178	MTP-179	MTP-180
MTP-265	MTP-266	MTP-267	MTP-268	MTP-269	MTP-270	MTP-271	MTP-272	MTP-273	MTP-274	MTP-275	MTP-276
MTP-361	MTP-362	MTP-363	MTP-364	MTP-365	MTP-366	MTP-367	MTP-368	MTP-369	MTP-370	MTP-371	MTP-372
MTP-457	MTP-458	MTP-459	MTP-460	MTP-461	MTP-462	MTP-463	MTP-464	MTP-465	MTP-466	MTP-467	MTP-468
MTP-553	MTP-554	MTP-555	MTP-556	MTP-557	MTP-558	MTP-559	MTP-560	MTP-561	MTP-562	MTP-563	MTP-564
MTP-649	MTP-650	MTP-651	MTP-652	MTP-653	MTP-654	MTP-655	MTP-656	MTP-657	MTP-658	MTP-659	MTP-660
MTP-745	MTP-746	MTP-747	MTP-748	MTP-749	MTP-750	MTP-751	MTP-752	MTP-753	MTP-754	MTP-755	MTP-756
MTP-85	MTP-86	MTP-87	MTP-88	MTP-89	MTP-90	MTP-91	MTP-92	MTP-93	MTP-94	MTP-95	MTP-96
MTP-181	MTP-182	MTP-183	MTP-184	MTP-185	MTP-186	MTP-187	MTP-188	MTP-189	MTP-190	MTP-191	MTP-192
MTP-277	MTP-278	MTP-279	MTP-280	MTP-281	MTP-282	MTP-283	MTP-284	MTP-285	MTP-286	MTP-287	MTP-288
MTP-373	MTP-374	MTP-375	MTP-376	MTP-377	MTP-378	MTP-379	MTP-380	MTP-381	MTP-382	MTP-383	MTP-384
MTP-469	MTP-470	MTP-471	MTP-472	MTP-473	MTP-474	MTP-475	MTP-476	MTP-477	MTP-478	MTP-479	MTP-480
MTP-565	MTP-566	MTP-567	MTP-568	MTP-569	MTP-570	MTP-571	MTP-572	MTP-573	MTP-574	MTP-575	MTP-576
MTP-661	MTP-662	MTP-663	MTP-664	MTP-665	MTP-666	MTP-667	MTP-668	MTP-669	MTP-670	MTP-671	MTP-672
MTP-757	MTP-758	MTP-759	MTP-760	MTP-761	MTP-762	MTP-763	MTP-764	MTP-765	MTP-766	MTP-767	MTP-768

Publications

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Standardization of Protocol for Isolation of Genomic DNA from Mature Leaves of *Vigna radiata* (L.) Wilczek)

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ABSTRACT

Green gram is a widely cultivated pulse crop rich in protein, high in vitamin-B content and essential aminoacids. It is easily digestable and low flatulence produced crop. The quality and quantity of DNA used for amplification by PCR is the key to reproducible results and success of genotyping. Especially, DNA purity is extremely crucial for obtaining clear and discriminate patterns. DNA extraction from mature leaves of Green gram is difficult due to presence of contaminants such as phenols and polysaccharides. Therefore, the present study was under taken to obtain high quality and pure DNA in mature leaves of Green gram. With few modifications three different DNA extraction protocols were tried in the present study to obtain high quality and pure DNA *viz.*,(i) Doyle and Doyle (1987), (ii) Modified Method of Murray and Thompson (1980), and (iii) using of spin column filter. Out of the three methods tried for DNA extraction, the method of Murray and Thompson (1980) was found most efficient, as the DNA obtained through this protocol was relatively pure which gave amplifying products in the PCR. The genotype used for the standardization was Co (Gg) 7.

