MAPPING AND CLONING OF OLIGO CULM *Osoc* AND SEEDLING LETHAL *Ossl* INSERTIONAL MUTANTS IN BASMATI

A THESIS

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By

P. OSMAN BASHA

DEPARTMENT OF BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE-247 667 (INDIA)

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INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **MAPPING AND CLONING OF OLIGO CULM *Osoc* AND SEEDLING LETHAL *Ossl* INSERTIONAL MUTANTS IN BASMATI** in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during the period from January 2004 to April 2008 under the supervision of Dr. H. S. Dhaliwal, Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee, India.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

P. Osman Basha

(P. OSMAN BASHA)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

H.S. Dhaliwal

(H. S. Dhaliwal)
Supervisor

Date: 21/04/08

The PhD Viva-Voce Examination of **Mr, P. Osman Basha** Research Scholar has been held on .....

Signature of Supervisor

Signature of External Examiner

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ABBREVIATIONS USED

T-DNA	Transfer Deoxyribonucleic Acid
Osoc	*Oryza sativa* Oligo culm
Ossl	*Oryza sativa* Seedling lethal
Ossg	*Oryza sativa* Small grain
T-RB	T-DNA Right Border
T-LB	T-DNA Left Border
hpt	Hygromycin Phosphotransferase
T-RB	T-DNA Right Border
T-LB	T-DNA Left Border
AP	Adaptor Primer
AD	Arbitrary Degenerate Primer
SPT	Streptomycin Phosphotransferase
NPT	Neomycin Phosphotransferase
F_1	First Filial Generation
F_2	Second Filial Generation
F_3	Third Filial Generation
PCR	Polymerase Chain Reaction
TAIL-PCR	Thermal Asymmetric Interlaced Polymerase Chain Reaction
iPCR	Inverse Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase –Polymerase Chain Reaction
Ds	Dissociation Element
RAP-DB	The Rice Annotation Project Database
KEGG	Kyoto Encyclopedia of Genes and Genomics
IRFGC	International Rice Functional Genomics Consortium
IRGSP	International Rice Genome Sequencing Project, 2005
SSR	Simple Sequence Repeats
BSA	Bulk Segregant Analysis
FST	Flanking Sequence Tags

NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment and Search Tool
EDTA	Ethylene Diamine Tetraacetic Acid
TBE	Tris Borate
TAE	Tris Acetate
TE	Tris EDTA
DEPC	Diethyl Pyrocarbonate
SAM	Shoot Apical Meristem
AM	Axillary Meristem
FM	Floral Meristem
CTAB	Cetyl Trimethyl Ammonium Bromide
PAGE	Poly-acrylamide Gel Electrophoresis
mRNA	Messenger Ribonucleic acid
cDNA	Complimentary DNA
DNase	Deoxyribonuclease
RNase	Ribonuclease
ppm	Parts Per Million
T_m	Melting Temperature
bp	Base Pair
cM	Centimorgan
%	Percentage
χ^2	Chi Square
df	Degree of Freedom
µg	Microgram
µm	Micrometer
mm	millimetre
Fig.	Figure
mg	Milligram
ng	Nanogram
ml	Millilitre
mM	millimole

°C	Degree Centigrade
U	Units
v/v	Volume/Volume
w/v	Weight/Volume
sec	Second
h	Hour
min	Minute
g	Gram
l	Litre
M	Molarity
μ	micron
α	Alpha
β	Beta

ABSTRACT

Insertional mutagenesis is one of the most powerful tools for annotating gene functions. Basmati 370 insertional mutants, Oligo culm (*Osoc*), Seedling lethal (*Ossl*) and Small grain (*Ossg*) mutants were isolated using *Agrobacterium tumefaciens* mediated transformation with binary plasmid containing Hm^R*Ds*. The Oligo culm (*Osoc*) mutant had 60-70% reduced tillering capacity and retarded growth of seminal roots. The Seedling lethal (*Ossl*) mutant showed 5-15% of albino plants which died soon after germination while among non-albino 10-20% died slowly without tillering after transplantation. The Small grain (*Ossg*) mutant had 10-15% reduced weight and length of grains. Southern hybridization of Oligo culm and Seedling lethal mutants indicated single T-DNA insertions.

The Oligo culm mutant (*Osoc*) was crossed with *indica* fine rice cultivar PR106 to develop F_2 mapping populations. Histogram for plant height and days to flowering phenotypic data showed nearly normal distribution while for tiller number it showed skewed distribution towards Oligo culm mutant. The observed χ^2 value for segregation of hygromycin resistance and *hpt* amplification in F_2 population gave a good fit to 3:1 segregation ratio indicating a single T-DNA insertion. The linkage between T-DNA insertion and Oligo culm mutant phenotype was confirmed in F_2 population of *Osoc*/PR106. Bulk Segregant Analysis (BSA) was performed to identify chromosomal location of *Osoc*. Uniformly distributed rice SSR markers on twelve chromosomes were selected. Out of 209 SSR markers used, 98 were found to be polymorphic between Basmati 370 and PR106. Polymorphic SSR markers RM 279 (6.37cM), RM236 (2.45cM) and RM12413 (1.47cM) mapped on rice chromosome 2 were found linked to

Oligo culm (*Osoc*) mutant and the T-DNA insertion was flanked by RM236 and RM12413 markers.

The T-DNA flanking sequences of Oligo culm (*Osoc*), Seedling lethal (*Ossl*) and Small grain (*Ossg*) mutants identified using Genome walking and TAIL-PCR, showed their T-DNA insertions on chromosome 2, 11 and 11, respectively. The positions of insertions were confirmed by designing T-DNA and rice genome specific primers on chromosome 2 and 11 for Oligo culm (*Osoc*) and Seedling lethal (*Ossl*), respectively.

In Oligo culm mutant the insertion was present within the exonic region of the putative zinc-binding protein. The function of this protein has been predicted to be like that of a transcription elongation factor (Elf1) in *Oryza sativa*. The gene starts at base 1819090 and stops at base 1816334 (complementary sequence) of chromosome number 2 with UTR regions, 4 exons and 4 introns. The length of the gene is 2,757bp with 875bp mRNA. One paralog was identified on rice chromosome 7 and the alignment results of paralogs using CLUSTALW program, showed high conservation between them. Orthologs of Elf1 were found throughout the living kingdom. This family of short proteins contains a putative zinc binding domain with four conserved cysteines.

Oligo culm (*Osoc*) gene transcription analysis studies were performed using RT-PCR. Total RNA was extracted from roots and shoots of Basmati 370 and Oligo culm after six and twelve days of germination and the cDNA was synthesized. The results of amplification using Oligo culm gene specific and actin1 (reference gene) primers confirmed that the *Osoc* was a knockout mutant phenotype. Oligo culm (*Osoc*) EST database suggested that the gene was expressing more in roots followed by callus, panicle

and stem. Five FSTs were found for *Osoc* gene but no phenotypic descriptions are available for these FSTs.

In the Seedling lethal (*Ossl*) mutant the T-DNA is inserted on chromosome number 11 where no gene has been predicted in *japonica* and *indica* rice. Basmati 370 might have an important gene at the insertion site, knocking out of which probably gave the seedling lethal phenotype.

In Small grain (*Ossg*) mutant the T-DNA was inserted in the intronic region of the Pectin methyl esterase gene also on chromosome 11. The total length of the gene was 2,185bp with five exons and two introns. The length of mRNA was 1749bp which coded for protein with 423 amino acid residues. The gene Pectin methyl esterase contains a putative pectinesterase domain. The EST database suggests that the Small grain (*Ossg*) gene is expressed in panicles and flowers.

The Oligo culm (*Osoc*) gene of Basmati 370 has been cloned using series of PCR primers based on Nipponbare sequence. Alignment results of Basmati 370 and japonica *Osoc* gene showed 23 SNPs at nucleotide level and 3 amino acid differences at protein level which coded for a protein with the predicted function of transcription elongation factor.

The techniques Bulk Segregation Analysis, Genome Walking, TAIL-PCR and RT-PCR techniques and bioinformatics tools led to the cloning of Oligo culm (*Osoc*) gene controlling tillering in rice. Detailed morphological and molecular analyses of Seedling lethal (*Ossl*) and characterization of Small grain (*Ossg*) mutants has been done. The cloned and identified genes with respective morphology can be validated by genetic complementation or RNAi approaches.

CHAPTER 1

INTRODUCTION

Among cereals, rice is the most important staple food for half of the world's population. Genus *Oryza* to which rice belongs includes 20 wild species and 2 cultivated species. The two cultivated species are *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice). *Oryza sativa* is now commercially grown in 114 countries covering all the continents. Half of the total world rice production is from India and China (www.irri.org/science/rice/stat). Rice provides 60% of food intake in South East Asia.

Basmati 370 is considered as the "crown jewel" among rice cultivars because of its extra long grains, fine texture, non-sticky nature, pleasant and distinct aroma. Basmati rice has a typical flavor caused by the compound 2-acetyl-1-pyrroline (Buttery *et al.*, 1983). The word Basmati originated form the Hindi word, '*bas*' meaning smell and '*mati*' meaning full of aroma. Cultivation of authentic traditional Basmati rice is restricted to regions along the Indus River in Pakistan and the Indo-Gangetic plains of Indian sub-continent. Consumers prefer traditional Basmati rice to the evolved Basmati, because the evolved rice falls short of quality traits of traditional Basmati. India is the largest grower and exporter of Basmati rice.

The size of the rice genome is 6 and 40 times smaller than that of maize and wheat genomes, respectively, thus making rice an excellent model plant for cereal genomics. In 2002, the draft genome sequences of two major rice subspecies *japonica* cultivar (Goff *et al.*, 2002) and *indica* cultivar (Yu *et al.*,2002) were published and made available in public database. Finally in 2005, the International Rice Genome Sequencing Project (IRGSP) finished the fine quality sequencing of 95% (372.1 Mb) out of 389 Mb by map-based sequence of the rice genome (IRGSP 2005, Jung *et al.*, 2007). The total number of transcripts or gene models was estimated to be 66,710. Out of these, 15,232 transposable element related gene models were removed. Out of

the remainig 41,478 non-transposable element related gene models, 33,882 gene models have been empirically validated through ESTs and full length cDNA sequences (Ohyanagi *et al.*, 2006; Ouyang *et al.*, 2007; Jung *et al.*, 2007).

The completion of the rice genome sequence provided a new platform for its functional genomics. Assigning functions to these genes will help greatly to improve both quality and quantity of rice. For assigning functions to the predicted genes various approaches such as insertional mutagenesis, microarray, TILLING and RNAi etc. have been used. Out of these, insertional mutagenesis is the most powerful, easiest and cost effective approach for analysing gene function on a large scale. Insertional mutagenesis, not only affects the gene function but also tags the gene, facilitating its isolation and characterization (Azpiroz-Leehan and Feldmann, 1997). Transposons and T-DNA have been used most widely as insertional mutagens (Suyoung *et al.*, 2003; Greco *et al.*, 2003). The T-DNA insertions in genome are random events and the inserted sequences are stable through subsequent generations.

Bulked segregant analysis (BSA) is a technique used for the identification of linked markers to any specific gene or genomic region as against analyzing the entire genome. Michelmore *et al.* (1991) developed BSA with RFLP or RAPD markers and now other markers are also being used for identifying linked markers of desired traits such as SSRs and AFLP (Ek *et al.*, 2005; Altinkut *et al.*, 2003). Microsatellite markers are distributed relatively uniformly all over the genome and detect a high level of allelic diversity in cultivated varieties and distantly related species of rice (reviewed by McCouch *et al.*, 1997). High density map with 2400 SSRs is available for rice (McCouch *et al.*, 2002).

Number of productive tillers per plant is an important yield component in rice and other cereals because tillers are the grain bearing branches. Tiller number is

controlled by QTLs. Branches are developed from vegetative axillary meristems. The axillary meristems are generated in the axils of the leaves and develop into branches. Therefore, the pattern of axillary meristem development is a key factor for determining plant architecture. Axillary meristem development involves two steps, axillary meristem formation and subsequent out growth. Mutants defective in axillary meristem have been characterized in various plants including rice, *Arabidopsis*, tomato, pea and maize. Mutants defective in axillary meristem can be broadly classified into two types, (i) mutants with single or reduced tiller number and (ii) high tiller number. The rice mutant *Moc1* typically produces a monoculm only (Li *et al.*, 2003b). Sequence analysis indicated that *Moc1* is an ortholog of tomato lateral suppressor (*Ls*) gene and *Arabidopsis* LATERAL SUPPRESSOR (*LAS*) gene (Schumacher *et al.*, 1999; Greb *et al.*, 2003). Another type of mutant teosinte branched1 (*tb*1) produces excessive branches in maize (Doebley *et al.*, 1995) and its ortholog *Ostb*1 (Takeda *et al.*, 2003) is present in rice. Tillering is a complicated process in which several genes must be finely regulated. The genetic regulation of tillering pattern is poorly understood. Plant growth and development require a high level of coordination between cell division and cell differentiation. In plants, the majority of the cell cycle regulators have been identified, but very little is known about their function and regulation during plant growth and development. Novel *Arabidopsis* mutant, cytokinesis defective 1(*cyd 1*) was identified by having partial or missing cell wall in stomata (Yang *et al.*, 1999). A *paa1* and *paa2* genes were involved in copper transportation. Double mutants of *paa1* and *paa2* genes were seedling-lethal showing the importance of copper in photosynthesis (Salah *et al.*, 2005).

Rice production can be increased with an intervention of biotechnology in improved conventional breeding. A high-quality rice genome sequence, molecular marker technology and assigning functions to genes will greatly help to develop high yielding rice varieties. Cloning of genes involved in yield related traits such as tillering, plant development and grain size can be very useful in order to increase rice production. Oligo culm (*Osoc*) Seedling lethal (*Ossl*) and small grain (*Ossg*) insertional mutants were isolated using independent *Agrobacterium* mediated transformation of Basmati 370 (Dhaliwal *et al.*, 2001).

The present study was undertaken with the following objectives.

1. Screening T-DNA insertional mutants for important phenotypic traits.
2. Confirmation of T-DNA insertions and identification of copy number by Southern hybridization.
3. Parental polymorphic survey of Basmati 370 and PR106 by rice SSR markers.
4. Construction of linkage map for oligo culm gene by Bulk Segregation Analysis.
5. Identification of T-DNA flanking genome sequence using TAIL-PCR and Genome walking.
6. Expressional analysis studies using qualitative RT-PCR for knockout genes.
7. Bioinformatic studies of identified knockout genes.
8. Cloning of Basmati 370 *Osoc* gene.

CHAPTER 2

LITERATURE REVIEW

Rice is one of the most important staple cereals for half the mankind. In India the production of rice exceeds 100 million tonnes annually because of increase in the area under cultivation and introduction of high yielding varieties since 1988. Out of 114 rice growing countries more than 50, have an annual production of more than 100,000 tonnes. More than 90% rice is produced in Asia, China and India, growing more than half the produce. The top 10 rice producing countries are China, India, Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Japan, Philippines, and Brazil (http://www.irri.org/science/ricestat/). In India rice is the staple food for 65% of the total population.

The relevant literature on the present study has been reviewed under the following heads.

2.1 Rice

2.2 Basmati Rice

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2.3.1 Rice molecular marker

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2.1 Rice

The genus *Oryza* has 2 cultivated and 22 wild species. The cultivated species are *Oryza sativa* and *Oryza glaberrima*. *Oryza sativa* is cultivated all over the world, where as *Oryza glaberrima* is cultivated in West Africa. The basic chromosome number of wild diploid and tetraploid species is x = 12, while the two cultivated species are diploid (2n=24). Two subspecies *japonica* and *indica* belonging to *Oryza sativa* occupy most of the cultivated area. *Indica* rice is generally found in the lowlands of tropical Asia, whereas *japonica* rice is found in the upland hills of

Southern China, North-East and South-East Asia and Indonesia as well as regions outside Asia (Khush, 1997).

There are considerable cultural differences for rice consumption which determine the quality of rice. Generally the Indians prefer non-sticky rice where as the Chinese and Japanese prefer sticky rice. The quality depends on chemical composition, cooking quality, gelatinization temperature and aroma. Aroma is the highest desired trait followed by taste and elongation of grain after cooking (Bhattacharjee *et al.*, 2002) for the Indian consumers.

Rice cultivation conditions in India are extremely diverse. India has the largest area of the world under cultivation of rice. About 28% area under rice cultivation is rain-fed low land, 46% irrigated, 12% rain-fed upland, and 14% flood-prone. The major rice cultivation area is rain-fed, and therefore the yield is strongly dependent on rainfall (http://www.irri.org/science/cnyinfo/india.asp).

2.2. Basmati Rice

Basmati 370 is one of the superfine varieties with extra long slender grains with pleasant fragrance, soft texture, sweet taste, low amylase content, dry and fluffy. It obtains 1.5 to 2 fold length wise elongation and the least breadth wise swelling on cooking and is intact and non-sticky in nature (Siddiq *et al.*, 1997). Because of its unique and high quality nature, Basmati is considered as the 'Crown jewel' of rice. Basmati rice is traditionally grown under Himalayan foot hills of India and Pakistan and the quality is associated with this geographical region (Bligh, 2000). Among Basmati cultivars, Basmati 370 variety is considered as having the highest rated quality. India and Pakistan are the largest growers and exporters of Basmati rice. Together, India and Pakistan export 1.3 million tonnes of Basmati rice. Every state in

India has its own distinct scented rice varieties. Basmati rice is cultivated in Pakistan (Punjab) and in India (Punjab, Haryana, Uttarakhand, Western Uttar Pradesh and Haryana). Production of Basmati rice in 2004-05 reached 11.2 lakh tonnes and in value terms, exports reached Rs. 2,741 crores compared to Rs. 1,990 crores in 2003. Cooking quality and aroma are greatly influenced by environmental conditions (Singh and Singh, 1997), time of transplanting, harvesting and storage (Ali *et al.*, 1991). The aroma of Basmati rice is largely because of a chemical compound 2-acetyl-1-pyrroline. The chemical is present in all rice cultivars varying from 0.04-0.06ppm, but is present in a concentration which is ten times higher in Basmati rice (Buttery *et al.*, 1983). In addition to 2-acetyl-1-pyrroline there are more than 100 volatile compounds responsible for aromatic nature (Hussain *et al.*, 1987).

2.3 Molecular markers

Molecular marker is a segment of DNA that is found at a specific site in the genome. As with alleles, the characteristics of molecular markers vary from individual to individual. Therefore, the distances between linked molecular markers can be determined by their recombination in segregating generation. The molecular markers are developed based on polymorphism in DNA sequences. Diversity analysis studies have been carried out using different marker series (Lakshmi *et al.*, 2002; Prashanth *et al.*, 2002; Selvi *et al.*, 2005; Singh *et al.*, 2006). Marker assisted selection is being used to pyramid genes in shortest period (Sanchez *et al.*, 2000; Singh *et al.*, 2001).

Different types of markers developed as based on Southern hybridization, include restriction fragment length polymorphisms (RFLPs), which were the first DNA based markers, that were used in various crops (Yu *et al.*, 2002; Balyan *et al.*,

2005; Gupta *et al.*, 2005; Parida *et al.*, 2006). The PCR based markers include random amplified polymorphic DNA (RAPDs), simple sequence repeats (SSRs) or microsatellites, sequence tagged sites (STSs), sequence characterized amplified regions (SCARs), amplified fragment length polymorphisms (AFLPs), inter-simple sequence repeat amplification (ISSR), cleaved amplified length polymorphic sequences (CAPs), DNA amplification fingerprinting (DAF) and single nucleotide polymorphism (SNP) (for a review, see Gupta *et al.*, 1999).

2.3.1 Rice Microsatellite markers

Microsatellites are the tandem simple sequence repeats of (SSR) of 1-5bp. SSRs are present throughout the genome. SSR can be amplified *in vitro* using PCR with specific primers. SSR markers are reliable and cost effective over RFLPs. These SSR markers have been used for constructing high resolution genetic maps in various species such as *Arabidopsis*, rice, *Drosophila*, human etc. (Innan *et al.*, 1997; Yang *et al.*, 1994; Goldstein and Clark, 1995; Schlötterer *et al.*, 1997; Bowcock *et al.*, 1994; Dib *et al.*, 1996). In rice, microsatellite markers were developed from genomic libraries, ESTs and DNA databases (Wu and Tanksley, 1993; Panaud *et al.*, 1996; Akagi *et al.*, 1996; Temnykh *et al.*, 1999).

McCouch *et al.* (2002) developed 2,240 microsatellite markers for rice and integrated them into the existing genetic maps. Approximately one SSR marker in every 157 kilo-base (kb) pairs was experimentally confirmed in the rice genome. Most of these studies were based on the analysis of either random rice DNA sequence databases including rice ESTs, and genomic sequences or rice SSRs-containing sequences isolated from several small-insert genomic libraries (Temnykh *et al.*, 2000).

Cho *et al.* (2000) evaluated the frequency of polymorphism for mapping in rice and detected that microsatellites derived from genomic libraries detected a higher level of polymorphism than those derived from ESTs contained in the GenBank database (83.8% versus 54.0%).

2.4 Molecular mapping

Most of the important traits including yield, quality, days taken to flower, biotic and abiotic stress tolerance and disease resistance are controlled by many genes with small effects called QTLs (Paterson *et al.*, 1988; Mackay, 2001; Morgante and Salamini, 2003; Flower *et al.*, 2002; Gu *et al.*, 2005; Gupta *et al.*, 2003). The availability of different DNA molecular markers in the recent years has led to considerable progress in QTLs and gene mapping in plants (Sharma *et al.*, 2005; Singh *et al.*, 2006; Singh *et al.*, 2007; Zhu *et al.*, 2005). The molecular markers have been extensively used for construction of genetic maps utilizing mapping populations, which could consist of an F_2, a backcross, doubled haploids (DHs) and recombinant inbred lines (RILs) (Kumar *et al.*, 2007). The markers exhibiting polymorphism among the parents of the mapping population can be used for constructing linkage maps, which are used for gene tagging and QTL analysis. High density genetic chromosome bin maps and physical maps are constructed in various plants by using different markers (Conley *et al.*, 2004; Goyal *et al.*, 2005; Hossain *et al.*, 2004; Kalavacharla *et al.*, 2006). Several genes have been mapped and cloned in various crop plants (Sharma *et al.*, 2005).

2.5 Bulked Segregant Analysis

Conventional QTL mapping and identification of linked markers for desired traits required determination of genotypes at several hundred polymorphic loci in several hundred individuals. Because only few of the markers are expected to show linkage, a simple screen of genetic markers to identify those showing linkage in pooled DNA samples can greatly facilitate gene identification. Bulked segregant analysis (BSA) has been widely used to identify molecular markers linked to any specific gene or genomic region. Originally BSA was developed with RFLP or RAPD markers (Michelmore *et al.*, 1991). Two bulks are prepared from pools which are genetically dissimilar for a trait, but heterozygous at remaining genomic regions.

The type of marker (dominant and codominant) and mapping population will determine the size of bulks. Smaller bulks will increase the false results (Michelmore *et al.*, 1991). Simplicity and low cost of BSA have led to its use for more traits in various plant species like rice, barley, pea and *Populus* (Wang *et al.*, 1995; Poulsen *et al.*, 1995; Ek *et al.*, 2005; Tabor *et al.*, 2000). The success of method depends on the marker type and the segregating population employed.

Wang *et al.* (1995) mapped the thermo-sensitive genetic male-sterile gene in rice (Oryza *sativa* L.) with RAPD molecular markers. Amplification with OPB-19 primer showed linkage and mapped on chromosome 8 with RIL population and was reconfirmed by remapping with a DHL population.

Poulsen *et al.* (1995) constructed a genetic linkage map for the leaf rust resistance region linked to *RphQ* (12cM) using the mapping population of barley accession Q21861 and an Australian barley variety Galleon. The markers were developed from PCR product of RAPD marker OPU-02 (Operon). Both the markers

RphQ and OPU-02 can be used for developing new barley variety resistant to leaf rust.

Ek *et al.* (2005) used BSA to determine the position of powdery mildew resistance gene on chromosome 6 of pea with SSR markers. Two pools of individuals (from the cross of resistance pea cultivar 955180 and susceptible pea cultivar Mojoret) were constructed according to resistance and susceptible homozygous lines. Out of 315 SSR markers two marker combinations were found linked and used for marker assisted breeding for powdery mildew resistance.

Tabor *et al.* (2000) utilized pooling of DNA to identify RAPD marker OPG10 and OPZ19 which were linked to *Lrd1* at estimated 2.6cM and 7.4cM distance respectively. These markers may be helpful in cloning of *Lrd1* gene and marker assisted selection of leaf rust resistance gene in *Populus deltoides*.

Altinkut *et al.* (2003) constructed a population of water stress tolerant and sensitive barley lines. Bulk segregant analysis was used to identify AFLP marker linked to stress tolerance in barley. Total 12000 AFLP fragments were amplified using 40 primer combinations. One primer combination amplification was present in tolerant parent/bulk but not in the sensitive bulk. This primer combination can be used in breeding program to select drought tolerant plants.

2.6 *Agrobacterium* Mediated Transformation

2.6.1 Characteristic features of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens and *Agrobacterium rhizogenes* are the most well known plant pathogens. *Agrobacterium tumefaciens* has the *Ti* (tumour inducing) plasmid and *Agrobacterium rhizogenes* has the *Ri* plasmid. These plasmids have the

same general features and can be interchanged between two species. Virulent bacteria contain one or more large plasmids.

A. tumefaciens has an ability to integrate a part of the Ti plasmid into the host plant genome, which causes the plant cells to form tumors. T-DNA sequence of bacterial *Ti* (tumor inducing) plasmid integrated into the plant genome leads to the genetic modification of the genome. Expression of T-DNA genes produce specific compounds called opines, which are utilized as a carbon source by *Agrobacterium*. Expression of several oncogenic (*onc*) genes leads to the formation of tumours (Gaudin *et al.*, 1994). The entire process is regulated and controlled by a set of genes known as *Vir* genes, which are activated by the detection of wounded plant phenolic compounds. The *Ti* plasmid contains the genes that determine the host range and the symptoms which the infection will produce. The T-DNA and the virulence (*vir*) region are the two distinct regions of all *Ti* plasmids, which are necessary for plant transformation. The T-DNA region contains genes for opine metabolism and phytohormone independence. The *Vir* region produces endonucleases and other essential proteins for the excision and transfer of *Ti* region into plant cells.

2.6.2 Research on *Agrobacterium tumefaciens*

Agrobacterium was first isolated by DelDott and Cavara, (1897) from tumors of infected grape plants. Smith and Townsend, (1907) were the first to discover that plants could be infected using a needle dipped in culture medium. It has been concluded that the bacterium requires a wound site in the plant in order to enter and induce a tumorous response. That is why *Agrobacterium tumefaciens* is present in many soil samples, yet relatively few plants are affected. The binary Ti vectors (able to replicate in *Escherichia coli* and *A. tumefaciens* contain an antibiotic resistance

gene for selection, whilst some of the more versatile Ti plasmids contain a series of *lacZ* restriction sites which allow complementation based screening for recombinant plasmids (Norrander *et al.*, 1983). Hellens *et al.* (2000) designed a new binary Ti vector system, which overcomes difficulties related to the plasmids, such as low recombination frequency, interchangeable selectable markers and in transfer of large fragments of DNA.

2.6.3 Mechanism

There are many different biological processes involved in transformation of T-DNA from bacterium to plant, such as intercellular signaling, cell-to-cell DNA transport and DNA integration into the host cell nucleus (Tzfira and Citovsky, 2000; reviewed by Gelvin, 2003).

2.6.3.1 Sensing plant signals

VirA and *VirG* proteins (Tzfira and Citovsky, 2000) are involved in sensing plant signals. *VirA* acts as a membrane sensor protein and *VirG* promotes activation of all the *Vir* genes. *VirA* functions as protein kinase and phosphotransferase (Tzfira and Citovsky, 2000). *VirG* is expressed both in the presence and absence of plant phenolic compounds (Stachel and Zambryski, 1986).

2.6.3.2 Attachment and penetration

Agrobacterium first weakly attaches to plant, and then synthesizes cellulose fibrils, which anchor them to the wounded plant cell surface. *chvA*, *chvB*, *pscA* and *att* bacterial genes are required for this process, as a mutation in any of these genes leaves the bacterium not capable to attach to the plant. A plant glycoprotein vitronectin,

which is a component of the plant extracellular matrix (ECM), is also thought to be involved in attachment process. The bacterial cell controls the transfer of the T-DNA complex into the plant cell. T-DNA is covered with proteins to prevent it from degradation out side the bacterial cell; it is referred to as T-complex. The T-complex is made up of single stranded T-DNA coated by VirE2 protein (Tinland, 1996) and VirD2 protein attached at the 5` end (Howard and Citovsky, 1990). The VirD1 and VirD2 protein also recognize and cut the left and right T-DNA borders. The T-complex is coated by VirE2 protecting it from nuclease attack when it enters the plant cell (Tinland, 1996). T-complex is exported into plant cell by the type IV secretion system. This system is assembled from proteins encoded by the virD4 gene and the virB operon (Tzfira and Citovsky, 2000). Eleven VirB proteins play a role in the transport of the T-complex across the membrane. VirB1 initiates the assembly, and VirB2 is the main structural protein in the pilus (Zupan *et al.*, 1998).

The T-DNA does not encode functions for transport and integration; it requires only right and left border sequences for integration. This property makes it so useful that any DNA sequence inserted between the T-DNA borders will be transferred into the plant genome, allowing the efficient production of transgenic plants. The insertion of T-DNA in the genome is a random process. The auxin and cytokinin biosynthetic genes are expressed in plant system, resulting in uncontrolled growth of the gall. Opines are synthesized and used only by the gall as its sole carbon source.

2.6.4 *Agrobacterium*-mediated transformation in rice

It was believed that monocots, including rice (*Oryza sativa* L.) cannot be used for *Agrobacterium* mediated transformation because these plants do not respond to

wounding. However, efficient transformation of rice mediated by *Agrobacterium* was developed after identification of phenolic stimulator acetosyringone (Hiei *et al.*, 1994; Rashid *et al.*, 1996). Hiei *et al.* (1994) substituted the wound response by adding acetosyringone. Co-cultivation of explant with *Agrobacterium* in the presence of acetosyringone at temperature (22-28^{0}C) was critical for rice transformation. The first transgenic rice was developed from both *japonica* (Toriyama *et al.*, 1988; Zhang and Wu, 1988; Zhang *et al.*, 1988) and *indica* rice (Peng *et al.*, 1992). Several reports are being made for successful plant regeneration with direct and indirect DNA delivery techniques in rice. Several researches have developed efficient transformation protocols in various cultivars such as semi dwarf *indica* rice cultivar ADT39, Bangladesi *indica* rice cultivar Binni, Pusa Basmati and Basmati 370 (Tyagi *et al.*, 2007; Forkan *et al.*, 2004; Mohanty *et al.*, 1999; Rashid *et al.*, 1996).

2.7 Rice as model crop for Functional genomics

It is estimated that productivity of the rice must be increased by 30% over the next 20 years to meet population explosion (Peng *et al.*, 1999). Rice production can be increased with the combination of conventional breeding and complete rice genome sequence information. The rice plant has been selected as a model crop because of the following features;

1. The size of the rice genome is 389 Mb (IRGSP, 2005) which is 6 and 40 times smaller than that of maize and wheat, respectively.
2. Molecular and Genetic information available about rice (ESTs, Molecular markers and physical maps available).
3. Efficient transformation systems are available.

4. Isolated important genes can facilitate cloning of homologs from other cereal crops.

2.8 T-DNA and transposon tagging as a tool for functional genomics

The availability of the high quality complete sequence of rice genome has resulted in explosion of information on 41,478 non-transposable element genes (Jung *et al.*, 2007). The term Functional Genomics can be defined as "development and application of global (genome-wide or system-wide) experimental approaches to assess gene function by making use of the information and reagents provided by structural genomics" (Hieter and Boguski, 1997). Biological function of these genes can be identified by various approaches such as insertional mutagenesis, microarray technology, RNAi and generation of point/deletion mutations by chemical agents (Koornneef *et al.*, 1982; Sundaresan *et al.*, 1996; Krysan *et al.*, 1999; Anand *et al.*, 2003). Out of these, insertional mutagenesis is a simple and powerful approach because insertions not only cause mutations but also tag the genes. Forward genetics begins with mutant phenotype and leads to identification of genotype. Reverse genetics begins with a mutant gene and goes towards identification of gene function (Krysan *et al.*, 1999).

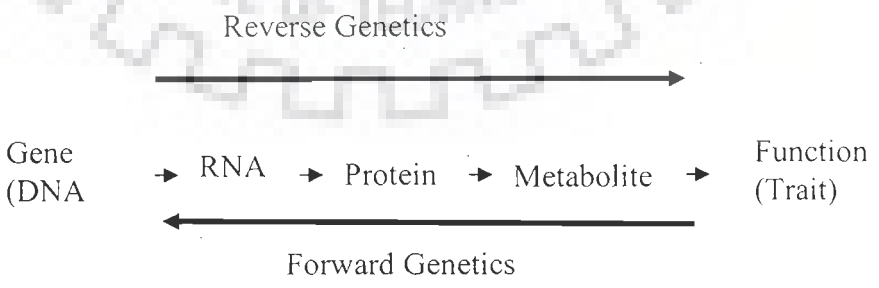


Fig. 2.1: Functional Genomics Approaches

Transposon and T-DNA have been used most widely for insertional mutagenesis for reverse genetics approach (Mathur *et al.*, 1998; Wisman *et al.*, 1998). T-DNA insertion is a random process and the insertion sequences are stable throughout multiple generations (Azpiroz-Leehan and Feldmann, 1997; Parinov and Sundaresan, 2000). Insertional mutagenesis has been used in rice (Chin *et al.*, 1999; Suyoung *et al.*, 2003; Sallaud *et al.*, 2004) and *Arabidopsis* (Krysan *et al.*, 1999; Tissier *et al.*, 1999). Low copy number insertions were observed (1.5 insert per genome) in rice and *Arabidopsis* (Feldmann, 1991; Jeon *et al.*, 2000). *Ds* transposon mutants can be easily confirmed by excision of the transposon from the disrupted gene in the presence of *Ac* element.

Flanking sequence of the gene in which T-DNA is inserted can be easily identified by using various methods such as TAIL-PCR, iPCR, Genome walking etc. (Liu *et al.*, 1995; Ochman *et al.*, 1988; Siebert *et al.*, 1995). Use of T-DNA as the insertional mutagen is more convenient than transposon tagging because T-DNA insertions will remain stable through multiple generations. However, transposon mutagenesis is useful because of its ability to transpose to nearby locations providing mutations within all of the members of a gene family (Krysan *et al.*, 1999).

2.8.1 Gene trap and Activation tagging

Insertional mutagenesis has the advantage that the inserted fragment acts as a tag for gene identification. However it has some limitations, for example identification of the function of redundant genes, genes involved in early embryogenesis or gametophyte development and it is also difficult to establish the linkage between visible phenotypes and the insertion.

Gene trap and Activation tagging systems were developed to overcome the limitation of the conventional insertional mutagenesis method. In activation tagging system multimerized cauliflower mosaic virus (CaMV) 35S enhancers were used to enhance the expression of genes (Hayashi *et al.*, 1992; Suzuki *et al.*, 2001). Insertion of multimerized CaMV 35S enhancers can effect transcriptional activation of the nearby genes. Over expression of genes may cause different phenotypes that can identify important genes such as redundant gene families and essential genes for survival. Gene trap system is one of the modified systems that involve fusions between the tagged genes and reporter genes like β-*glucuronidase* (*gus*) or green fluorescent protein. Fusion of tagged gene with reporter gene (*gfp*) facilitates identification of novel genes based on their expression patterns (Sundaresan *et al.*, 1995; Springer, 2000). Insertion of the promoter-less reporter gene not only destroys normal gene function but also activates expression of the reporter gene. The gene trap system is convenient for observing mutant phenotype because the reporter gene activation indicates location and time of expression of the knockout gene.

The T-DNA approach is very difficult in those plants where the transformation protocol has not been developed. The integration of the T-DNA is more complex and at the time of integration loss of border sequences, generally left border sequences makes it difficult for further molecular analysis. Transposable insertional mutagenesis is the promising method to overcome such limitations (Ramachandran and Sudaresan, 2001). Barbara McClintock discovered the transposable elements activator and dissociator (*Ac/Ds*) in maize, which are also present in other eukaryotic genomes. The size of autonomous *Ac* elements is 4563 base pairs in length which codes for transposase containing 807 aminoacids (Pohlman *et al.*, 1984; Kunze *et al.*, 1987). The transposase stimulates the movement of *Ac* element. The non autonomous *Ds*

element has lost the ability to produce transposase. The transposase that is encoded by *Ac* elements can move throughout the cell and excise any *Ds* or *Ac* element (McClintock., 1948) in *cis* and *trans*. The *Ac* and *Ds* system are used to isolate genes from various plant species such as maize and *Snapdragon* (Fedoroff *et al.*, 1984; reviewed by Ramachadran and Sudaresan, 2001).

In the two-element transposon tagging system, the whole genome can be saturated with *Ds* element with few transformations. The *Ds* element can be moved to other positions by crossing with a plant carrying the *Ac* gene; therefore from the single cross, different mutants can be isolated. *Ds* elements preferentially transpose to sites closely linked to the donor sites (Smith *et al.*, 1996; Machida *et al.*, 1997).

2.9 Mutants defective in Tillering and Branching

Number of tillers per plant is an important yield component in cereals including rice. Rice tillering ability is controlled by QTLs as well as environmental conditions such as light and temperature. The plant hormones, auxins and cytokinins have a major role in controlling tillering and branching process. Auxin has an inhibitory effect on axillary bud growth, whereas cytokinin promotes axillary bud outgrowth. The outgrowth of axillary buds is influenced mainly by hormonal signals as well as environmental conditions. The development of lateral shoots is inhibited by signals that are derived from the main shoot tip, a phenomenon named apical dominance. Auxin produced in the main shoot tip was believed to be the main repressor and cytokinin produced in the roots was considered to be the main activator of lateral bud (shoot) development.

The control of axillary shoot growth is poorly understood. One focus of research has been the control by plant hormones, mainly auxin and cytokinins. Cline, (1996)

pointed that the auxins synthesized in growing shoot apices and transported basipetally showed an inhibitory effect on axillary meristem. Cytokinins, transported acropetally from the root, may act as activators directly within the axillary shoot. The shoot branching process generally involves two developmental stages:

1. Formation of axillary meristem.
2. Out growth of axillary meristem.

Mutants that are defective in early steps of axillary meristem (AM) initiation have been characterized in different plant species, including tomato, *Arabidopsis*, rice and maize. AM formation is part of the general lateral organ initiation program at the shoot apical meristem (SAM). List of mutants defective in various plant species are explained below and given in the Table 2.1.

2.9.1 *Arabidopsis revoluta* mutant

In *Arabidopsis*, *revoluta* (*rev*) mutant (Otsuga *et al.*, 2001), lateral meristems in the axils of rosette and cauline leaves are often not initiated and flowers frequently fail to develop. *REV* is required for initiation of both lateral shoot meristem (LSMs) and flower meristem (FMs). In *Arabidopsis thaliana*, the lateral meristems include flower meristems (FMs), which form on the anks of the shoot apical meristem (SAM), and lateral shoot meristems (LSMs), which develop in leaf axils. The gene *rev* is expressed at the earliest stages of LSM and FM formation. The *revoluta* gene belongs to a subfamily of Homeodomain-Leucine-Zipper transcription factors. The gene *rev* is expressed in vascular bundles as well as in the SAM and axillary meristems. Comparative expression studies in wild type and *las*-4 plants have demonstrated that *rev* acts downstream of LAS in the initiation of axillary meristems. Dominant mutations in the two closest *rev* relatives, PHABULOSA (*PHB*) and PHAVOLUTA

Table 2.1: List of tillering and branching genes cloned in different plant species

S. No.	Gene name	Species	Homologs in other species	Gene product	Function
1	*Revoluta*	*Arabidopsis*		HD-ZIP transcription factor	Controls the initiation of AMs at both vegetative and reproductive phases
2	*Blind*	Tomato		Myb transcription factor	Required for the initiation of AMs at both vegetative and reproductive phases
3	*Super Shoot*	*Arabidopsis*		Cytochrome P450	Involved in axillary bud initiation and outgrowth
4	*BUD1*	*Arabidopsis*		MAP KINASE KINASE7	Negatively regulates polar auxin transport
5	*Teosinte Branched1*	Maize	*OsTB1*	TCP transcription factor	Controls lateral bud outgrowth
6	*Monoculm1*	Rice	*Ls, LAS*	GRAS transcription factor	Controls rice tiller bud initiation and out growth
7	*MAX1–MAX4*	*Arabidopsis*	Pea *RMS*, Petunia *DAD*1	Cytochrome P450 (MAX1), F-box LRR (MAX2), plastidic dioxygenase (MAX3), auxin-inducible polyene dioxygenase (MAX4)	Produce carotenoid-derived regulators of axillary bud outgrowth

(*PHV*), lead to a complete deaxilisation and radialisation of leaves and to the formation of ectopic meristems at the abaxial leaf base. The activity of *rev*, *PHB*, and *PHV* is restricted by microRNAs, and dominant *PHB* and *PHV* alleles contain point mutations in their miRNA target sites that render them insensitive to miRNA-regulated degradation. Currently, it is not clear, if the *rev*, *PHB* and *PHV* genes control the formation of lateral meristems directly or by their influence on the development of vascular elements.