Key words *DNA, CTAB, Vigna radiata, Spin column filter, Co(Gg)7*

Pulses are major constituents of human diet and are mainly grown in marginal lands under rainfed conditions. Among the pulses, blackgram and mungbean are the major pulses contributing much for the total production. Mungbean [*Vigna radiata* (L.) Wilczek] (2n=22), primarily an inbreeding species is cultivated throughout tropical Asia including India which accounts for 45% of the world production. *Vigna* species are an important source of protein for people, particularly in tropical Africa and Asia and several *Vigna* species have been domesticated in Asia. Species in Leguminosae (Fabaceae) genus *Vigna* subgenus *Ceratotropis* are called the Asian *Vigna* because of their natural distribution.

The quality and quantity of DNA used for amplification by PCR is the key to reproducible results and success of genotyping. Especially, DNA purity is extremely crucial for obtaining clear and discriminate patterns. Genus *Vigna* have high amount of polyphenol, orthohydroxyphenols and polysaccharides. These are powerful oxidizing agents to interfere with genomic DNA. Some varieties are recalcitrant to inhibit the PCR amplification.DNA isolation protocols for *Cicer* have been reported by Chakraborti *et al*., 2006 and from nodules of legumes have been reported by Krasova-Wade, 2007. Presence of phenols and other contaminants offer difficulty in pipetting DNA and make DNA unamplifiable in PCR reaction by inhibiting *Taq* DNA polymerase. Therefore, the present study was taken to obtain high quality and pure DNA in mature leaves of green gram.

MATERIALS AND METHODS

Chemicals used: (as per the protocol)

1) Extraction buffer, which is made by mixing of 0.7M Nacl, 50 mM Tris cl, 10mM EDTA, 2% CTAB, 0.2% â-mercapto-ethanol, 2% Poly Vinyl Pyrolidine and required amount of distilled water.
2) Tris EDTA (TE) buffer : 10 mM Tris Cl, 1 mM EDTA
3) 95% cold ethanol, 70% Ethanol (70ml ethanol+30ml Milliq water) and absolute ethanol.
4) Ice cold Isopropanol.
5) C.I: Chloroform: Iso amyl alcohol, 24: 1v/v).
6) C.O: (Chloroform: Octanol, 24: 1).

Table 1. Values of DNA concentration extracted through three different methods (assessed by measuring OD@ 280nm with multimode reader by employing the software *i-control* of TECAN)

SAMPLES	EXTRACTION METHODS					
	CTAB		Murray and Thompson (1980) with few modifications		Binding buffer loaded with silica using spin column filters	
	Quantity of DNA (ng/µl)	Absorbance $_{260/280\ nm}$	Quantity of DNA (ng/µl)	Absorbance $_{260/280\ nm}$	Quantity of DNA (ng/µl)	Absorbance $_{260/280\ nm}$
1	162.2	1.35±0.10	386.5	1.46±0.10	526.3	1.85±0.021
2	225.8	1.42±0.12	286.9	1.56±0.12	412.5	1.90±0.026
3	186.5	1.46±0.08	320.7	1.61±0.08	471.2	1.92±0.024
4	282.2	1.48±0.07	369.8	1.53±0.07	425.1	1.89±0.07192
5	120.7	1.39±0.11	211.2	1.54±0.11	532.5	1.91±0.074
AVERAGE	195.48	1.42±0.096	315.02	1.54±0.096	473.52	1.894±0.043

7) 10mM ammonium acetate
8) RNase A (10 mg/ml).
9) Silica Powder (Celite 545 silica)
10) 2M Guanidine Hydrochloride
11) 5M NaCl

Preparation of Silica Powder-DNA Binding Solution

- Fill silica powder (Celite 545 silica) into 15 mL Falcon-tube (approx. 100mg)
- Add 3 mL dH2O
- Shake vigorously (vortex and invert)
- Let slurry settle for approx. 15 min
- Remove (pipette off) the liquid
- Repeat 2 times (a total of 3 washes)
- After last washing step: resuspend the silica powder in about the same amout of water (up to about 5 mL)
- Add 8ml of DNA binding buffer (2M Guanidine Hydrochloride dissolved in absolute ethanol)
- Add 2 ml of TEbuffer (PH-8.00)
- Suspend the silica powder in DNA binding buffer