2.9.2 Tomato *Blind* Gene (*bl*) and torosa gene

During vegetative development, axillary buds are not formed in 40–90% of their leaf axils, whereas wild-type plants develop side shoots in more than 99% of their axils. In addition, *bl* and *to* mutants have a tendency to terminate shoot growth after formation of an inflorescence (Schmitz *et al.*, 2002). Furthermore, inflorescences of *bl* and *to* mutants are highly reduced, producing only 1 to 4 flowers per truss, compared with 7 to 12 in the wild type. The recessive mutants *bl-1*, *bl-2*, and *to-1* were crossed with each other and also with *lateral suppressor* (*ls-1*). F1 and F2 plants from crosses between *bl-1*, *bl-2*, and *to-1* showed reduced side shoot formation and mutant inflorescences, supporting the conclusion that they were allelic.

2.9.3 *Arabidopsis* bushy and dwarf1 (*bud1*)

Polar auxin transport (PAT) plays a crucial role in the regulation of many aspects of plant growth and development. *Arabidopsis* bushy and dwarf1 (*bud1*) mutant (Dai *et al.*, 2006), which generates more branches than wild type at the late developmental stage and leads to the loss of apical dominance, results from a deficiency in polar auxin transport. Molecular genetic analysis indicated that the *bud1*

phenotype is a result of increased expression of *Arabidopsis* MAPKINASE KINASE7 (*MKK7*). The *bud1* gene encodes the *Arabidopsis* MAP KINASE KINASE7 (*MKK7*), a member of the *Arabidopsis* MAPkinase cascade. *MKK7* is the first identified component of the MAP kinase cascade to regulate IAA transport, thus affecting apical dominance; however, increasing evidence suggests that auxin is not a direct and exclusive effector of apical dominance. Determination of tritium-labeled indole-3-acetic acid transport showed that the increased expression of MKK7 in bud1 or the repressed expression in MKK7 antisense transgenic plants causes deficiency or enhancement in auxin transport, indicating that MKK7 negatively regulates PAT.

2.9.4 Maize *Teosinte Branched1* and *Oryza sativa teosinte branched1*

The outgrowth of axillary buds is affected by maize TEOSINTE BRANCHED 1 (*tb1*), Changes in *tb1* is crucial to the evolution of maize from its wild ancestor teosinte (Doebley *et al.*, 1995). In the maize mutant, *teosinte branched1* (*tb1*), tillers and secondary and tertiary branches grow out, leading to a bushy architecture. The gene *tb1* encodes a bHLH transcription factor of the TCP family. It has been proposed that changes in the expression of *tb1* caused the suppression of tillering that occurred during the evolution of maize from teosinte.

The rice ortholog of *TB1* was reported to be involved in growth suppression after AM initiation. Over expression of *OsTB1* under the control of the actin promoter led to a suppression of tillering in rice. Moreover, the *fine culm1* (*fc1*) mutation has increased tillering due to a loss-of-function mutation in *OsTB1*. The initiation of tiller buds is not affected in *OsTB*1 over expression transgenic lines. These findings indicate that the pivotal role of *OsTB*1 is to control the outgrowth of rice tiller buds rather than the initiation of tiller buds (Takeda *et al.*, 2003). *OsTB*1 expression has

been found throughout the axillary bud as well as in the basal part of the shoot apical meristem (SAM), in vascular tissues in the pith and in the lamina joint. These data suggest that, in rice as in maize, *TB1* functions as a negative regulator of shoot branching. *OsTB1* appears to be regulated by *MOC1*. It is not yet clear whether Tb-related genes have a similar role downstream of *LAS/MOC1* in eudicot branch development (Takeda *et al.*, 2003).

2.9.5 *MONOCULM*1, *Lateral suppressor (ls)* and *LATERAL SUPRESSOR* (LAS)

Mutants that are impaired in early steps of axillary meristem initiation have been characterized in different plant species, including tomato, *Arabidopsis* and rice (Schumacher *et al.*, 1999; Greb *et al.*, 2003; Li *et al.*, 2003). The *ls* gene regulates the formation of axillary meristem (AM) during the vegetative phase of development in tomato. After the transition to reproductive development, axillary shoots can be formed and inflorescence branching is not affected in these mutants. Mutations in the orthologous *Arabidopsis* gene (*LAS*) also reduce the number of axillary shoots. Rice mutations in the *Ls/LAS*-orthologous gene MONOCULM1 (*MOC1*) show defects in the formation of tillers. *MOC*1 mutants have almost no tillers because of their failure to form tiller buds. Increase in *MOC1* expression increases tiller number, implying that *MOC1* promotes AM outgrowth and initiation.

The similarity of the *Ls/LAS/MOC1* mutant phenotypes and the transcomplementation experiment suggested that the *Ls/LAS/MOC1* genes encode key regulators of axillary meristem initiation that are conserved over large evolutionary distances. The *Ls/LAS/MOC*1 genes encode putative transcriptional regulators of the plant-specific GRAS family. Thus, the basic control mechanism in the process of side-shoot development is conserved among three diverse species tomato, *Arabidopsis*

and rice. *LS/LAS/MOC1* plays a conserved role in maintaining the potential for AM initiation in the leaf axils of both monocots and dicots.

2.9.6 More Axillary growth (MAX), Ramosus (RMS) and Decreased Apical Dominance1 (DAD)

The recessive mutants *ramosus* (*RMS*) in pea and *more axillary growth* (*MAX*) in *Arabidopsis* show premature and enhanced outgrowth of lateral shoots. The four recessive *Arabidopsis MAX* loci have phenotypes that are similar to those caused by the RMS mutants (Stirnberg *et al.*, 2002; Sorefan *et al.*, 2006; Booker *et al.*, 2005). These phenotypes include increased branching, slightly reduced stature and root growth, and rounder leaves with shorter petioles. Grafting studies have shown that *MAX*1, *MAX*3 and *MAX*4 shoots, like those of *RMS*1, *RMS*2 and *RMS*5, are restored to wild type by grafting to wild type root stocks. Reciprocal grafts involving wild type shoots and mutant roots were also wild type, showing that the presence of wild type *MAX*1, *MAX*3 and *MAX*4 in either root or shoot is sufficient to inhibit shoot branching. *MAX*4 encodes a member of the polyene chain dioxygenase superfamily. Other members of this superfamily include the *Arabidopsis* carotenoid cleavage dioxygenases (CCDs), which are related to the ABA biosynthetic protein VP14.

*MAX*4 and *RMS*1 are expressed at extremely low levels, and this expression appears to be upregulated by auxin. In pea, the upregulation of *MAX*4/*RMS*1 is rapid and occurs at the node, suggesting a mechanism by which the *RMS*1-dependent signal could mediate the inhibition of bud growth by auxin. Decreased Apical Dominance1 (*DAD*1) genes have been identified as functional homologs of *MAX*4 and the rice gene D3 as homologue of *MAX*2, demonstrating an evolutionary conservation of this pathway.

2.10 Flanking genome sequencing

After the completion of total genome sequence of the various species, the next step is assigning functions to all identified genes. Insertional mutagenesis is one of the promising tools to identify gene function. Insertion not only creates mutation but also tags the gene. Various techniques have been developed to identify flanking genome sequencing such as inverse PCR (Ochman *et al.*, 1988) and hemispecific or one-sided PCR methods (Frohman *et al.*, 1988; Isegawa *et al.*, 1992), TAIL-PCR (Liu *et al.*, 1995; Liu and Whittier, 1995) and Genome Walking (Siebert *et al.*, 1995).

2.10.1 TAIL-PCR

TAIL-PCR is a simple and powerful tool for the recovery of DNA fragments adjacent to known sequences. Thermal asymmetric interlaced (TAIL)-PCR was developed by Liu *et al.*, (1995). TAIL-PCR uses nested sequence-specific primers and shorter arbitrary degenerate primer. Amplification of specific product is thermally controlled. Researchers use this method to identify flanking genome sequencing of T-DNA and transposon insertions (Liu *et al.*, 1995; Smith *et al.*, 1996), and genes from humans and plants (Hahn *et al.*, 1996; Tsugeki *et al.*, 1996). TAIL-PCR is suitable for identifying flanking sequence from a large number of samples, because no other manipulations are required except PCR.

TAIL-PCR utilizes three nested specific primers (melting temperature 57^0-62^0C) in successive reactions together with an arbitrary degenerate (AD) primer (melting temperature 44^0-46^0C). The relative amplification efficiencies of specific and non-specific products can be thermally controlled. In the primary reaction, one low-stringency PCR cycle is allowed to anneal throughout genome including targeted sequence. Specific amplification is then preferentially amplified over non specific

ones by proceeding one reduced-stringency PCR cycle with two high-stringency PCR cycles. In the secondary nested PCR, specific amplification achieved by two high stringency cycle followed by one low stringency cycle. Higher annealing temperature favours the specific primer amplification resulting in linear amplification of desired fragment. In the secondary and tertiary reactions, amplification of specific products is favoured.

2.10.2 Genome walking

Several different genome walking strategies have been reported by researcher such as inverse PCR (Ochman *et al.*, 1988) and adapter-specific PCR (Universal Genome Walking Kit, Clontech, Palo Alto, CA, USA). Inverse PCR includes genomic DNA digestion and self ligation, vectorette PCR includes genomic DNA digestion and ligation to vectorette and genome walking method includes digestion and ligation to specific adaptor. Genome walking is an effective procedure that allows the identification of unknown regions from a known DNA sequence based nested PCR amplification. The ligated product is referred to as genome walking library and is used as a template for amplifying flanking regions using nested gene and adapter specific primers. The technique is reliable and efficient due to the use of two-rounds of PCR with nested gene and adapter specific primers in the second PCR. Universal Genome Walking Kit from Clontech is fast, reliable and relatively easy to use. Xiao-Dong *et al.* (2002) used Universal Genome Walking Kit (Clontech, Palo Alto, CA, USA) to amplify promoter regions of rubber hevein gene. Connors *et al.* (2002) characterized the promoters of American chestnut. Trindade *et al.* (2003) isolated and characterized the stolon specific promoter from *Solanum tuberosum*. The genome walking strategy has a limitation in amplifying smaller fragments when restriction sites are present

close to known sequence and larger fragments where restriction sites are present far away from upstream sequence. These problems can be overcome by construction of different genome walker libraries using different restriction enzymes and long PCR strategies, respectively (Rishi *et al.*, 2004).

2.11 RNA interference

RNAi is a powerful approach to identify gene functions in plants, and invertebrates etc. (Chuang *et al.*, 2000; Kennerdell *et al.*, 1998; Misquitta *et al.*, 1998). The simultaneous expression of sense and antisense sequences corresponding to the desired target gene form double-stranded RNA (dsRNA) and trigger sequence-specific gene silencing in a wide variety of organisms. RNA interference in plants also known as post-transcriptional gene silencing (PTGS) is thought to provide defence against viruses (reviewed by Waterhose and Wang, 2001) and regulate endogenous genes (reviewed by Kidner and Martienssen, 2003). The enzyme DICER degrades the dsRNA into approximately 21-nucleotide long 'small interfering RNAs' (siRNAs). These siRNAs then provide specificity to the endonuclease-containing, RNA-induced silencing complex (RISC), which targets homologous mRNAs for degradation (reviewed by Pickford and Cogoni, 2003). The mechanism of RNA interference (RNAi) is not well understood, but it provides an effective approach to discover gene function.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The plant material and chemical obtained, generated and used in the present work are mentioned in the following sections.

3.1.1 Plant materials

Three independent insertional mutants viz; Oligo culm (B-1-10), Seedling lethal (B-2-11) and Small seed size (B-2-1) in the present study were generated from superfine quality *indica* rice cultivar Basmati 370 by transformation through *Agrobacterium tumefaciens* strain EHA101 containing Hm^R*Ds* cloned in *Ti* plasmid. These T-DNA insertional Basmati 370 transgenic lines were obtained from PAU Ludhiana (Dhaliwal *et al.*, 2001). The details of Hm^R*Ds* construct used for transformation of Basmati 370 are given in (Fig. 3.1).

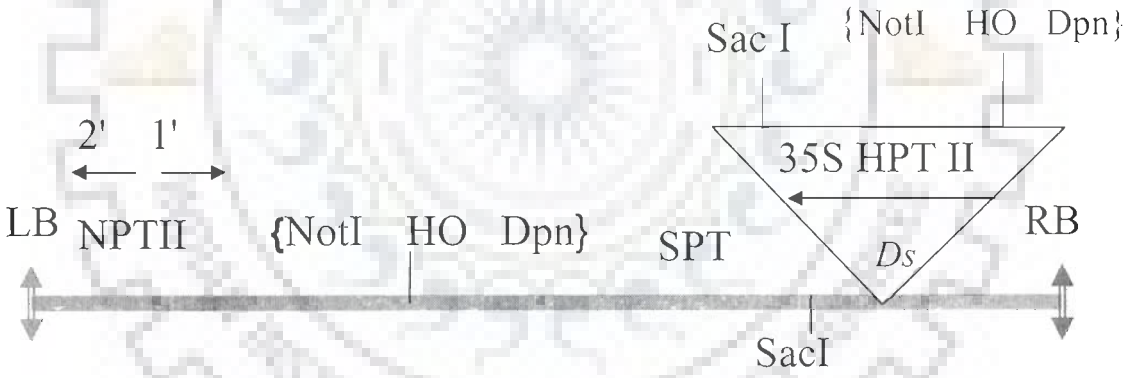


Fig. 3.1: Schematic presentation of the Hm^R*Ds* construct that was used to transform Basmati 370 via *Agrobacterium*. LB: Left border,RB: Right border, SPT: streptomycin phosphotransferase, NPT: Neomycin phosphotransferase, HPT: Hygromycin phosphotransferase. *Not*I, *HO* nuclease and *Dpn/Cla*I methylase: Rare cutter sites within the T-DNA and within the *Ds* element. *Sac*I is the unique restriction site in the construct.

Hm^R*Ds* was made by replacing the internal HindIII (position 1783) - XhoI (Position 3557) fragment of *Ac* with a 1.6Kb 35S - hygromycin phosphotransferase (*hpt*II) - OcS 3' fusion. The 'simple *Ds*' and 'Hm^R*Ds*' were cloned into the binary vector pCLODIII (Dean *et al.*, 1992) which carries the left border (T-LB) and right

border (T-RB) of the *Agrobacterium* T-DNA, NPTII (neomycin phosphotransferase), selectable marker and a 35S-SPT fusion. The Hm^R*Ds* also carried three rare cutter sites, *Not*I, HO, nucleas and *Dpn/Cla*I methylase within the T-DNA and within the *Ds* element.

3.1.2 Generation of mapping population

Crosses were made between T-DNA insertional Oligo culm mutant (*Osoc*) with distantly related rice cultivar PR106. The F_1 plants were selfed to get F_2 and selfing continued upto F_4 generations at IIT Roorkee under containment conditions with the recommended package of practices for Basmati rice. The F_2 seeds were screened for hygromycin resistance during germination in petriplates at a concentration of 80 ppm hygromycin.

3.1.3 Molecular Biology Kits

QIAquick gel extraction kit	Qiagen, Valencia, CA, USA
TRIzol plus RNA Purification kit	Invitrogen-life technologies, CA
RNeasy MinElute cleanup kit	Qiagen, Valencia, CA, USA
Superscript™ II RT kit	Invitrogen- life technologies, CA
Sequencing Quick Start Kit	Beckman Coulter, Fullerton, CA

3.1.4 Enzymes

Restriction endonucleases	New England Biolabs, Ipswich, MA
Taq DNA polymerase	Bangalore Genei, Bangalore
Reverse transcript, Superscript II	Invitrogen- life technologies, CA
DNAseI	Promega Madison, WI, USA

T4 DNA ligase	Bangalore Genei, Bangalore

3.1.5 Antibiotics

Hygromycin	Sigma- Aldrich, St Louis, MO, USA

3.1.6 Molecular weight Markers

1Kb, 500bp and 100bp DNA ladders	Bangalore Genei, New England Biolabs

3.1.7 Membrane

Hybond-N	GE Biosciences, Piscataway, NJ, USA

3.1.8 Radioisotopes

Radio labelled α-P^{32} dCTP was handled in accordance to the North Dakota Radiological Health Rules, Section 33-10-04.1-14, "Waste Disposal" North Dakota State University, ND, USA.

3.1.9 Buffers and solutions

10X TBE buffer	1L 108g Tris 55g Boric acid 40ml of 0.5M EDTA (pH 8.0)
TE buffer	10mM Tris-HCl (pH 8.0) 1mM EDTA (pH8.0)
20X SSC (pH 7.0)	1L 175.3g NaCl 88.2g Sodium citrate pH was adjusted to 7.0 and autoclaved
Denaturation buffer (For Southern)	1.5M NaCl 0.5N NaOH These were made from sterile 5M NaCl and 10N NaOH stocks respectively
Neutralization buffer (For Southern)	1.5M NaCl 1.0M Tris (pH 7.4)

	These were made from sterile 5M NaCl and 10N NaOH stocks respectively
Pre-hybridization buffer	6X SSC 5X Denhardt's reagent
Plant genomic DNA extraction buffer	2% Cetyl Trimethyl Ammonium Bromide (CTAB) 50mM EDTA (pH 8.0) 100mM Tris-HCl (pH 8.0) 1.4M NaCl Autoclaved and 1% B-mercaptoethanol was added before use
Phenol: Chloroform	Equal quantities of Tris saturated phenol (pH 7.6) and chloroform were mixed and stored in amber coloured bottles.
3M Sodium acetate (pH 5.2)	40.8g of $CH_3COONa .3H_2O$ was initially added to 80ml water and the volume adjusted to 1L and sterilized by autoclaving.
5X RNA gel loading buffer	0.2M MOPS (pH 7.0) 36% Formaldehyde 75% Deionized Formamide
10X DNA loading dye	0.4% bromophenol blue 0.4% Xylene cyanol FF 50% Glycerol in distilled water

3.2 Methods

3.2.1 Recording of phenotypic data

Five plants from each row of parents and 210 F_2 plants were scored for following traits as per Xiao *et al.*, 1998.

Tiller number: As the number of tillers bearing grain filled panicles of a plant.

Plant height: Average height (cm) from the ground to the tip of the panicle excluding the awns.

Days to heading: Number of days from date of sowing in the field until 10% of the panicles had emerged.

3.2.2 DNA isolation

Young leaves were collected from 5-6 week old plants from field. DNA was isolated by CTAB method as described by Rogers and Bendich (1988) with little modifications. Approximately 5g of leaf tissue was washed and dried properly followed by grinding to fine powder with liquid nitrogen using grinding shaft and transferred to 30ml polypropylene tubes. 15ml of Pre-warmed extraction buffer was added to frozen tissue mixed thoroughly and incubated at 65^0C for 45min with intermittent shaking followed by addition of Equal volume of Phenol:chloroform:isoamyl alcohol (25:24:1) which was mixed by inversion for 15min an then centrifuged at 8,000 x g for 10min at 25^0C. The clear upper aqueous phase was transferred to fresh polypropylene tube and DNA was precipitated with 0.7 volumes of isopropanol. The spool of DNA was removed, air dried and dissolved in high salt TE buffer. This DNA solution was given an RNase treatment (with DNase free RNaseA) at 37^0C for 1hrs and extracted with Chloroform: Isoamylalcohol (24:1). The DNA was reprecipitated with 2 volumes of ethanol and dissolved in TE buffer then checked on a 0.8% agarose gel in 1X TAE [40mM Tris-acetate, 1mM EDTA (pH 8.0)] buffer.

3.2.3 *hpt* amplification

hpt fragment was amplified with 20ng of DNA, 0.2mM of each of the dNTP's, 10pmole of forward and reverse primer (5′ GTC TGT CGA GAA GTT TCT GAT CG 3′ and 5′ GCG AGT ACT TCT ACA CAG CCA T 3′), 0.6U of Taq DNA polymerase

and 1X PCR buffer (50mM KCl, 10mM Tris-HCl, 1.5mM $MgCl_2$) in a total reaction volume of 20µl. The PCR amplification was performed in PTC-200 thermalcycler (M J Research, USA), initial denaturation step of 94°C for 5min, followed by 25 cycles of denaturation at 94°C for 30sec, annealing at 58°C for 20sec and extension at 72°C for 1min with final extension of 72°C for 5min.