Preparation of Wash Buffer:

- 1mL of 5M NaCl was added to 99mL of 95% EtOH

Three protocols (enlisted below) were followed for standardizing of DNA isolation from mature leaves of mungbean. For this leaves of about 250mg were used. Of the three protocols used, the DNA extraction method described by Murray and Thompson, 1980 could give good quality DNA enable to PCR analysis. The genotype used for standardization was Co (Gg)-7. The details of three protocols tried in the present study are given below:

1. CTAB method of DNA extraction by (Doyle and Doyle, 1987)
2. DNA extraction by Murray and Thompson, 1980 with few modifications
3. Use of SPIN Column filters.

CTAB method of DNA extraction (Doyle and Doyle, 1987)

Mature leaf samples were collected directly in eppendorf tubes were ground into fine powder with the help of micro pestle by freezing in liquid Nitrogen which facilitates easy grinding**.** To the powder 400 ìl CTAB extraction buffer with 0.2% â- mercaptoethanol was added and the sample tubes were kept in water bath at 60 ^{0}C for 30-60 min with occasional and proper mixing after every 5-10 minutes, such that to see not to form any clumps at the bottom. Tubes were removed from water bath and then contents were allowed to cool to room temperature. Equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added and mixed thoroughly by gentle inversion for 15 minutes until clear separation of three layers is attained. After that contents were centrifuged at 12000 rpm for 12 minutes at 24^{0}C temperature. The clear aqueous phase (supernatant) was carefully pipetted out into

new tubes. The chloroform: isoamyl alcohol (24:1 v/v) step was repeated twice to remove the organic contaminants in the supernatant. To the supernatant, cold isopropanol of about 0.5 to 0.6 volume (2/3rd of pipette volume) was added and kept it for overnight. Then the contents were mixed gently by inversion. Subsequently the tubes were centrifuged at 12000 rpm for 12 min at 24^0C temperature to pellet out DNA. The supernatant was discarded gently and the DNA pellet was washed with 70% ethanol and centrifuged at 10,000 rpm for 4-5 min. This step was repeated twice. The tubes were allowed for air drying until the pellet gets completely dried and then the pellet was dissolved in 40 ìl TE buffer. DNA was stored in -20^0C for further use. Results of CTAB method of DNA extraction by (Doyle and Doyle 1987) were furnished in (fig.1).

DNA extraction method of Murray and Thompson, 1980 with few modifications

Mature leaves were ground to fine powder in liquid nitrogen using a pestle and mortar. To the powder 500 ìl of extraction buffer was added. Suspended the clumps with a spatula then transferred into eppendorf tubes and incubated for 1 hr with frequent mixing in a water bath maintained at 65°C. 500 ìl of chloroform: octonal (24:1) was added and mixed gently for 10 min. The tubes were centrifuged at 13000 rpm for 15 min and the supernatant was collected into new eppendorf tubes. This step was repeated twice.DNA was precipitated by adding 2/3rd volume of ice-cold Isopropanol followed by gentle mixing and incubated for overnight at -20°C followed by centrifugation at 13000 rpm for 15 min at 4°C. The resultant supernatant was discarded and DNA pellet was washed with 1 ml of 70% ethanol then allowed for air drying and was dissolved in 100ìl of TE buffer. Results of DNA extraction method of Murray and Thompson (1980) with few modifications furnished in (fig 2).

DNA extraction method using spin column filters:

Mature leaves were ground to fine powder in liquid nitrogen using a pestle and mortar. To the powder 500 ìl of extraction buffer was added. Suspended the clumps with a spatula then transferred into eppendorf tubes and incubated for 1 hr with frequent mixing in a water bath maintained at 65°C. Equal volume of Silica based binding buffer (Preparation given above) was added to each centrifuge tube mixed well then transferred to spin column filter. Centrifuge the spin column filter at 11000rpm @2 min and then discard the substance collected in collection tube. Add 500µl of Washing buffer (Preparation given above) to each filter then centrifuge for 11000rpm@ 2 min. Repeat the washing procedure for second time also. Dry spin was allowed to remove the excess ethanol in the filtrate. Eluted the DNA with sterile water by adding 100 µl to each tube by centrifuge at 11000rpm for 2 min