3.2.4 Southern hybridization

1.5µg of genomic DNA of Oligo culm, Seedling lethal and wild type Basmati 370 were digested with the 10U of four restriction enzymes (*HindIII*, *DraI*, *EcoRV* and *EcoRI*) separately at 37°C overnight. The resultant fragments were separated by electrophoresis through 0.9% agarose gel, using the 1X TAE buffer at 20V for 24hrs until the bromophenol blue dye had migrated to the appropriate position. The DNA was electrophoresed at low voltage for longer period for better separation of fragments. The gel was stained with ethidium bromide, rinsed in distilled water and photographed. The gel was destained in distilled water and DNA was purinated in 0.25N HCl for ten minutes and then neutralized with 0.4N NaOH. The DNA from the gel was transferred to a Biodyne/Hybond N^+ membrane (Amersham Inc.) by the disposable gel transfer system using 0.4N NaOH solvent. Three sheets of Whatman filter papers (3MM) were placed in such a way that the edges were dipped into the tray containing 0.4N NaOH for capillary transfer. Gel was placed on filter paper followed by Biodyne/Hybond N^+ membrane and three sheets of Whatman filter papers presoaked in 0.4N NaOH and paper towels (15cm thick) overnight. This arrangement allowed the DNA to transfer to the membrane. *hpt* (950bp) probe was labelled from specific PCR product amplified by using *hpt* specific primers (5′ GTC TGT CGA GAA GTT TCT GAT CG 3′ and 5′ GCG AGT ACT TCT ACA CAG

CCA T 3'). The PCR product was eluted form gel and purified using the QIAGEN QIAquick PCR purification kit (USA). PCR product (25ng) was made up to 8µl with sterilized double distilled water for 10min to denature. 2µl of hexanucleated buffer; 3µl of dNTP (AGT); 2µl of Klenow fragment and 5µl of radiolabelled α-P^{32} dCTP were added to the PCR product and incubated for 1hr and purified through spin columns containing Sephadex G50. The blot was prehybridized with prehybridization buffer at 65°C for 2hr. 30µl of buffer was added to the Radio labelled probe and denatured it by adding equal volume of 0.4N NaOH. Denatured probe was added to the blot and incubated for 20hr at 65°C. Hybridization membrane was washed at 65°C for 30min each in 2X SSC and 1X SSC followed by 1hr in 0.5X SSC. All washing solutions contained 0.1% (w/v) SDS. The hybridized blot was exposed to X-Ray film for 7 days which revealed the specific hybridization signals.

3.2.5 Bulk Segregation Analysis

3.2.5.1 Detection of polymorphic SSR markers

Two hundred and nine rice microsatellites SSRs (simple sequence repeats) primer pairs were selected representing all the 12 rice chromosomes covering both arms for initial parental polymorphism between Basmati 370 and PR106. Information regarding chromosomal location, SSR sequence of primers was given by Temynkh *et al.* (2000 and 2001) and Cho *et al.* (2000) (Appendix: 1). PCR was carried out in 20µl reaction containing 50ng of DNA, 0.1mM of each of the dNTPs, 0.25µM of forward and reverse primers, 0.6U of Taq DNA polymerase and 1X PCR buffer (50mM KCl, 10mM Tris-HCl, 1.5mM $MgCl_2$) using 94°C for 5min, followed by 35 cycles of denaturation at 94°C for 30sec, annealing at 55°C for 30sec and extension at 72°C for 2min with final extension at 72°C for 7min.

3.2.5.2 Resolution of amplified SSRs product

After the completion of the reaction 2µl of 10X loading dye (0.4% w/v bromophenol blue, 0.4% w/v Xylene Cyanol FF, 50% Glycerol) was added. The PCR product was loaded on 2.5% LMP high resolution superfine agarose (Promega) prepared with 0.5X TBE buffer. Gels were stained with 1µg/ml ethidium bromide and then visualized under UV light and photographed using UVP Gel documentation system (Model GDS 7600) with GRAB-IT software programme (Annotating Grabber 32-Bits).

3.2.5.3 Identification of linked microsatellite markers

Two bulks were made from the selected rows of segregating F_3 mapping population of *Osoc* and PR106. Bulks were prepared from 12 homozygous *hpt* +ve Oligo culm F_3 progenies (bulk 1) and *hpt* -ve high tillering lines (bulk 2). An equal concentration of DNA from these lines was pooled. These two bulks and parents were used to identify putative linked markers to Oligo culm mutant. The microsatellite markers distinguishing parents and bulks were used to analyse individual progenies constituting each bulk. Putative markers were applied on the F_2 segregating population. Linkage analysis was done using recombination frequency between marker and the mutant phenotype at each locus.

3.2.6 Genome Walking

3.2.6.1 Restriction digestion

Highly purified and intact genomic DNA of *Osoc*, *Ossl* and Basmati 370 was used for restriction digestion. Five different blunt end restriction enzymes were used

to generate genome walking libraries. For complete digestion, 500ng of genomic DNA was restricted with 30U of *EcoRV, DraI, RsaI, HpaI* and *HaeIII* separately at 37°C overnight. The digested DNA was ethanol precipitated after Phenol: Chloroform (1:1) extraction. The DNA pellet was re-suspended in water and 500ng of completely digested DNA was used to ligate with the "Genome Walker Adaptor".

3.2.6.2 T-DNA specific Primers

Following are the T-DNA specific primers used for Genome Walking experiment. The specificity of each primer was confirmed by comparing its sequence with all the predicted rice coding sequences using the BLAST tool at NCBI to ensure that each pair targets a unique site within the set of predicted rice coding sequences.

T-LB 1: (5`-TGGGTATCTGGGAATGGCGAAATA-3`)

T-LB 2: (5`- CAAGGCATCGATCGTGAAGTTT-3`)

T-LB 3: (5`- AATGTAGACACGTCGAAATAAAGA-3`)

T-LB 4: (5`- CATGTAGATTTCCCGGACATGAAGCCATTT-3`)

T-LB 5: (5`- TCGCAAAGTATTTGAACGCAGGTACAATCG-3`)

T-RB 1: (5`-GGGGCATCGCACCGGTGAGTAAT-3`)

T-RB 2: (5`-AGCGAATTTGGCCTGTAGACCTCA-3`)

T-RB 3: (5`- TATTCGGGCCTAACTTTTGGTGTG-3`)

T-RB 4: (5`-GGCCTGTAGACCTCAATTGCGAGCTTTCTA-3`)

T-RB 5: (5`-GGCCTAACTTTTGGTGTGATGATGCTGACTG-3`)

3.2.6.3 Genome walker adaptor

Genome walker adapter was constructed by mixing 48bp sequence and 3` modified 8bp sequence followed by heating at 95°C for 10 minutes and then kept at room temperature for one hour.

Genome Walker Adaptor:

5'–GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT–3'
3'–**H2N**-CCCGACCA-PO4–5'

Adaptor-specific primers:

AP1: (5' -GTAATACGACTCACTATAGGGC-3')

AP2: (5' -ACTATAGGGCACGCGTGGT-3')

3.2.6.4 Ligation

Completely digested 500ng of DNA was used to ligate with the "Genome Walker Adaptor". The ligated products were made to final volume of 80µl, out of which 1µl containing approximately 6.25ng of template DNA was used for the first Genome walking PCR.

3.2.6.5 Genome walking PCR Conditions

The PCR reactions were carried out in a total volume of 20µl containing the following to a final concentration: 1X Taq DNA polymerase buffer, 1.5mM $MgCl_2$, 0.2mM of each (dATP, dTTP, dCTP, dGTP), Taq polymerase and 0.2µM of each primer. T-DNA specific primer (T-RB1: 5' GGGCATCGCACCGGTGAGTAAT 3') and Adaptor specific primer (AP1: 5' GTAATACGACTCACTATAGGGC 3') were used for the primary PCR. The PCR product was separated on agarose gel. The primary PCR product was diluted fifty fold and 1µl of diluted PCR product was used as template for the secondary PCR.

The secondary PCR reaction was carried in a total volume of 50µl containing the following to a final concentration; 1X Taq DNA Polymerase buffer, 1.5mM $MgCl_2$, 0.2µM of each primer (adaptor specific and T-DNA specific), 1µl diluted first PCR reaction as template and Taq DNA polymerase. The T-DNA specific primer

Left Border Sequence

Right Border Sequence

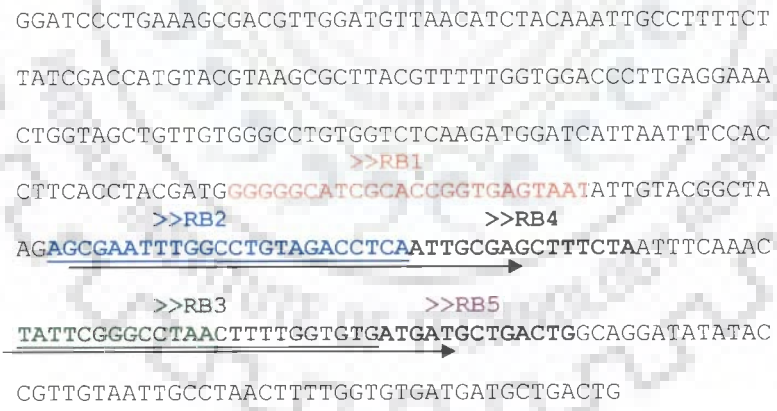


Fig. 3.2: Sequence of T-DNA left and right border and positions of T- DNA nested specific primers.

(T-RB2: 5′ AGCGAATTTGGCCTGTAGACCTCA 3′) and an adaptor-specific primer (AP2: 5′ ACTATAGGGCACGCGTGGT 3′) were used. The amplified product from the secondary PCR reaction was resolved using 1% (w/v) agarose gel and 1X TBE buffer.

Tertiary PCR was performed using T-DNA specific primer (T-RB3: 5′ TATTCGGGCCTAACTTTTGGTGTG 3′) and an adaptor-specific primer (AP2: 5′ ACTATAGGGCACGCGTGGT 3′) and template of fifty fold diluted secondary PCR product with similar set of PCR conditions using Applied Biosciences 2720 Thermal Cycler, USA. The schematic presentation of Genome Walking method is given in Fig. 3.3. The PCR conditions for Genome Walking are given in Table 3.1.

Table 3.1: PCR Cycles used for Genome Walking.

Reaction	Cycle number	Thermal cycling conditions
Primary	1	94^0C (3min)
	10	94^0C (30sec), 72^0C - 67^0C (45sec) (-0.5^0C / per cycle), 72^0C (2.5min)
	30	94^0C (30sec), 67^0C (45sec), 72^0C (2.5min)
	1	72^0C (7min)
	1	4^0C (Forever)
Secondary	1	94^0C (3min)
	10	94^0C (30sec), 72^0C - 67^0C (45sec) (-0.5^0C / per cycle), 72^0C (2.5min)
	30	94^0C (30sec), 67^0C (45sec), 72^0C (2.5min)
	1	72^0C
	1	4^0C (Forever)
Tertiary	1	94^0C (3min)
	35	94^0C (30sec), 72^0C - 67^0C (45sec) (-0.5^0C / per cycle), 72^0C (2.5min)
	1	72^0C
	1	4^0C (Forever)

3.2.7 TAIL-PCR

T-DNA specific primers T-RB1, T-RB2 and T-RB3 and arbitrary degenerate primers (AD) were used for TAIL-PCR experiment.

Sequence of arbitrary primers

AD1: 5' NTCGASTWTSGWGTT 3' (64-fold degeneracy)

AD2: 5' NGTCGASWGANAWGAA 3' (128-fold degeneracy)

AD3: 5' WGTGNAGWANCANAGA 3' (256-fold degeneracy)

Where, N= A/C/G/T, S= C/G, and W= A/T

3.2.7.1 TAIL-PCR procedure

20μL of primary TAIL-PCR reactions contained 0.75X PCR buffer (10mM Tris-HCl, pH 8.3, 1.5mM $MgCl_2$), 200μM each of dNTPs, about 200ng of genomic DNA, 0.8 unit of Taq polymerase, 0.15μM T-RB1 and a given AD primer (1.5μM for AD1, 2μM for AD2 and AD3). 1μL of the primary PCR product was diluted with 49μL distilled water and 2μL of diluted DNA was used as template for secondary reactions. 20μL of secondary reaction contained 1X PCR buffer, 0.6 unit of Taq polymerase, 200μM each of dNTPs, 0.2μM T-RB2 and the same AD primer used in the primary reaction (1.5μM for AD1, 2μM for AD2 and AD3). 100μL of tertiary PCR mixture contained 1X PCR mixture, 0.6 unit of Taq polymerase, 200μM each of dNTPs, 0.2μM T-RB2 and the same AD primer used in the primary reaction (1.5μM for AD1, 2μM for AD2 and AD3). 1μL of the secondary PCR product was diluted with 9μL distilled water and 2μL of the diluted DNA was used as template DNA in tertiary PCR reactions. The schematic presentation of TAIL-PCR is given in Fig. 3.4. Primary, secondary and tertiary reactions were executed as summarized in Table 3.2 in Biorad thermal cyclers.

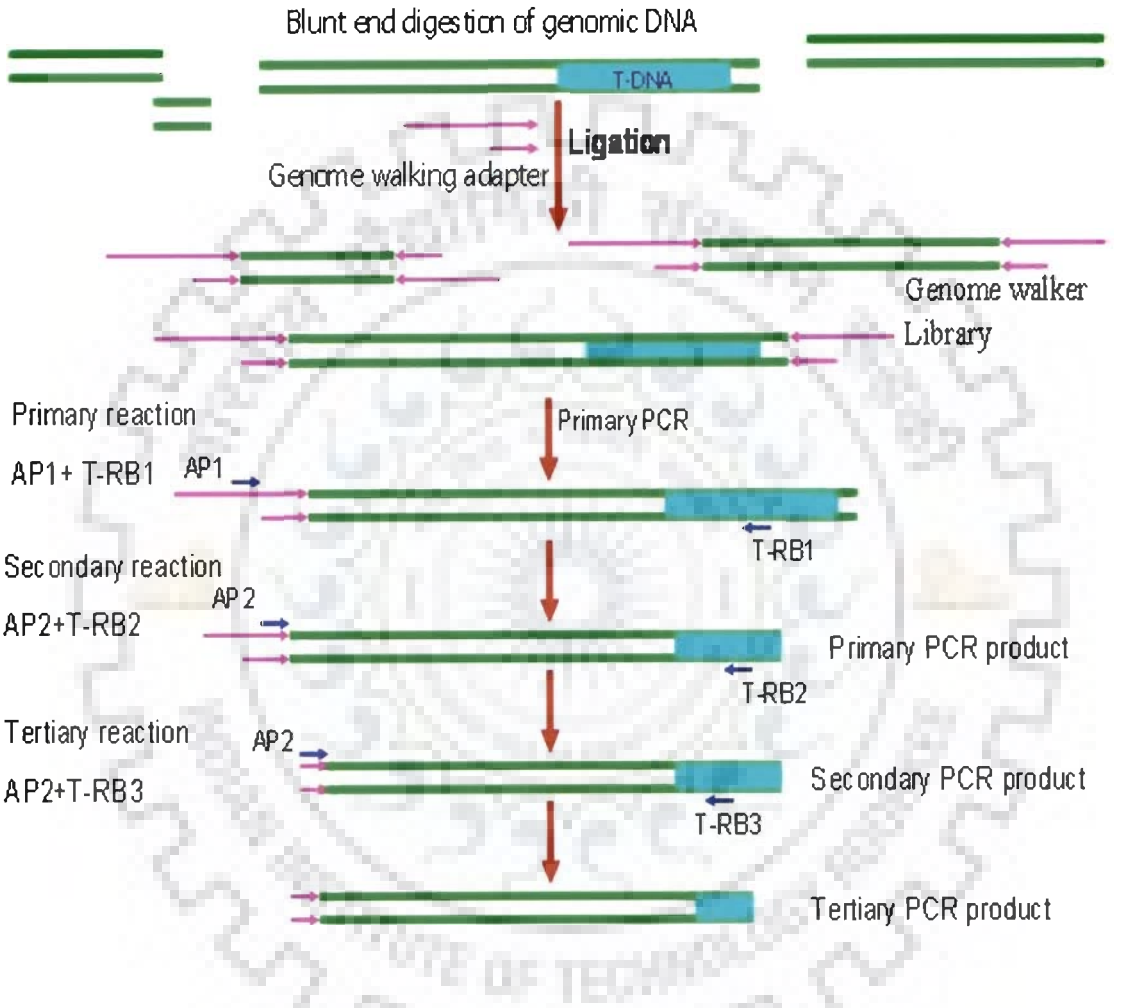


Fig. 3.3: Diagrammatic presentation of Genome walking. T-RB: T-DNA right border specific primers, AP: Adapter specific primers.

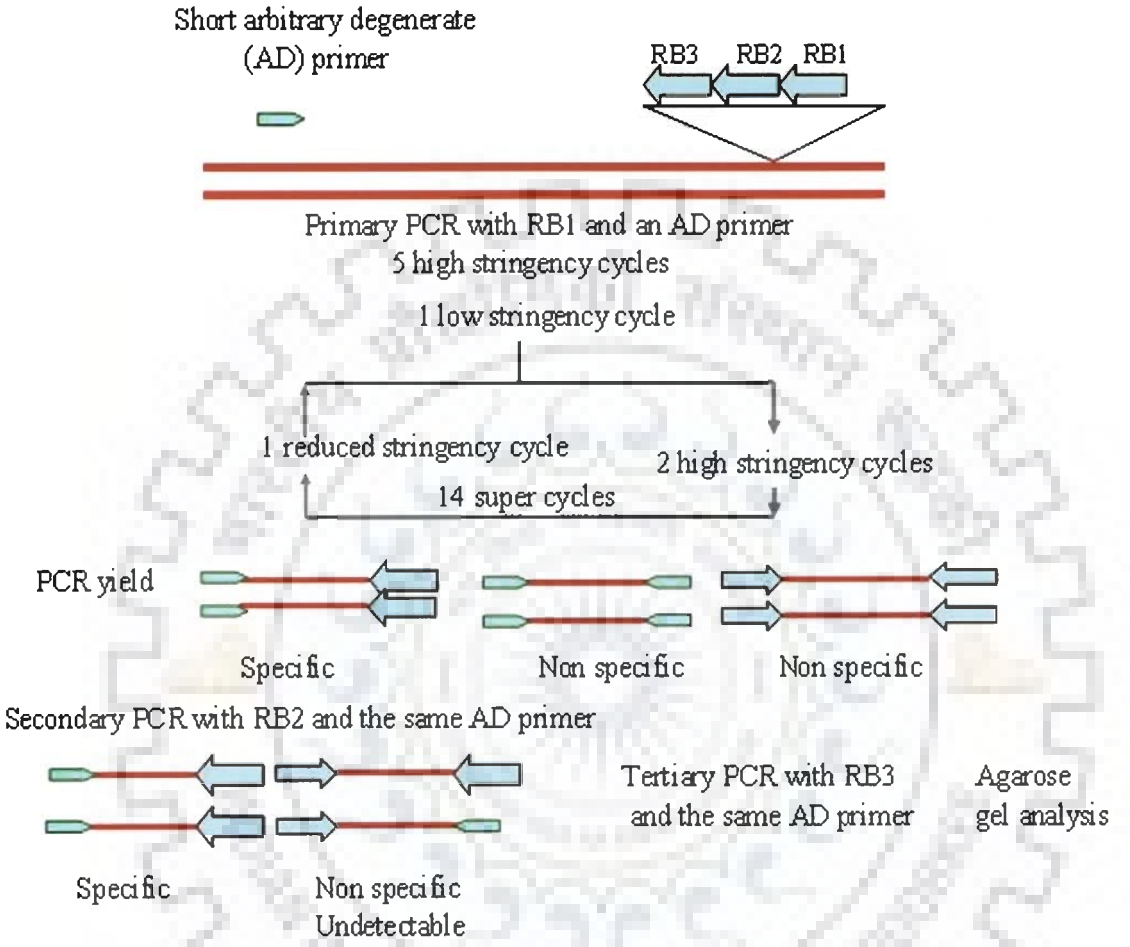


Fig. 3.4: Diagrammatic presentation of TAIL-PCR experiment.

Table 3.2: PCR Cycle conditions used for TAIL-PCR.

Reaction	No. of cycles	Temperature(^{0}C)	Time
Primary reaction	1	93 95	1min. 1min.
	5	94 62 72	1min 1min. 2.30min.
	1	94 25 72	1min. 3min. 2.30min.
	15	94 68 72 94 68 72 94 44 72	30sec. 1min. 2.30min. 30sec. 1min. 2.30min. 30sec. 1min. 2.30min.
	1	72	5min.
Secondary reaction	1	94	1min.
	15	95 63 72 94 63 72 94 44 72	10sec. 1min. 2min. 10sec. 1min. 2min. 10sec. 1min. 2min.
	1	72	5min.
Tertiary reaction	1	95	3min.
	30	94 44 72	20sec. 1min. 2min.
	1	72	10min.