RESULTS AND DISCUSSION

Proper choice of the leaf tissue is very important for DNA extraction (Lodhi *et al.*, 1994). In this research, leaf tissue harvested from five - week-old plants was used for DNA extraction because for research programme like TILLING we should take leaves after pod setting. Generally, mature plant tissues contain high concentrations of polysaccharides, polyphenols, and other secondary metabolites (Dabo *et al.*, 1993; Zhang *et al.*, 2000). Three different DNA extraction protocols were tried with few modifications (composition and components of extraction buffer) in order to obtain high quality and pure DNA.

The highest DNA yield was obtained by using the spin column filter followed by the method of Murray and Thompson, 1980 with few modifications and then Doyle and Doyle, 1987 method. The DNA concentration extracted through three different methods was assessed by measuring OD@ 280nm with multimode reader by employing the software *i-control* of TECAN multimode reader and their respective yield were listed in Table 1.

The integrity, i.e. presence of high molecular genomic DNA, was determined by electrophoresis on a 0.8% agarose gel. High molecular DNA bands were obtained from spin column filter method (Fig 3) indicating that the DNA was pure and intact. Out of the three methods tried DNA extraction, the spin column filter method was found most efficient, as the DNA obtained through this protocol was relatively pure which gave amplifying products in the PCR.

After evaluating the yield, purity and functionality among the three protocols, the spin column filter method was considered an ideal protocol to isolate DNA from mature leaves of *Vigna radiata* .The quantity and the quality of the DNA extracted by this method were high enough to perform hundreds of PCR-based reactions. In addition, it had the added advantage of not requiring any phenol.

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Generation of TILLING resources in Mungbean (*Vigna radiata* (L.) Wilczek.)

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Abstract:

Mungbean (*Vigna radiata* (L.) Wilczek) is one of the food legume in worldwide, making it an important target for novel approaches of genetic analysis. This study evaluated the use of ethyl methane sulfonate (EMS) for the generation of a mutant population for targeted induced local lesions in genomes (TILLING) in Mungbean. TILLING is a powerful reverse genetics approach that uses a large mutant population for identification of mutants in loci of interest. Based on kill curve analysis 70 mM EMS was found to be an appropriate concentration for the generation of a mutant population in Mungbean variety CO7. Based on TILLING results from other species, a population of 3744 lines is estimated to be sufficient for saturation of the Mungbean genome.

Key words: ***Vigna radiata*, Ethyl Methane Sulphonate, TILLING, Mutagenesis, Co 7.**

Introduction:

Mungbean is diploid in nature with 2n=2x=22and a small genome size estimated to be 0.60 pg/1C (579 Mbp), which are similar to those of the other Vigna species (Somta & Srinives, 2007).Its small genome, with a low incidence of duplications, makes it suitable for sequencing and genomic applications and as a reference for the elucidation of genomes of more complex legume species such as soybean (Gepts et al., 2005).Recent advances in large-scale genome sequencing projects have opened up new possibilities for the application of conventional mutation techniques in not only forward but also reverse genetics strategies. TILLING (Targeting Induced Local Lesions IN Genomes) was developed a decade ago as an alternative to

insertional mutagenesis. It takes advantage of classical mutagenesis, sequence availability and highthroughput screening for nucleotide polymorphisms in a targeted sequence. As the gene and genomic sequences from the model legumes is becoming available in the public data bases, it is high time for the legume scientists and breeders to cash the benefits of these resources by applying them to their routine research and breeding programmes. Among the methodologies available currently to extend the benefits of genomics from models up to the level of functional genomics in crops TILLING seems to be the most viable option especially for grain legumes, as it does not require the transgenic procedures to create functional knockouts. In the present scenario of dwindling production and productivity of grain legumes in the country, it is very essential to use the functional genomic tools like TILLING to create targeted mutations and perform functional analysis of the putative genes involved in the morphogenesis, flowering and meiotic control. TILLING has been shown to be effective in legumes such as soybean (Cooper et al., 2008), Lotus japonicus (Perry et al., 2003), and Medicago trucatula (Javotet al., 2007).

Materials and Methods:

Plant material:

Seeds of the four mungbean varieties (Co7, Co6, VRM (Gg) 1 and VBN (Gg) 3) were presoaked for 6 hours in water. Then the seeds were immersed for six hours in phosphate buffer along with the requisite concentration (0.00, 0.10, 0.20, 0.30, 0.40, 0.50, 0.60, 0.70, 0.80, 0.90 and 0.100Ml/l) of EMS and with intermittent shaking. Immediately after the completion of treatment duration, the treated seeds were thoroughly washed with 1 % sodium thio-sulphate then washed in running tap water for half an hour to eliminate the residual effect of the chemical and the excess moisture in the seed coat was removed by using folds of blotting paper. Each EMS treatment was composed of three replicates planted in a randomized complete block design with the experimental unit being a seedling tray containing 60 seeds. Germination data were collected 7 and 15 d after planting. A kill curve analysis for fixing a variety/EMS dose combination that will lead to the production of a TILLING population with optimal density of point mutations (G>A transitions).

Mutant Population Development:

12,000 seeds of Co (Gg) 7 variety were treated with EMS dose of 70mM and raised during summer 2012 (D.S : 29/02/2012) with spacing of 30x10cm . Out of which 3744 M_1 plants survived till maturity were individually harvested for raising M_2 population. Lethality, injury and other morphological variations recorded in M_1 generation (Fig 1a-p). Among the 3744 M1 plants harvested, seeds of 1872 M_1 plants were separated as Set I and was sown during Kharif 2012 (D.S: 13.08.2012) on a plant to progeny basis (Fig 2). Individual plants were harvested on single plant basis and seeds of about 1746 families were banked in PGR Gene Bank, TNAU, Coimbatore. Leaves of individual plants were collected and DNA were extracted and stored for further molecular analysis. Another set of 1872 M_2 seeds were separated as Set II and was sown during Rabi 2012 (D.S. : 13.12.2012) on a plant to progeny basis. Individual plants were harvested on single plant basis and seeds of about 1182 families were banked in the Gene Bank of PGR TNAU, Coimbatore. So far we have generated 2928 MTP (Mungbean TILLING Population) in 2 batches as detailed below (Table 1).

Phenotypic evaluation of mutant population:

It was interesting to observe novel robust branching and early flowering mutants in the M_2 generation (Fig 3). Eventhough this study aims in identifying allelic variants in genes coding for plant type traits using the reverse genetic approach, but the frequency of ideal plant types and variation in flowering time that we observed in the M_2 families was very encouraging or the discovery of many allelic series in Candidate Genes governing plant form and flowering duration in mungbean. Phenotypic variability in M_2 population was presented in Fig 4a-d.

Results and Discussion:

Effect of EMS concentration on plant survival:

EMS concentrations on the germination of four varieties of mungbean (Co7, Co6, VRM (Gg) 1 and VBN (Gg) 3) seeds indicated that high concentrations of EMS resulted in a dramatic reduction in germination. Evaluation of the plants 15 d after germination was found to more accurately reflect actual germination after EMS treatment. Significant differences in germination at 15 d were found between no treatment and EMS concentrations of 70 mM or higher EMS-treated seedlings that survived after germination showed less than 30% survival (Table 2). The

varieties used, EMS doses tested and the data on survival is furnished in Table 2 and Fig 5. The variety which shows relatively higher tolerance (high survival %) to EMS doses is suitable for creating TILLING populations as these varieties can harbour more mutations with in a manageable population size than the varieties that cannot survive high EMS doses.