3.2.8 Confirmation of T-DNA insertion positions

The position of T-DNA insertion was confirmed using T-DNA specific and genome specific primers using Basmati 370 and mutant DNA template. For these five T-DNA specific primers (T-RB1, T-RB2, T-RB3, T-RB4 and T-RB5) and two nested genome-specific primers Oligo-RB1, Oligo-RB2, Oligo-LB1 and Oligo-LB2 for Oligo culm and Seedling lethal-RB1, Seedling lethal-RB2, Seedling lethal-LB1 and Seedling lethal-LB2 for Seedling lethal mutant were used. Conditions were 94°C for 3min; [Touchdown PCR 94°C, 30sec; 70°C to 65°C (-0.5°C /cycle), 45sec; 72°C, 150sec] for 10 cycles; [94°C for 30sec; 65°C for 45sec; 72°C for 150sec] for 30 cycles and an additional cycle of 72°C for 7min using Applied Biosciences 2720 Thermal Cycler, USA.

Oligo culm and Seedling lethal mutants Genome-specific nested primers sequence:

Oligo-RB1: (5`-GGCATAGAATTTGCAGGTCATGAAG-3`)

Oligo-RB2: (5`-TGTAAATCCGATGGTTCAACAAACC-3`)

Oligo-LB1: (5`-CTGTTTACAATTCGTGCTGCATTTG-3`)

Oligo-LB2: (5`-AAGTTTCCGCTCGGTATACAACTCC-3`)

Seedling lethal-RB1: (5`-TGCAACCCAACCTCGCAATC-3`)

Seedling lethal-RB2: (5`-TTTGCGCTCAAGTTCGACACA-3`)

Seedling lethal-LB1: (5`-CCAACCCTGGCACTAATTCCTGA-3`)

Seedling lethal-LB2: (5`-GCACACGCGGTATGCATGGT-3`)

3.2.9 Cloning of Basmati 370 Oligo culm gene.

Seven sets of primers were designed based on Nipponbare sequence. PCR was performed using Oligo culm gene specific primers and Basmati 370 genomic DNA as the template. The PCR conditions were 94°C for 5min; 35 cycles of 94°C, 30sec;

55°C, 30sec; 72°C, 120sec; and final extension at 72°C for 7min using Applied Biosciences 2720 Thermal Cycler, USA. PCR products were eluted from gel and sequenced using both forward and reverse primer.

Primer sequence designed for sequencing of Oligo culm gene in Basmati 370:

Oligo F1: 5'-TACTGCTAGCTGCAGCGTCT-3'

Oligo R1: 5'-TCAAGCGGTTGCACTTAAAA-3'

Oligo F2: 5'-GTTTTGGGTACGGTCAGAGC-3'

Oligo R2: 5'-GCGCCACAAAGAGACAAAGT-3'

Oligo F3: 5'-TCTATGTGTTTCCTTCATGCTG-3'

Oligo R3: 5'-TGCTCGATTGCAAAGTTCAC-3'

Oligo F4: 5'-ACCATGTGGCCATTCGTTT-3'

Oligo R4: 5'-TCCAAAAGGACTAGCGAGGA-3'

Oligo F5: 5'-CCCTCCAAACCTGTCATTTG-3'

Oligo R5: 5'-GCTGGCATTGTCATCTTCAT-3'

Oligo F6: 5'-GTGAACAACCCTGAGGAGGA-3'

Oligo R6: 5'-TCAGGCACGACGACATGTTA-3'

3.2.10 Gel elution and purification of PCR product

The DNA fragment was excised from the agarose gel with a clean and sharp scalpel. 3 volumes of Buffer QG (provide in the QIAquick gel extraction kit) was added to 1 volume of gel and incubated for 10min at 50°C. After the gel slice had dissolved completely, 1 gel volume of isopropanol was added. QIAquick spin column was placed in the 2ml collection tube and centrifuged for 1min. Added 0.5ml Buffer QG and centrifuged for 1min followed by addition of 0.75ml Buffer PE (provided in the kit) and centrifuged for 1min with and additional run of 1min. Placed the column in the clean 1.5ml microcentrifuge tube. Elution of DNA was done adding 20µl of

water was added to the centre of the QIAquick membrane and centrifuged the column for 1 minute.

3.2.11 Direct Sequencing of PCR product

The sequencing reactions were carried in a total volume of 20µl using ECQ Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter) containing the following to a final concentration; 700bp and 260bp gel eluted fragments of second Genome Walking PCR (65ng-130ng); sequencing primer T-RB2/AP2 (5µM) and DTCS Quick Start master mix (8µl). The thermal cycling program was, 96°C for 30sec, 50°C for 20sec, 60°C for 4min for 30 cycles. 5µl of stop solution/ Glycogen mixture [2µl of 3M Sodium Acetate (pH 5.2), 2µl of 100mM Na_2EDTA (pH 8.0) and 1µl of 20mg/ml of glycogen] was added to the sequencing PCR product. For ethanol precipitation added 30µl of cold 95% v/v ethanol followed by centrifugation at 14,000rpm at 4°C for 15 minutes. Rinsed with 70% (v/v) ethanol and vacuum dried; re-suspended the sample in 40µl of the sample loading solution (provided in the kit). The re-suspended samples were transferred to the appropriate wells of the CEQ sample plate overlaid with a drop of light mineral oil. The sample plate was loaded into the Beckman Coulter sequencing machine.

3.2.12 RT-PCR

3.2.12.1 Total RNA isolation

Roots and shoots of the Oligo culm mutant and Basmati 370 were freeze dried after 6 and 12 days of seed germination. The frozen specimen was removed from the freezer and placed into TRIZOL reagent in 1.5ml eppendorf and homogenized using a tissue homogenizer and incubated for 5min at room temperature. Added 0.1ml

chloroform and was shaken vigorously for 15-30sec by hand and incubated at room temperature for 2-3min. Centrifuged at 12000X g for 15min at 4°C, removed the upper colourless layer into a fresh tube. Added 1/10 volume of sodium acetate and two volumes of ethanol followed by incubation for 30min at 20°C. Centrifuged at 12000X g for 15min at 4°C, pellet was washed with 70% ethanol and resuspended in 50µl RNase free water. RNA was quantified by using Nanodrop method.

3.2.12.2 DNase treatment and cleaning of RNA

DNA was removed by DNase treatment. 10µl reaction consisted of 8µl RNA in DEPC treated water, 1U/µg RQ1 RNase free DNase and 1µl 10X reaction buffer was incubated at 37 °C for 30min. Resulted RNA was Phenol:chloroform extracted and precipitated with sodium acetate and ethanol. RNA was pelleted and washed with 70 % ethanol and resuspended in DEPC-treated water.

RNA was further cleaned up using RNeasy MinElute cleanup kit. 350µl buffer RLT was added to 100µl of RNA and mixed thoroughly, followed by addition of 250µl of 100% ethanol and mixed thoroughly. Added 700µl of sample to an RNeasy MinElute spin column in a 2ml collection tube; centrifuged for 15sec at 8000X g. Discarded the flow through and added 500µl of 80% ethanol to the spin column; centrifuged for 2min at 8000X g; discarded the flow through and opened the cap of the spin column and centrifuged at high speed for 5min and discarded the flow through. Transfer the spin column in new 1.5ml collection tube, pipetted out 14µl of RNase free water directly on to the centre of the silica gel membrane, centrifuged to the maximum speed to elute RNA and quantified by using Nanodrop method (NanoDrop Tech).

3.2.12.3 Resolution of total RNA

The electrophoretic unit was treated with 1N NaOH overnight. All the solutions were prepared in DEPC treated water. 2µl of total RNA mixed with 2µl of STS loading dye was loaded on the gel and run at 50V. Gel was visualized and photographed.

3.2.12.4 cDNA synthesis

Total mRNA was reverse transcribed using the superscript^{TM} II RT kit (Invitrogen). 20µl reaction was set using 1µg of total RNA; 500µg/ml oligo (dT) $_{12\text{-}18}$ and 1µl dNTP mix (10mM each) was heated to 65°C for 5 min and quick chilled on ice. Followed by brief centrifugation, added 4µl of 5X first strand buffer; 2µl of 0.1M DDT; 40U/µl RNase OUT. Mixed the contents of the tube thoroughly and incubated at 42°C for 2 minutes. Added 1µl (200U) of Super Script II RT and mixed by pipetting gently up and down and incubated at 42°C for 90min. Inactivated the reaction by heating at 70°C for 15min. 2µl of this cDNA was used for the RT-PCR.

3.2.12.5 RT-PCR conditions

PCR reaction was carried out in 20µl volume containing 20ng of DNA; 0.2mM of each of the dNTP's; 10pmole of forward and reverse primers; 0.8U of Taq DNA polymerase and 1X PCR buffer (50mM KCl, 10mM Tris-HCl, 1.5mM $MgCl_2$). The PCR amplification was performed in PTC-200 Thermalcycler (M J Research, USA) with the thermal profile; initial denaturation step of 94°C for 5min, followed by 35 cycles of denaturation at 94°C for 20sec, annealing at 62°C for 20sec and extension at 72°C for 30sec with final extension of 72°C for 5min.

Oligo culm gene specific primer sequence

RTOligo-F: 5′ CGACATATACAGCGAATGGATC 3′

RTOligo-R: 5′ TCTTGTTCAGGCACGACCAC 3′

Actin (Reference) gene specific primer sequence

Actin F- 5'ATCCTTGTATGCTAGCGGTCGA 3'

Actin R- 5' ATCCAACCGGAGGATAGCATG 3'

3.2.13 Scanning electron microscopy

For Scanning Electron Microscopy (SEM), Seedling lethal and Basmati 370 leaf samples were fixed in FAA (20% formaldehyde, 5% acetic acid and 60% alcohol) fixative solution at 4°C. Leaf samples were dehydrated in a graded 2.5% glutaraldehyde/ethanol series. The samples were mounted on scanning electron microscopy stubs and coated with gold (Bozzola and Russell *et al.*, 1999).

3.2.14 Bioinformatics tools and Database

Various bioinformatics tools used for homology search (BLAST) (Altschul *et al.*, 1990), retrieving selected genomic region of rice (mapview and entrez), searching for paralog and orthlogs (dpget-bin), retriving EST profile data for specific gene (UniGene), prediction of introns, exons and amino acid sequence from Oligo culm gene sequence (GENSCAN), alignment of protein sequence (ClutalW) and designing primers (Primer3) are listed below.

http://www.ncbi.nlm.nih.gov/blast

http://www.ncbi.nlm.nih.gov/mapview/maps.

http://www.ncbi.nlm.nih.gov/sites/entrez

http://www.genome.jp/dbget-bin

http://www.ncbi.nlm.nih.gov/UniGene/

http://genes.mit.edu/GENSCAN

http://www.frodo.wi.mit.edu/

http://www.tools.neb.com/NEBcutter2

http://signal.salk.edu/

CHAPTER 4

RESULTS

The present study was undertaken with a view to characterize the Basmati 370 insertional mutants. Three phenotypic mutants Oligo culm (*Os*oc), Seedling lethal (*Os*sl) and Small grain (*Ossg*) were identified among twenty four independent transformants. The results of molecular mapping and cloning of the Oligo culm gene and molecular characterization of Seedling lethal (*Ossl*) and Small grain (*Ossg*) mutants are presented below.

4.1 Confirmation of the presence of T-DNA in Mutants

The T-DNA containing Hm^R*Ds* was used to generate insertional mutants of Basmati 370. *Ds* element contained *hpt* (hygromycin phosphotransferase) gene as the selectable marker. The insertional mutant seeds were checked for the presence of *hpt* gene which showed resistance to hygromycin. The Basmati 370 seeds were germinated in a petri plate in water containing different concentrations of hygromycin (10-100ppm). The Basmati 370 seeds did not germinate at 80ppm hygromycin concentration. All the transgenic plants germinated at a concentration of 80ppm hygromycin (Fig. 4.1), indicating the presence of *hpt* gene in the insertional mutants. The presence of *hpt* gene was further confirmed by PCR amplification of 950bp fragment by *hpt* gene specific primers. Expected size of amplification was observed in the insertional mutants and no amplification was obtained in wild type Basmati 370 (Fig. 4.2).

4.2 Oligo culm (*Osoc*), Seedling lethal (*Ossl*) and Small grain (*Ossg*) mutants of Basmati 370

Basmati 370 insertional mutants, Oligo culm, Seedling lethal and Small grain size mutants were selected for molecular characterization and identification of candidate genes, because of their importance in grain yield and plant development. The Oligo culm mutant has 60% reduced tillering capacity. Field investigation of Oligo culm at different conditions showed 1-4 tillers per plant as compared 12-18 tillers per plant in the wild type Basmati 370 (Fig. 4.3 a and b). Tillering in rice is one of the important grain yield component, because tillers are grain bearing branches. In addition to few tillers the Oligo culm mutant has retarded growth of seminal roots (Fig. 4.3c) and reduced plant height with normal seed set. The seminal roots originate from the embryonic axis opposite to coleoptile.

In Seedling lethal mutant a proportion (5-15%) of albino seedlings (Fig. 4.4a) die soon after germination and Seedling lethal (10-20%) plants with highly retarded growth usually with single tiller and pale yellowish colour die (Fig. 4.4b) within a month of transplantation in soil. Scanning electron microscope results of leaf surface of Seedling lethal mutant and Basmati 370 showed cell death in the lethal seedling (Fig. 4.5). Grain weight is one of the most important yield components controlled by quantitative trait loci (QTLs) with complex genotype x environment interaction. The T-DNA insertional mutant, Small grain (*Ossg*) has 10-15% reduced grain weight and length compared to wild type Basmati 370 (Fig. 4.6).

4.3 Southern hybridization

Southern hybridization was performed to identify the copy number in Oligo culm and Seedling lethal mutants. Genomic DNA of mutants and Basmati 370 was

Fig. 4.1: Germination of Oligo culm (*Osoc*) insertional mutant seeds on 80ppm hygromycin. a- Oligo culm mutant, b- Basmati 370.

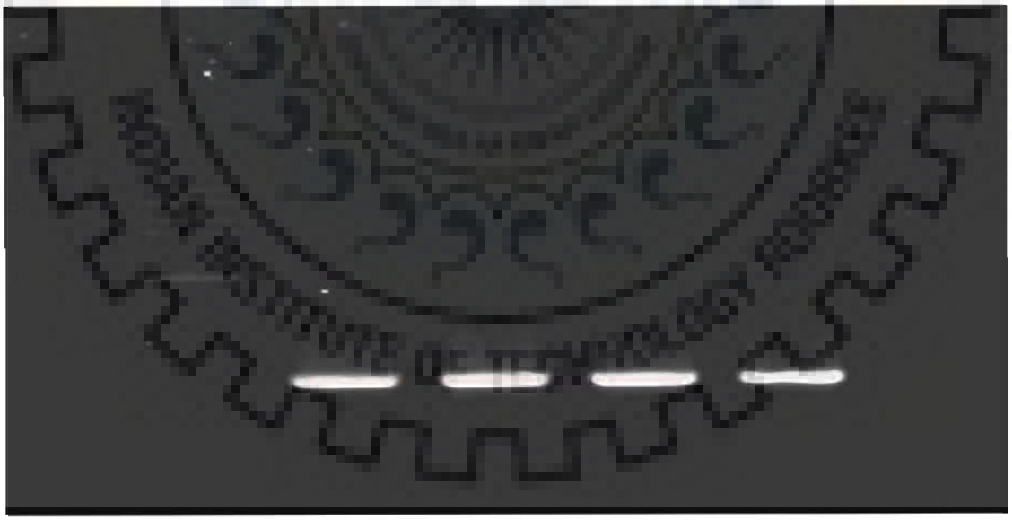


Fig. 4.2: PCR amplification of 950bp *hpt* fragment to confirm T-DNA insertion. M-100bp ladder, 1-Oligo culm mutant. 2- Seedling lethal mutant, 3&4- Positive controls, 5- Basmati 370.

Fig. 4.3: Morphological characterization of Oligo culm mutant (*Osoc*). a and b: Oligo culm plants showing few tillers and wild-type showing high tiller number. Fig. c: Retarded growth of seminal roots in Oligo culm and normal growth of seminal roots in wild type Basmati 370.

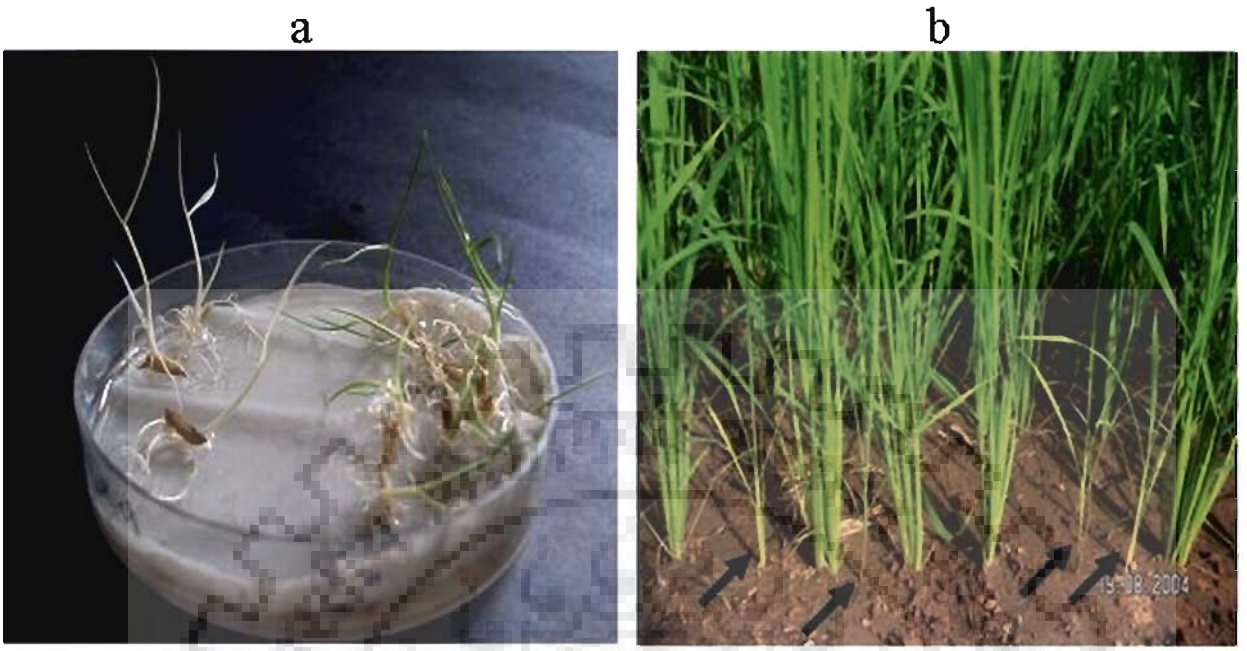


Fig. 4.4: Morphological characterization of Seedling lethal (*Ossl*) mutant. a: 10-25% albinos seedling during germination (left). b: Seedlings lethal (arrow) within a month of transplantation in soil.

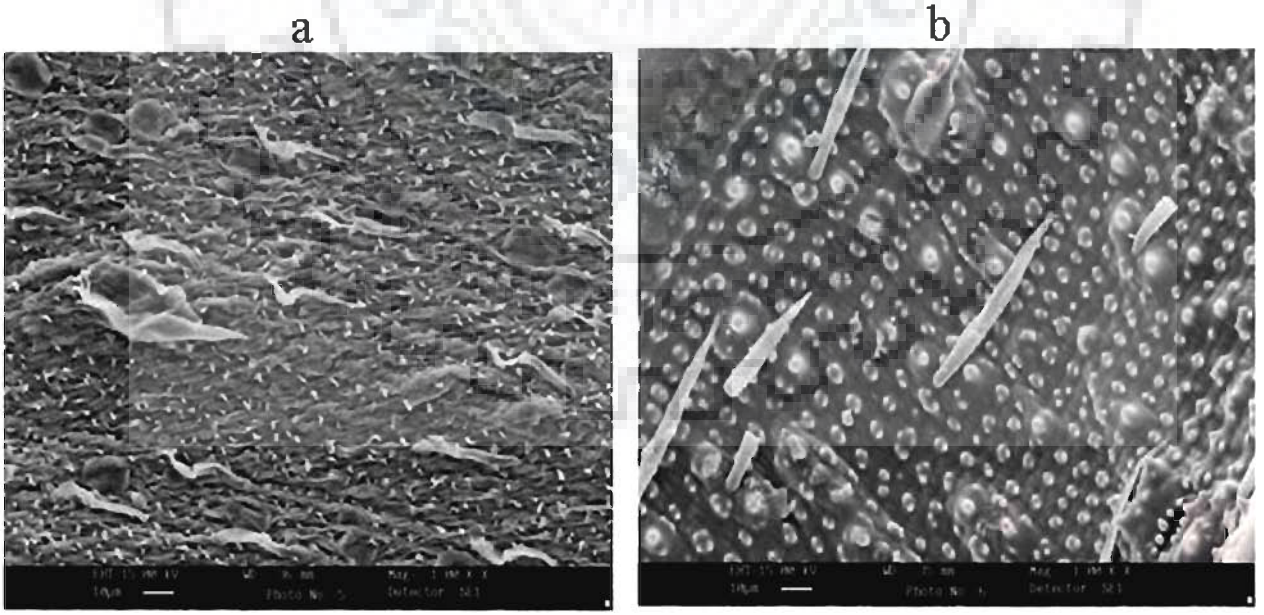


Fig. 4.5: Scanning electron Microscopic of leaf surface. a: Seedling lethal mutant. b: Wild type Basmati 370.

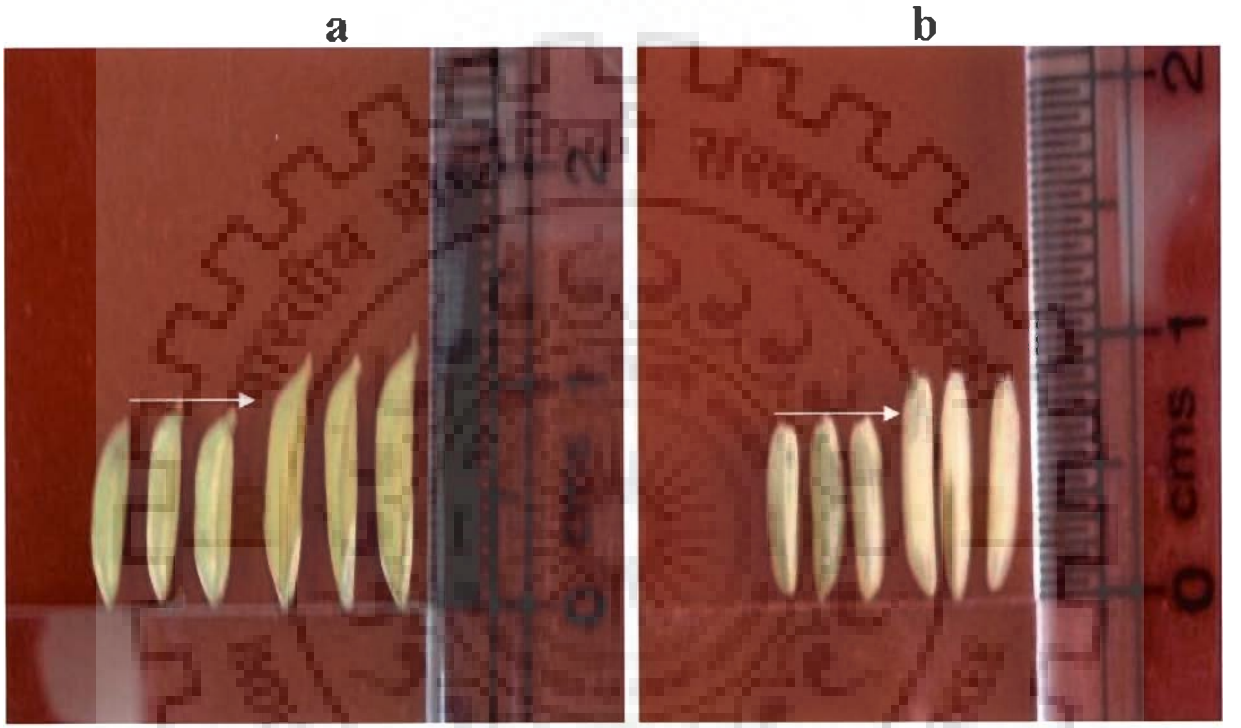


Fig. 4.6: Morphological characteristics of Small grains (*Ossg*) mutant a: With hull b: Without hull. Small grain (*Ossg*) mutant (left) and Basmati 370 (right).

digested with four different restriction enzymes *Eco*RV, *Eco*R1, *Dra*1 and *Hind*111 and hybridized with α-P^{32} dCTP radio labeled *hpt* amplified gene. Single band was observed in all the four restriction digestions of both mutants where as no hybridization was observed in wild type Basmati 370, indicating a single copy T-DNA insertions in the mutants (Fig. 4.7).

4.4 Inheritance pattern of *hpt* in F_2 population

Oligo culm mutant was crossed with Basmati 370 and distantly related fine rice variety PR106. The F_1 plants of Oligo culm mutant with Basmati 370 and PR106 showed 12-18 tillers per plant equal to that of the wild type Basmati 370 (Fig. 4.8), indicating that the mutant responsible for Oligo culm is recessive.

The *hpt* specific primers were used to study inheritance of T-DNA insertion in F_2, the population was derived from a cross of Oligo culm mutant and PR106. Out of 100 F_2 seeds 69 seeds germinated at 80ppm hygromycin and 31 seeds did not germinate. The ratio of germinated and non germinated seeds showed Mendelian segregation in 3:1 ratio (Table: 4.1). The germination data was further confirmed by *hpt* specific amplification in F_2 plants. Out of 204 plants, 141 plants showed *hpt* amplification (Fig. 4.9) and 63 plants were *hpt* negative. The goodness of fit of the observed number of positive and negative plants was tested against the expected segregation ratio for single insertion using $\chi 2$ test (Table: 4.2). The data showed a good fit to the expected ratio 3:1 suggesting that there was single T-DNA/*hpt* insertion in *Osoc* mutant. Phenotypic data for tiller number, days to flowering and plant height was collected from F_2 plants (Appendix: 2). Frequency distribution shows normal distribution for plant height suggesting that the absence of segregation distortion in the population (Fig. 4.10a). The frequency distribution for tiller number

in the F_2 plants (Fig. 4.10b) was skewed toward lower tiller number indicating the partial dominance of reduced tillering of Oligo culm (*Osoc*) mutant.

Table 4.1: Germination of F_2 seeds of *Osoc*/PR106 on 80ppm hygromycin.

Germination on 80ppm hygromycin			χ^2 value	
Geminated	Non germinated	Total	Observed (at 3:1 ratio)	Table (p>0.05) at 1*d.f.*
69	31	100	1.92	3.84

Table 4.2: *hpt* amplification in F2 plants of *Osoc*/PR106.

hpt amplification			χ^2 value	
hpt +ve	*hpt* -ve	Total	Observed (at 3:1 ratio)	Table (p>0.05) at 1*d.f.*
141	63	204	3.76	3.84

4.5 Bulk Segregant Analysis

4.5.1 Parental polymorphism

A total of 209 SSR markers uniformly spread over 12 chromosomes of rice were selected based on the rice maps of Temynkh *et al.* (2000) and McCouch *et al.* (2002). These markers were tested for parental polymorphism between Basmati 370 and PR106. Out of 209 SSR markers 98 (46.88%) markers were polymorphic. Schematic presentation of polymorphic SSR markers location on different rice chromosomes is given in Fig. 4.11.

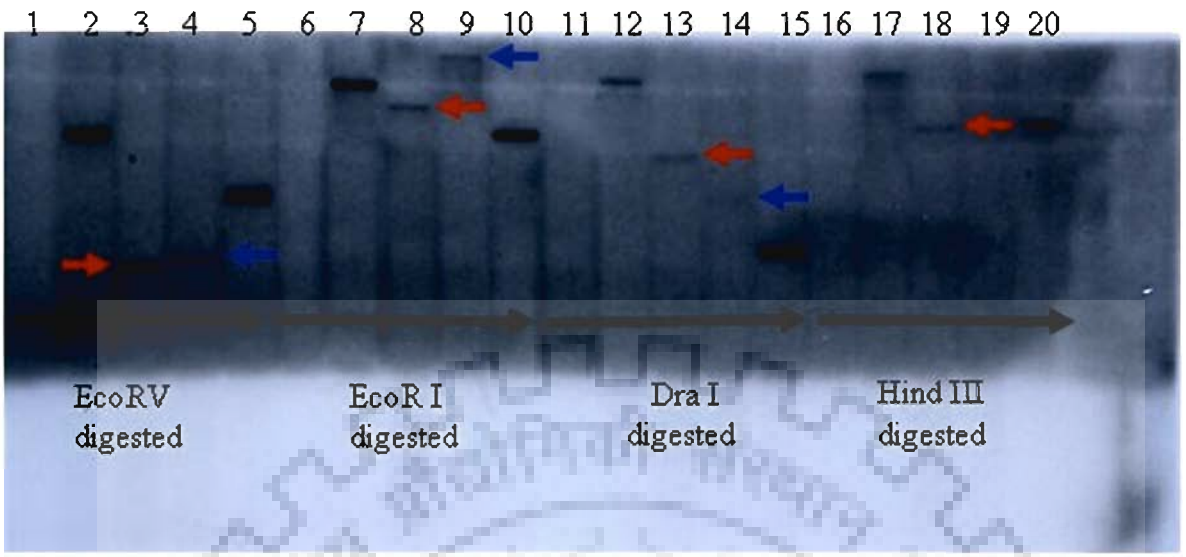


Fig. 4.7: Southern hybridization in insertional mutants including *Osoc* and *Ossl* with four restriction enzymes and with *hpt* DNA labeled α-P32 labeled dCTP. Basmati 370 lanes 1, 6, 11, 16 with no *hpt* signal, while Oligo culm mutant lanes 3, 8, 13&18 (blue arrows) and Seedling lethal mutant lanes 4, 9, 14 & 19 (red arrows) showing single copy insertions.

Fig. 4.8: Inheritance of Oligo culm mutation.

Fig. 4.9: 950bp *hpt* specific amplification in F2 plants of *Osoc*/PR106. M- 1kb Ladder, Lanes 1 to 21 F2 plants.

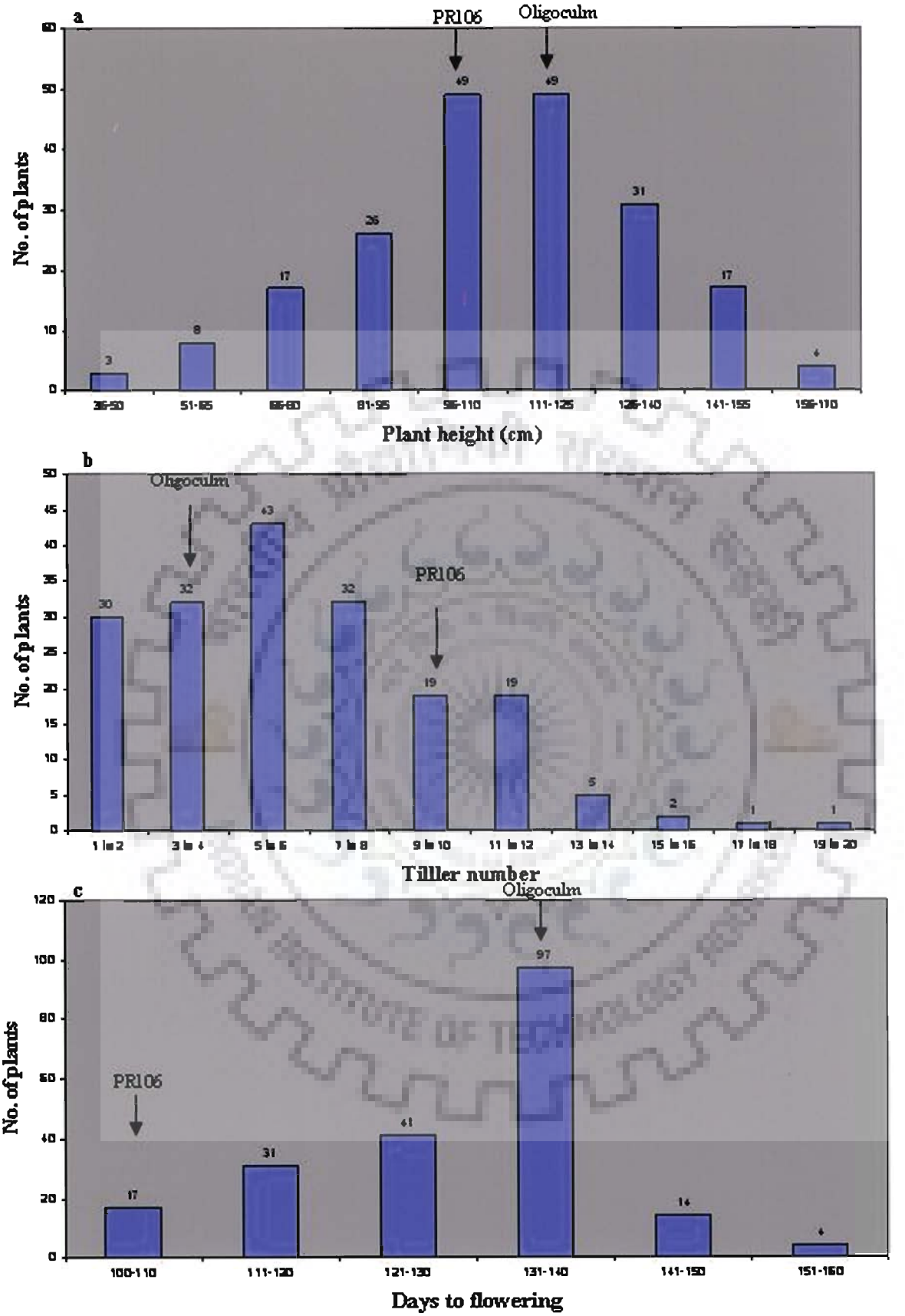


Fig. 4.10: Frequency distribution of Osoc/PR106 F_2 plants for plant height (a), tiller number (b) and days to flowering (c).

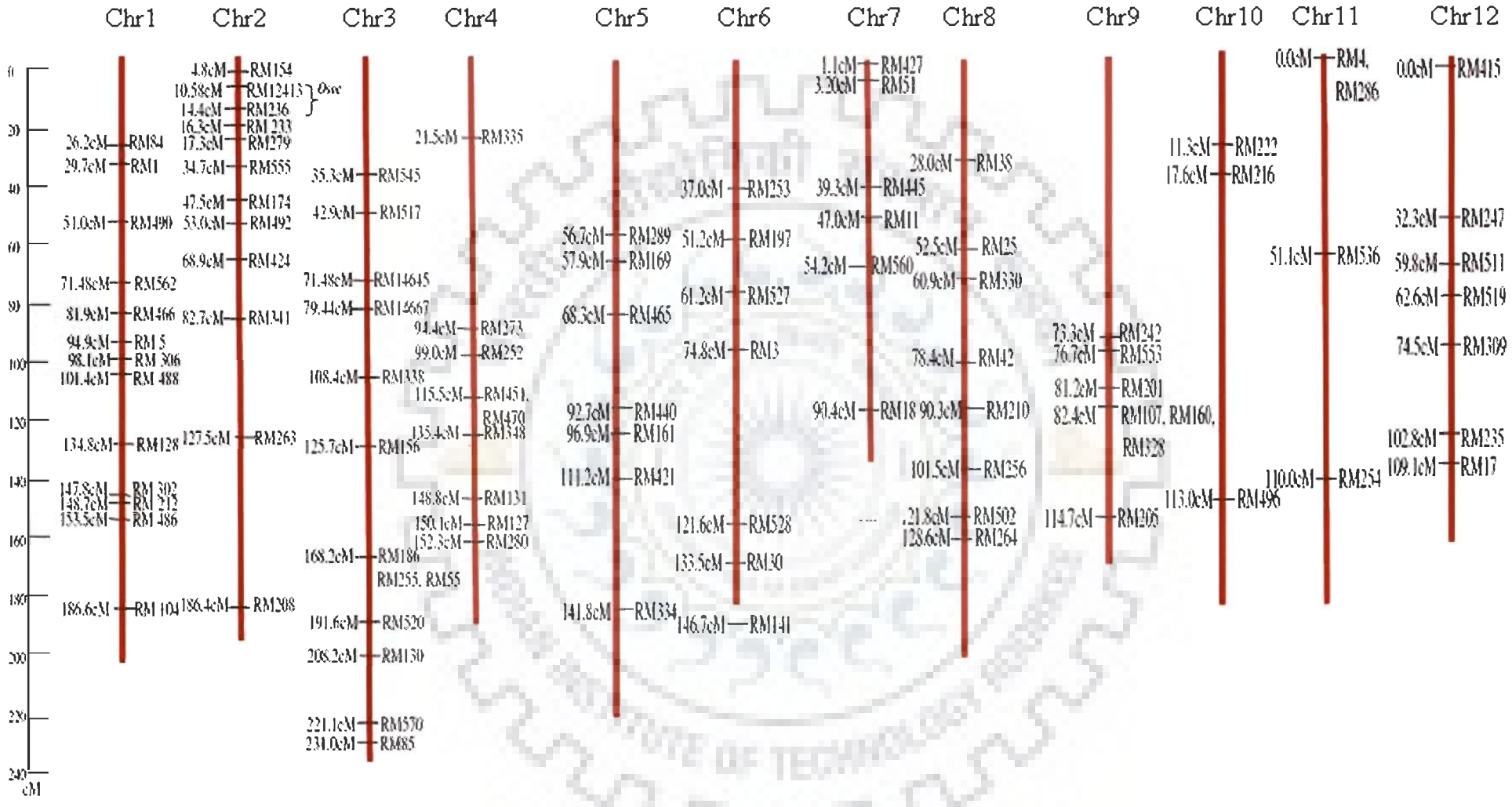


Fig 4.11: Map position of SSR markers showing polymorphism between Basmati 370 and PR106.

4.5.2 Chromosomal location and mapping of Oligo culm mutant

Mapping population was developed by crossing Oligo culm with distantly related variety PR106. Mapping population was developed not only for mapping purpose but also for identification of linkage between T-DNA insertion and mutant phenotype. The Oligo culm homozygous F_3 progenies were *hpt* +ve indicating that the Oligo culm (*Osoc*) phenotype is due to T-DNA insertion.

The chromosomal location and mapping of the knockout gene with respect to molecular markers especially co-dominant SSR markers can be very useful for further analysis. F_3 homozygous lines that were Oligo culm and *hpt* +ve were included in one bulk and the equal number of *hpt* –ve progenies with high tillering were included in the other bulk. Each bulk consisted of 12 F_3 lines and each line consisted of ~15 plants. All the 98 polymorphic SSR markers were genotyped on four DNA samples i.e. Oligo culm mutant, PR106, bulk 1 (Oligo culm and *hpt* +ve) and bulk 2 (high tillering and *hpt* -ve). Out of 98 polymorphic markers two markers RM279 and RM236 showed linkage with Oligo culm mutant. Considering the physical location of RM279 and RM236, five IRGPC primers RM12413, RM12404, RM12400, RM12398 and RM12394 were synthesized. Out of five primers, RM12413 showed polymorphism between parents and linkage to Oligo culm mutant. The gel pictures showing polymorphism between bulks with RM279, RM236 and RM12413 markers are given in Figs. 4.12.1, 4.13.1 and 4.14.1, respectively.

4.5.3 Construction of linkage map

The candidate markers showing polymorphism between bulks and considered as the potential SSR markers associated with Oligo culm (*Osoc*) mutant were genotyped on debulks. In bulk 1, five heterozygous and one recombinant were

observed out of 12 plants with RM279 (Fig. 4.12.2), no heterozygous and recombinants were observed with RM236 and RM12413 (Fig. 4.13.2, 4.14.2), while in the bulk 2, three heterozygous and no recombinants were observed with RM279 and no heterozygous and recombinants were found with RM236 and RM12413 (Fig. 4.13.3, 4.14.3). The banding pattern obtained on debulks indicated that the three markers of linkage maps of chromosome 2 are closely linked to the Oligo culm mutant. The data generated by BSA indicated that the gene responsible for Oligo culm is located on chromosome 2. The SSR markers that showed linkage with Oligo culm mutant were used for genotyping F_2 population. The genotype analyses observed 13 recombinants with RM279, 5 recombinants with RM236 and 3 recombinants with RM12413 out of 204 F_2 plants (Table 4.3).

The genetic distance between SSR markers and T-DNA insertion position was calculated based on recombination frequency. The total number of recombinants was calculated with respect to T-DNA/*hpt* and flanking marker. The genetic distance between SSR markers and T-DNA insertion position was calculated as 2.45cM, 6.37cM and 1.47cM for RM236, RM279 and RM12413 respectively (Fig. 4.15). The T-DNA insertion in Oligo culm (*Osoc*) is flanked by the closely linked markers RM12413 and RM236.