The results of kill curve analysis shows that the variety Co (Gg) 7 shows relatively better tolerance to EMS treatment as compared to the other three varieties Viz., Co(Gg)6, VRM (Gg) 1 and VBN (Gg) 3. Among the doses the concentration of 70mM was found to be the optimal EMS dosage for achieving 30% kill for the variety Co (Gg) 7. Hence combination of Co (Gg) 7 with 70mM EMS were fixed for generating Mungbean TILLING Populations (MTP).

Mutant population development:

Effective TILLING requires near saturation of the haploid complement of the genome in a large mutant population. Mutation frequency and tolerance to mutation load varies significantly from species to species (Table 3) and may be influenced by ploidy (Comai and Henikoff, 2006; Henikoff and Comai, 2003; Stadler, 1932). Based on the average mutation frequency of 2.6 or 2.7 mutations/ Mb, _4.6 to 5.0 million mutations were generated in the L. japonicus or M. truncatula TILLING populations, respectively. With the goal of generating greater than 5 million mutations in mungbean and assuming a mutation frequency of 2 to 3 mutations/Mb, like with L. japonicus and M.truncatula, a population of 5600 lines would generate between 5.9 and 8.9 million mutations. This number of total mutations falls between that of diploid genomes such as A. thaliana, L. japonicus, and M. truncatula and the more complex, polyploidy cereal genomes (Table 3). The cereal genomes such as barley, maize (Zea mays), and wheat (Triticum aestivum) have a higher content of repetitive DNA, more duplication, and larger, polyploidy genomes, thus requiring higher mutation density.

DNA has been extracted and stored for TILLING from over 2928 M_2 families. Phenotypic screening of 3744 M_2 plants has shown stable mutations have been observed for leaflet modification, tall, indeterminate, and dwarfing (fig-6) and are generally inherited as recessive mutations. Late-maturing and twiny (fig 4) growth mutants have also been observed but at a lower frequency.

Based on kill curve analysis for survival, 70 mM EMS is an appropriate concentration for the generation of a mutant population suitable for TILLING in variety Co7. Using this

concentration, a maximum number of mutations can be generated with a minimum population size and with relatively high efficiency. Higher concentrations of EMS resulted in overall survival rates of less than 10%, which are considered inadequate for efficient mutagenesis.

In comparison with mutation frequencies and effective population sizes in other species, a population of 3744 mutant lines is expected to be adequate for genome saturation and effective TILLING of Vigna radiata variety Co 7. This estimate is based on the predicted mutation frequency of two to three mutations/Mb per haploid complement for such diploid plants as A. thaliana, L. japonicus, and M. truncatula as well as that of the more complex, polyploid cereal genomes. Using concentration of 70 mM EMS, over 2928 M_2 families have been developed that show morphological and phenological mutations at reasonable frequencies. The actual mutation frequency for Co 7 will be calculated based on TILLING experiments at specific loci with natural rates of polymorphism in mungbean. Functional redundancy in specific species such as A. thaliana could confer greater tolerance to high mutation load as has been found with yeast (Saccharomyces cerevisia) (Gu et al., 2003). If mungbean is similar to other small genome legumes such as M. truncatula and L. japonicus with relatively low functional redundancy, it is expected to be less tolerant to high mutation load. If identified, beneficial mutations from the population could be directly incorporated into breeding programs using the same molecular tools such as DNA sequence and polymerase chain reaction primers developed as part of the TILLING protocol. Furthermore, because EMS-generated mutant lines are not transgenic, novel lines with agronomically beneficial traits could be incorporated into breeding programs without additional hurdles. Once the Co 7mutagenesis population developed from this research is completed and the TILLING protocol is optimized for Mungbean, the population will be used for genetic analysis and will be made available to the research community for the evaluation of specific loci and traits of interest.

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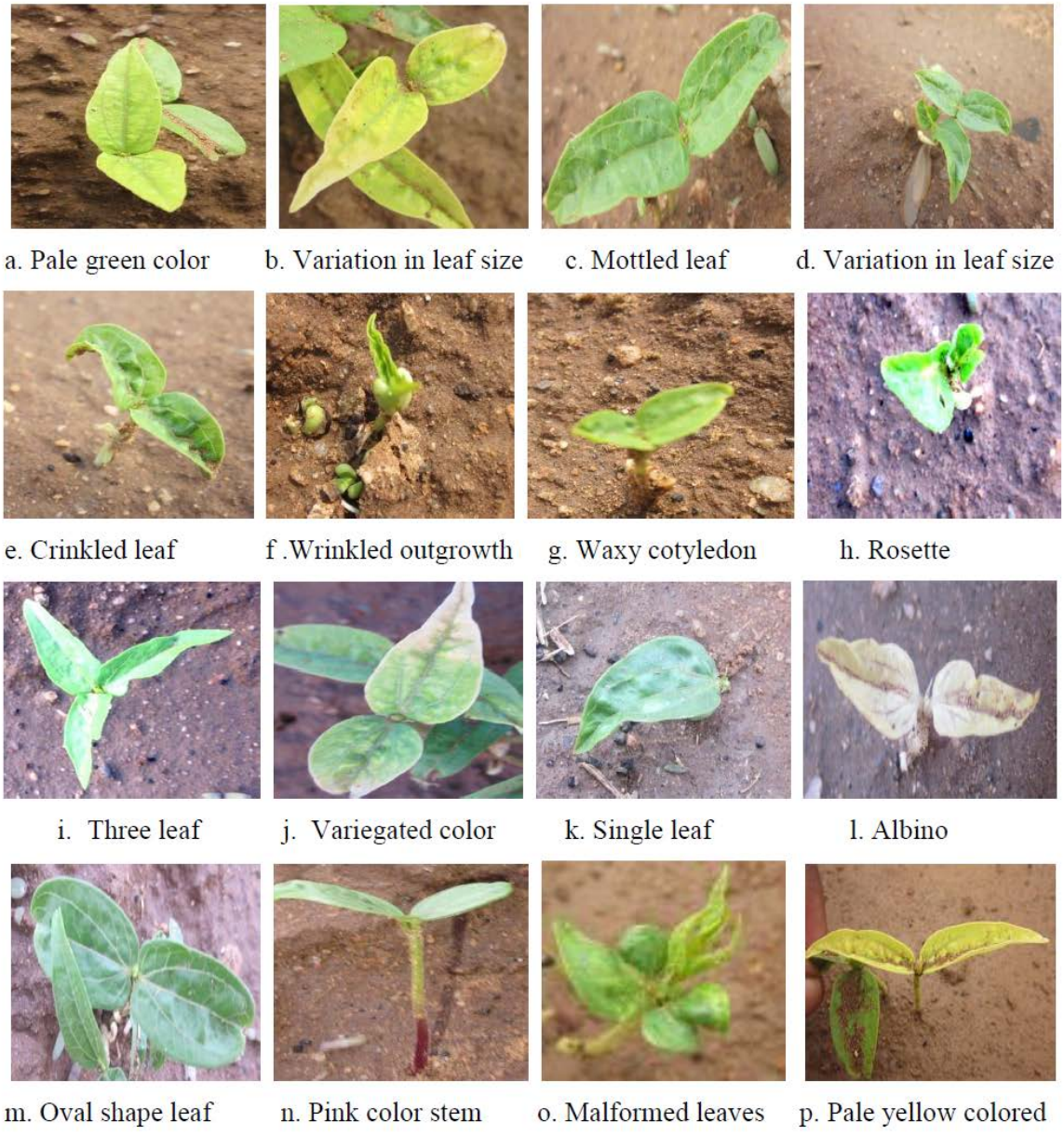