4.6 Identification of T-DNA flanking genomic sequence

4.6.1 Genome walking method

*Eco*RV, *Dra*I, *Hpa*I, *Hae*III and *Rsa*I restriction enzymes were used to create GenomeWalker libraries with Oligo culm mutant. Genomic DNA was digested with five blunt ended restriction enzymes, then purified and ligated to the adaptor. Three T-DNA specific primers (T-RB1, T-RB2 and T-RB3) and two adapter specific

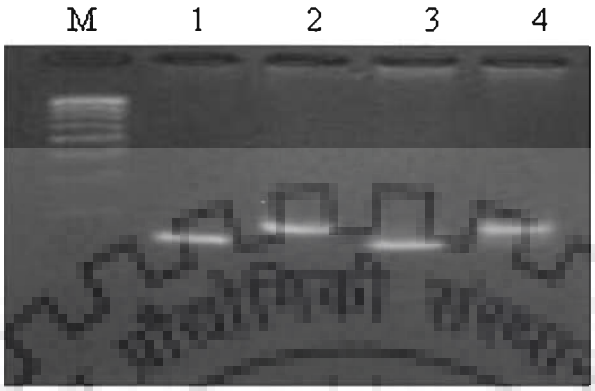


Fig. 4.12.1: BSA of Oligo culm with RM279 on bulks. M-100bp Ladder, 1- Oligo culm mutant, 2- PR106, 3- Oligo culm homozygous (*hpt* +ve) bulk, 3- High tillering F3 homozygous (*hpt* –ve) bulk.

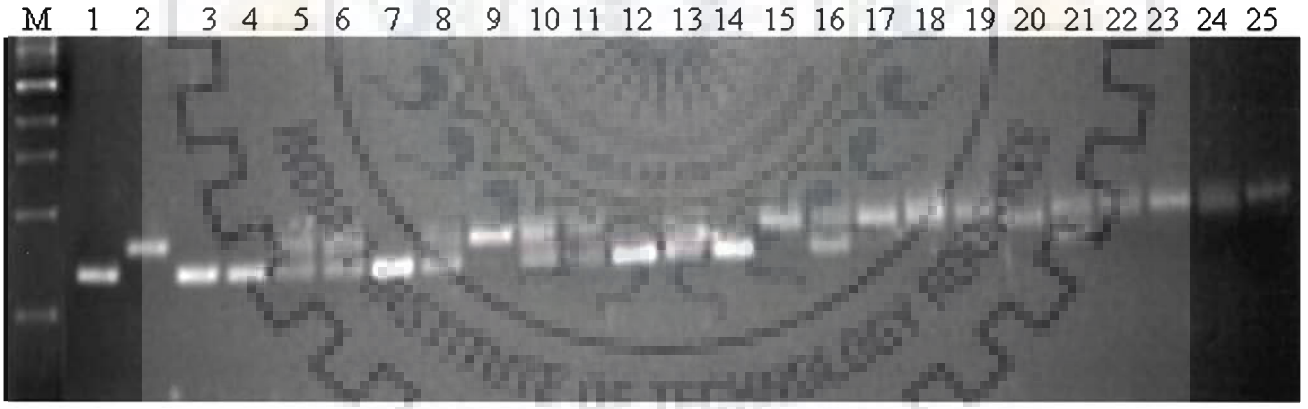


Fig. 4.12.2: BSA of Oligo culm with RM279 on debulks. M- 100bp Ladder, 1- Oligo culm mutant, 2- PR106, 3 to 14- Oligo culm homozygous (*hpt* +ve) debulks, 15 to 25- High tillering F3 homozygous (*hpt* –ve) debulks.

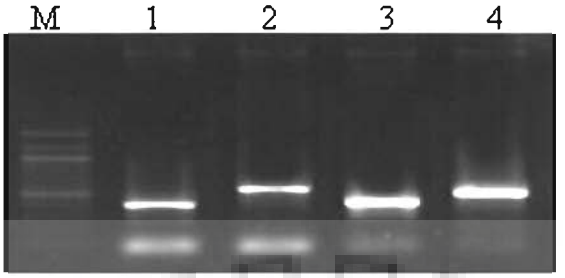


Fig. 4.13.1: BSA of Oligo culm with RM236 on bulks. M- 100bp Ladder, 1- Oligo culm mutant, 2- PR106, 3- Oligo culm homozygous (*hpt* +ve) bulk, 3- High tillering F3 homozygous (*hpt* –ve) bulk.

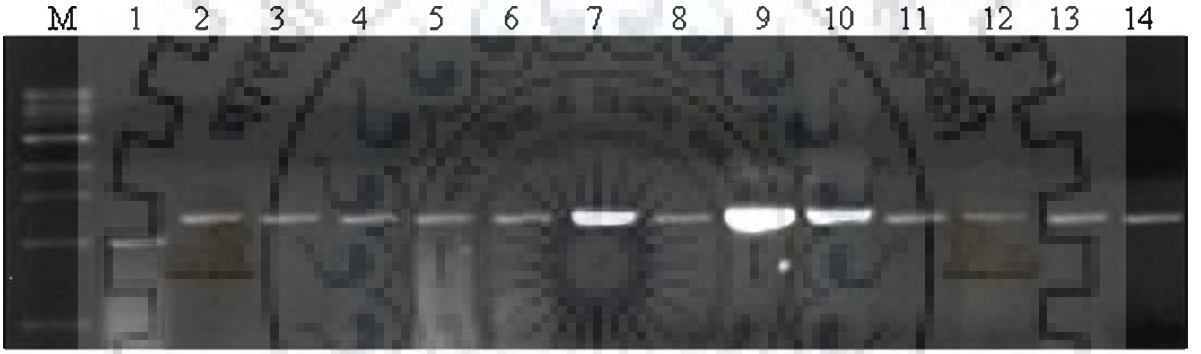


Fig. 4.13.2: BSA of Oligo culm with RM 236 on *hpt* -ve debulks. M- 100bp Ladder, 1- Oligo culm mutant, 2- PR106, 3- High tillering F3 homozygous (*hpt* – ve) debulk.

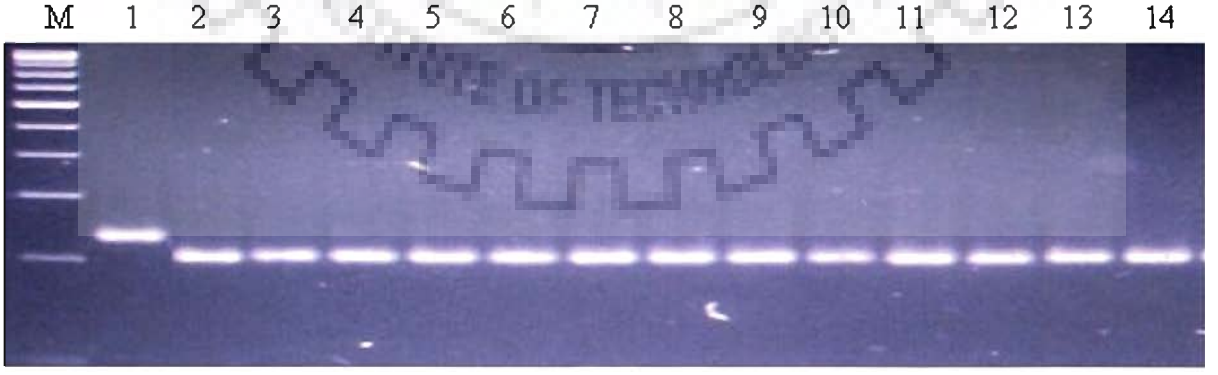


Fig. 4.13.3: BSA of Oligo culm with RM 236 on *hpt* +ve debulks. M- 100bp Ladder, 1-PR106, 2- Oligo culm mutant, 3- Oligo culm F3 homozygous (*hpt* +ve) debulk.

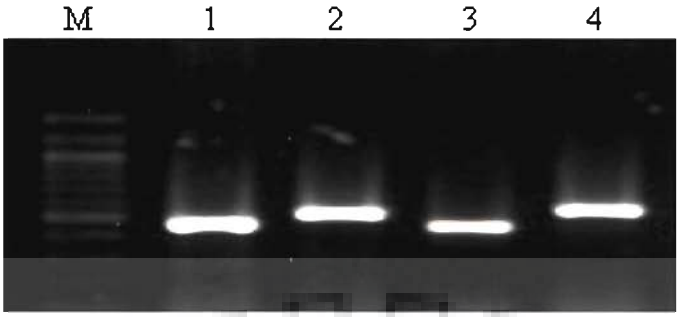


Fig. 4.14.1: BSA of Oligo culm with RM12413 on bulks. M- 50bp Ladder, 1- PR106, 2- Oligo culm mutant, 3- Oligo culm (*hpt* +ve) bulk, 4- High tillering F3 homozygous (*hpt* –ve) bulk.

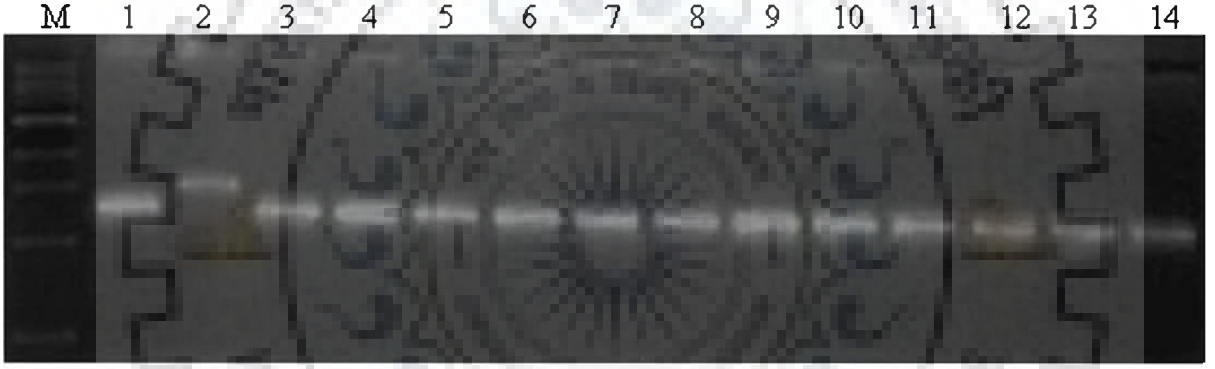


Fig. 4.14.2: BSA of Oligo culm BSA with RM12413 on +ve debulks. M- 100bp Ladder, 1- Oligo culm mutant, 2- PR106, 3 to 14- Oligo culm (*hpt* +ve) debulks

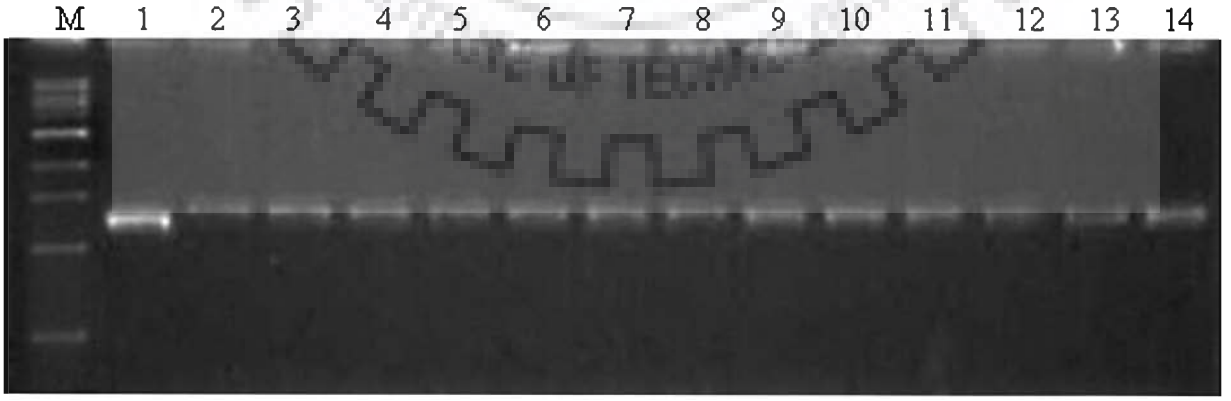
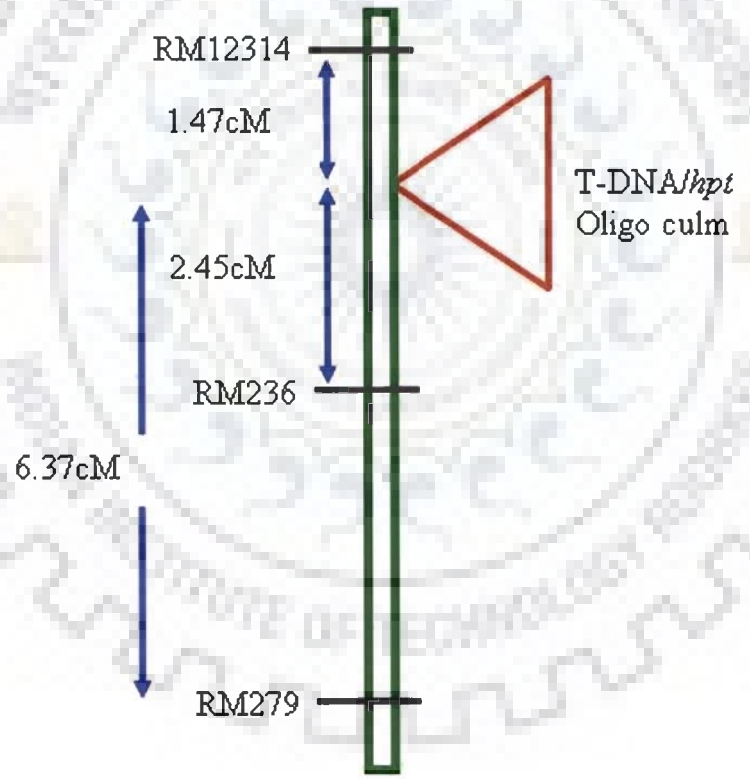


Fig. 4.14.3: BSA of Oligo culm with RM 12413 on debulks. M- 100bp Ladder, 1- Oligo culm mutant, 2- PR106, 3- High tillering F3 homozygous (*hpt* –ve) debulk.

Table 4.3: Recombination frequency in 204 F2 plants for three linked molecular markers.

Marker	Recombinants		Total number of recombinants	Recombination frequency (%)
	Homozygous for Basmati allele but *hpt* -ve	Homozygous for PR106 allele but *hpt* +ve		
RM236	1	4	5	2.45
RM279	2	11	13	6.37
RM12314	1	2	3	1.47



Chromosome 2

Fig. 4.15: Linkage map showing the genetic distances between markers and Oligo culm (*Osoc*).

primers (AP1 and AP2) were used for genome walking to identify flanking genomic sequences. The primary PCR reaction was carried out by outer adaptor specific primer (AP1) and the outer T-DNA specific primer (T-RB1). PCR cycling conditions are given in Table 3.1. The primary PCR gave multiple bands with a background smear (Fig. 4.16). Secondary PCR, which was performed using the internal primers (T-RB2 and AP2) and the 50 folds diluted primary PCR product as the template, selectively amplified the desired product in the subsequent nested PCR resulting in a single bright band. The *Dra*I, *Hae*III, *Eco*RV and *Hpa*I libraries resulted in amplification of 150bp, 300bp, 700bp and 150bp, respectively. No amplification was observed in the *Hae*III library. Tertiary reaction was also carried out by using T-RB3/AP2 and 50 folds diluted secondary PCR product (Fig 4.16). The resultant 700bp PCR product from *Eco*RV library being intense was eluted and purified using the QIAGEN QIAquick PCR purification kit (USA). The gel eluted PCR product was sequenced with T-RB2 and AP2 primers. The sequencing with T-RB2 and AP2 showed the 300bp and 551bp sequence results respectively.

To identify flanking sequence of Seedling lethal mutant *Eco*RV and *Dra*I Genome Walker libraries were constructed. Primary and secondary reactions were carried out as described above. PCR amplified product of 300bp was observed from *Eco*RV library (Fig. 4.17) and the resulting fragment was sequenced with T-RB2 and AP2 primers.

4.6.2 TAIL-PCR

Three T-DNA specific primers (T-RB1, T-RB2 and T-RB3) and arbitrary degenerate primer 1 (AD1) were used for TAIL-PCR to identify flanking genomic sequences of Oligo culm, Seedling lethal and Small grain mutants. Total genomic

DNA was used for amplification and procedure is as described by Liu *et al.* 1995. The fidelity of TAIL-PCR amplification was judged by the appearance on 1.2% agarose gels of secondary and tertiary PCR products shorter by 30-50 nucleotides as expected from the location of nested T-DNA specific primers. The product sizes of the tertiary TAIL-PCR was ~250bp for Oligo culm, Seedling lethal and Small grain mutants (Fig. 4.18). Amplified product from tertiary TAIL-PCR was run on 1.2% agarose gel, eluted and purified using the Qiagen QIAEXII gel elution kit (USA). The PCR product was eluted in 30µL of water. About 80ng of the purified PCR-amplified DNA was used for sequencing with the T-DNA specific T-RB3 primer (5'-TATTCGGGCCTAACTTTTGGTGTG-3`; Tm=57.4^{0}C) under the conditions specified by the manufacturer (Applied Biosystems, Foster City, CA.).

4.6.3 Chromosomal location of T-DNA insertion in Oligo culm mutant

The sequences obtained by Genome Walking and TAIL-PCR were used for BLAST (Basic local Align and Search tool) (Altschul, 1990) alignment with the rice genome sequence in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih) site. Oligo culm (*Osoc*) mutant T-DNA flanking sequence alignment results showed positions of the single BLAST hits in the *Japonica* rice genome (Nipponbare) on chromosome 2 with 97% identity and 8 gaps (Fig. 4.19). Similar alignment result was obtained at the same location of chromosome number 2 with the amplified flanking sequence by TAIL-PCR amplified product. The T-DNA insertion position was identified at 1818808bp on chromosome 2 which is in the exonic position of the predicted gene (Os02g0134300) (Fig. 4.20).

4.6.4 Confirmation of the T-DNA insertion position on chromosome 2 of Oligo culm mutant

To confirm the position of T-DNA insertion on chromosome 2, primers were designed based on the Nipponbare rice genome (Rice genome-specific primers: Oligo-RB1, Oligo-RB2, Oligo-LB1 and Oligo-LB2). The positions of T-DNA insertion and rice genome specific primers are given in Fig. 4.21. The expected sizes of amplifications are given in Table 4.4.

PCR was performed with the combination of T-DNA specific primers (T-RB1, T-RB2, T-RB3, T-RB4 and T-RB5) and genome-specific primers (Oligo-RB1 and Oligo-RB2). Amplification of expected size was obtained in Oligo culm (*Osoc*) mutant and no amplification was obtained in Basmati 370 (Fig. 4.22).

PCR with rice genome-specific primer combinations gave amplification in Basmati 370 but not in Oligo culm (*Osoc*) mutant (Fig. 4.23). Approximately 10Kb insertion is present between genome specific primers in Oligo culm mutant, which cannot be amplified by the Taq used for the PCR.