Fig 1.Lethality, Injury and Morphological variations in M_1 Generation

Fig 2. Field View of M_2 generation

Fig 3. Robust mutant families identified in M_2 generation

a. Variations in shoot and root length

b. Mutants with variable Branching patterns

c. Mutants with Twining Habit

d- Mutants with Terminal flowering and pod setting

Fig 4.(a-d): Phenotypic variability in M_2 population

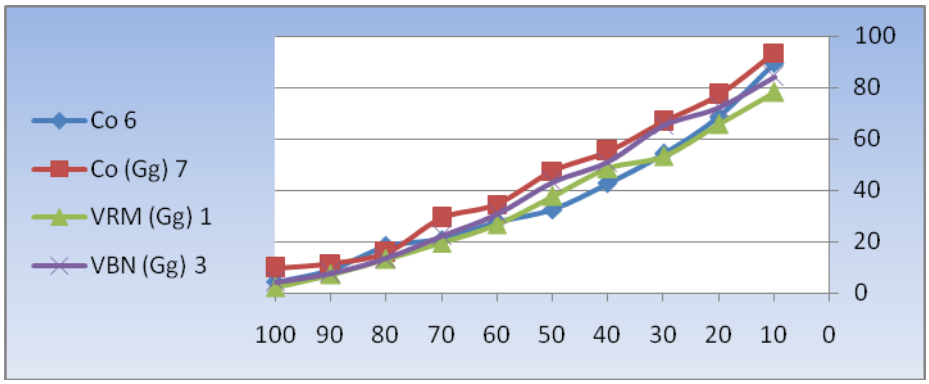

Fig 5. Graphical representation of results of kill curve analysis

a. Narrow **b. Round** **c. Triangular** **d. Trilobate**

e. Tall **f. Intermediate** **g. dwarf**

Fig 6. Variation in Leaf shape (a-d) and variation in plant height (e-g)

Table 1. Details on generation of Mungbean TILLING Population

No of seeds treated in M_1	No. of M_1 plant harvested	Batch No	No. of M_2 families grown	No of M_2 plants tagged for TILLING
12000	3744	1	1872	1746
		2	1872	1182
			Total	**2928**

Table 2: Results of kill curve analysis. Data on survival (%) observed after 15 days of treatment as comapred to untreated control

	EMS doses in mM									
Varieties	10	20	30	40	50	60	70	80	90	100
Co 6	89.4	68.5	54.1	42.7	32.5	27.6	21.0	18.2	8.9	4.1
Co (Gg) 7	93.4	77.1	66.8	55.2	47.4	34.5	29.2	15.6	11.4	9.8
VRM (Gg) 1	78.3	65.7	53.3	48.5	37.7	26.7	19.4	13.3	7.3	2.1
VBN (Gg) 3	84.1	72.3	65.3	51.0	43.1	30.6	22.1	13.4	7.8	4.2

Table 3. Estimation of chemically induced(EMS) point mutations in different plant mutant populations based on published mutation frequencies.

Species	Common name	Ploidy	*Mutation frequency (no/megabase)	#Genome size (megabase)	Estimated mutation per haploid genome (no.)	Mutatant population size (no.)	Estimated mutataion in population (million)	Reference
Hordeum vulgare	Barley	2	1.0	5439	5439	4600	25.0	Caldwell et al., 2004
Sorghum bicolor	Sorghum	2	2.0	750	1500	1600	2.4	Xin et al., 2008
Zea mays	Maize	2	2.0	2671	5342	10000(goal)	53.0	Wu et al., 2005
Oryza sativa	Rice	2	2-3	490	980-1470	6000(goal)	5.9-8.8	Till et al., 2007; Wu et al., 2005
Lotus japonicas	Miyakogusa	2	2.6	466	1212	3800	4.6	Udvardi et al., 2005
Medicago truncatula	Strong-spined medick	2	2.7	466	1236	4032	5.0	Javot et al., 2007
Arabidopsis thaliana	Mouse-ear cress	2	5.9	157	926	3000	2.8	Greene et al., 2003
Triticum turgidum	Durum wheat	4	25.0	12030	300750	768	231.0	Slade et al., 2005
Triticum aestivum	Bread wheat	6	40.0	16979	679160	1152	782.4	Slade et al., 2005

*Mutation frequency per megabase calculated based on mutation frequencies in referenced publications

#Plant genome size information from data presented by Bennett and Bennett and Leitch (2005)