Rice genome specific and T-DNA specific primer combination (T-RB1 and Oligo-RB2) was used on F_2 mapping population. The *hpt* +ve 12 homozygous lines showed amplification and no amplification was observed in the 12 *hpt* -ve homozygous lines. Subsequently T-DNA specific and genome specific primers were applied on the entire F_2 population. Out of 204 F_2 individuals 143 showed amplification (Fig. 4.24) indicating the linkage between T-DNA insertion and Oligo culm mutant phenotype.

```
>osoc.D10_070830173C
CGACCGTTGTGTCCCTGTTATCCAGACATAGAGACACTAATATGCAGAACTAACATCATCCTATAGATT
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CCAAAAACCCTCTGCTCTGAAATTCCCTAATCAACCCCAGCGATTTCATGCACCGTGAGTAAAATCACT
ACAAAATTCTAGCAGCCTAAGCACAAACGAACAGCAAAATCCAAATACTACCTCCCGACCTTCATCCGC
ATTTTACAAACCTAT

Identities = 531/546 (97%), Gaps = 8/546 (1%)

Query  19       TATC-CAGACATAGAGACACTAATATGCAGAACTAACATCATCCTATAGATTTGCAATAT  77
                ||||  || |||| ||| ||||||||||||||| ||||||||||||||||||||||||
Sbjct  1818268  TATCTGA-ACATTGAG-CACTAATATGCAGAACAAACATCATGCTATAGATTTGCAATAT  1818325

Query  78       TTTTCAATCAAATCACAAGGTGTTGTTGGTTCAATGCAACAATTTAGCATCTATACAGGC  137
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1818326  TTTTCAATCAAATCACAAGGTGTTGTTGGTTCAATGCAACAATTTAGCATCTATACAGGC  1818385

Query  138      CTCCTAAAGTCACAATCTAAGTTGTTGGCTCAAAATTTCAAATGCACAGCATATGCCCAG  197
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1818386  CTCCTAAAGTCACAATCTAAGTTGTTGGCTCAAAATTTCAAATGCACAGCATATGCCCAG  1818445

Query  198      CCAAATGATAATAGAAGCGGCAATTAAGCATCTATACAAACCCCCTCAAAATCACAACCG  257
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1818446  CCAAATGATAATAGAAGCGGCAATTAAGCATCTATACAAACCCCCTCAAAATCACAACCG  1818505

Query  258      AAGTCACCTTTTAAGAAACCTCACAATCTAAGCATCTAACAACAGCCTATCTGCTTAGCT  317
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1818506  AAGTCACCTTTTAAGAAACCTCACAATCTAAGCATCTAACAACAGCCTATCTGCTTAGCT  1818565

Query  318      TCGATTATCAACTTCTTCTCttttttttttttCTTAAAGAATTCACTAAAATTCCATCAAGC  377
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1818566  TCGATTATCAACTTCTTCTCTTTTTTTTTTCTTAAAGAATTCACTAAAATTCCATCAAGC  1818625

Query  378      GGT-GTTTTTAAAATAGCTCTGACCGTACCCAAAACCCCCAAAAACCCTCTGCTCTGAAA  436
                ||| |   ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1818626  GGTTGCACTTAAAATAGCTCTGACCGTACCCAAAACCCCCAAAAACCCTCTGCTCTGAAA  1818685

Query  437      TTCCCTAATCAACCCCAGCGATTTCATGCACCGTGAGTAAAATCACTACAAAATTCTAGC  496
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1818686  TTCCCTAATCAACCCCAGCGATTTCATGCACCGTGAGTAAAATCACTACAAAATTCTAGC  1818745

Query  497      AGCCTAAGCACAAACGAACAGCAAAATC-CAAATACTACCTCCCGACCTTCATCCGCATT  555
                |||||||||||||||||||||||||||| |||||||||||| |||| | ||||||||||
Sbjct  1818746  AGCCTAAGCACAAACGAACAGCAAAATCACAAATACTACCTCGCGAC-T-CATCCGCATC  1818803

Query  556      TTACAA  561
                |  ||||
Sbjct  1818804  T-ACAA  1818808
```

Fig. 4.19: T-DNA flanking genome sequence in Oligo culm (*Osoc*) mutant through Genome Walking and its BLAST alignment with Nipponbare sequence.

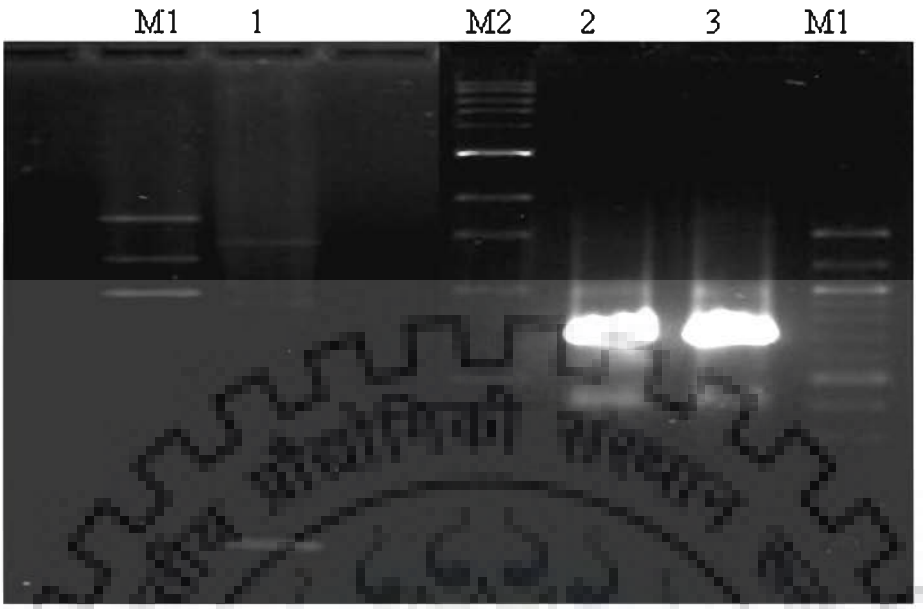

Fig. 4.16: Genome walking PCR amplification of Oligo culm from EcoRV digested library. M1- 100bp ladder, M2- 1kb ladder, 1- Primary PCR product with T-RB1/AP1 primers, 2- Secondary PCR product with T-RB2/AP2 primes, 3- tertiary PCR product with T-RB3/AP2 primers.

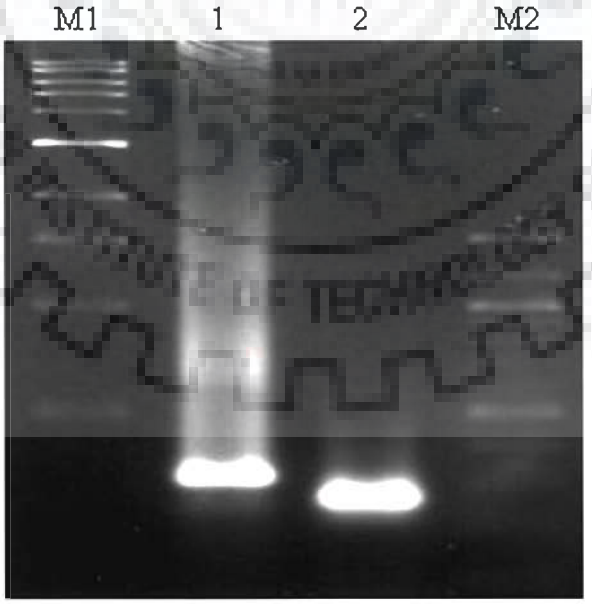

Fig. 4.17: Genome walking PCR amplification of Seedling lethal from EcoRV digested library. M1- 1Kb ladder, 1- Secondary PCR with T-RB2/AP2 primers, M2- 100bp ladder, 2- Tertiary PCR with T-RB3/AP2 primers.

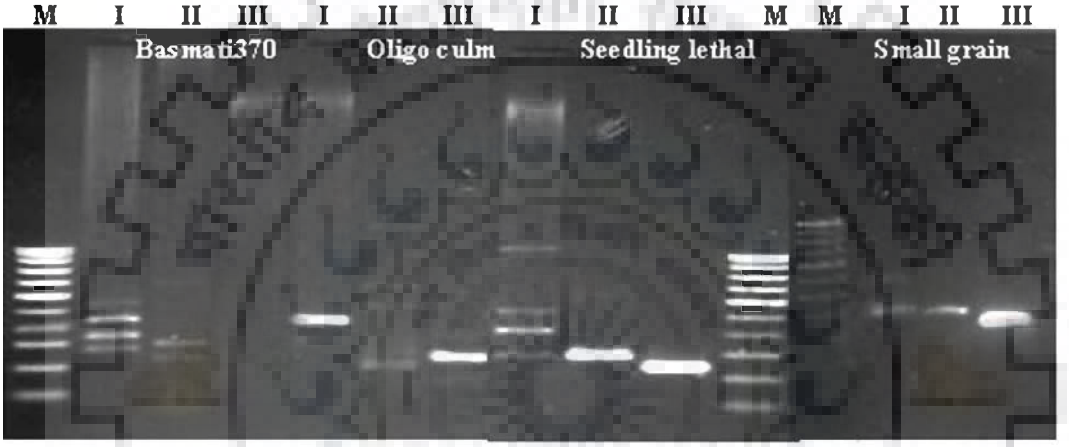

Fig. 4.18: Analysis of TAIL-PCR products amplified from T-DNA insertion mutants and Basmati 370 with T-RB1, T-RB2, T-RB3 and AD1 primers. Lane designations: M- 100bp DNA ladder; I- primary reaction product, II- secondary reaction amplification, III- tertiary reaction product.

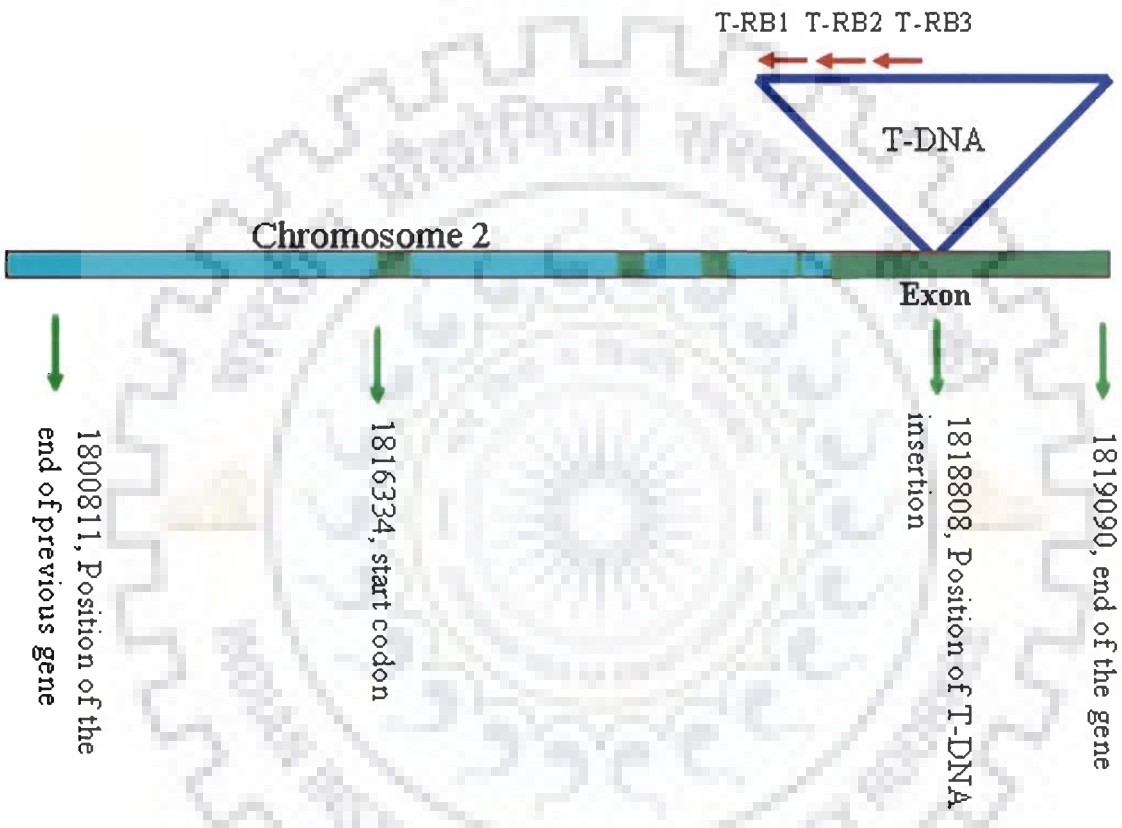

Fig. 4.20: Diagrammatic presentation of T-DNA insertion position on chromosome 2 of Oligo culm mutant.

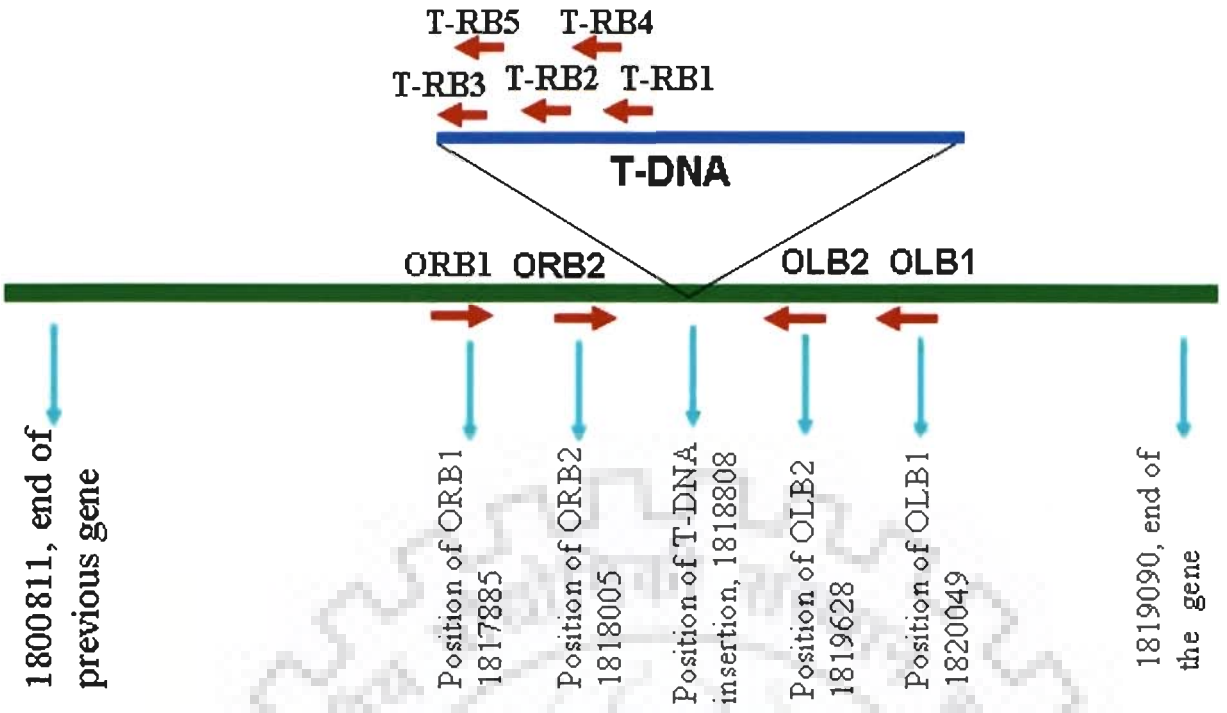

Fig. 4.21: Diagrammatic presentation of designing rice genome specific primers to confirm the position of T-DNA insertion in Oligo culm mutant

Table 4.4: Primer combination and expected size of PCR product in *Osoc* and Basmati 370.

Primer combination	Expected size in Basmati 370 (bp)	Expected size in Oligo culm mutant(bp)
Oligo-RB1 + T-RB1	-	1080
Oligo-RB1 + T-RB2	-	1026
Oligo-RB1 + T-RB3	-	986
Oligo-RB1 + T-RB4	-	1016
Oligo-RB1 + T-RB5	-	980
Oligo-RB2 + T-RB1	-	943
Oligo-RB2 + T-RB2	-	903
Oligo-RB2 + T-RB3	-	863
Oligo-RB2 + T-RB4	-	893
Oligo-RB2 + T-RB5	-	857
Oligo-RB1 + Oligo-LB1	2164	-
Oligo-RB1 + Oligo-LB2	1743	-
Oligo-RB2 + Oligo-LB1	2044	-
Oligo-RB2 + Oligo-LB2	1623	-

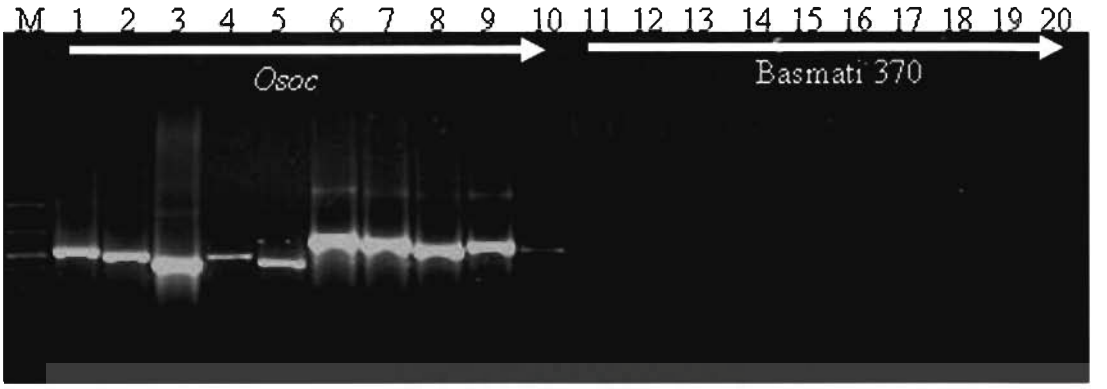

Fig. 4.22: Confirmation of T-DNA insertion position of Oligo culm with T-DNA and rice genome specific primers combination. M- 100bp ladder, Line 1 to 5 & 11 to 15- (Oligo-RB2/ T-RB1, T-RB2, T-RB3, T-RB4, T-RB5) and Line 6 to 10 & 16 to 20- (Oligo-RB1/ T-RB1, T-RB2, T-RB3, T-RB4, T-RB5).

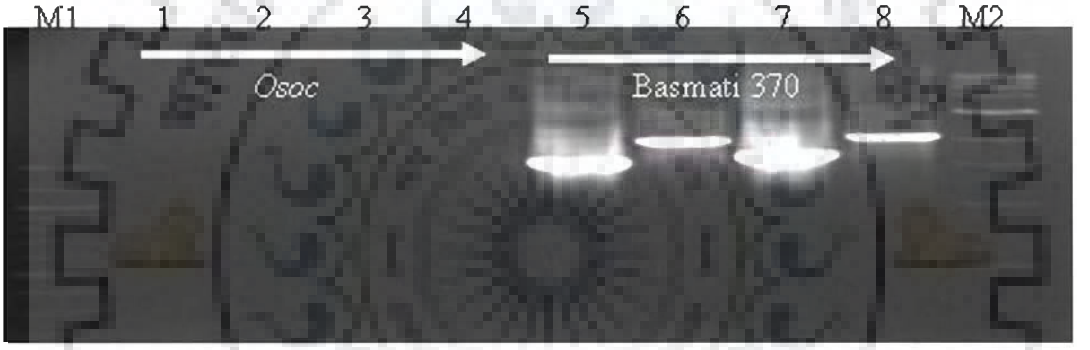

Fig. 4.23: Confirmation of T-DNA insertion position of Oligo culm mutant with rice genome specific primer combination. M1- 100bp ladder, M2- 1kb ladder, 1&5-(Oligo-RB2/Oligo-LB2), 2&6- (Oligo-RB2/Oligo-LB1), 3&7- (Oligo-RB1/Oligo-LB2), 4&8- (Oligo-RB2/Oligo-LB1).

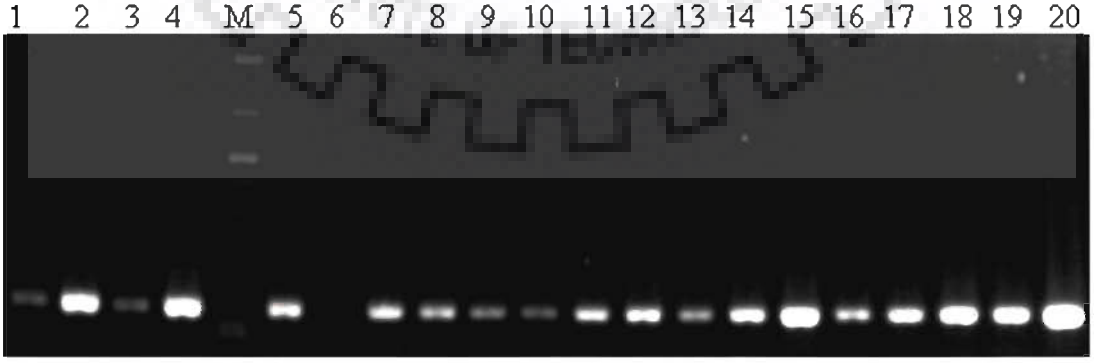

Fig. 4.24: Amplification of 943bp fragment by using T-DNA and rice genome specific primer (T-RB1/Oligo-RB2) on *Osoc*/PR106 population. M- 1kb Ladder, 1 to 20- amplification with individual plant DNA.

4.6.5 Chromosomal location of T-DNA insertion in Seedling lethal mutant

The similarity search of T-DNA flanking sequence of the Seedling lethal with Genome walking and TAIL-PCR was used for BLAST alignment. All showed significant alignment with the *Oryza sativa* (*japonica* cultivar Nipponbare) with just a single hit at chromosome 11 (Fig. 4.25). The minor differences in the sequences are likely to be attributed to either the sequencing error, errors in the sequences deposited in the database, or polymorphism between *japonica* and Basmati 370.

4.6.6 Confirmation of the T-DNA insertion position of Seedling lethal on chromosome 11

To confirm the insertion position in seedling lethal mutant on chromosome 11, primers were designed on rice genome (Genome-specific primers, Seedling lethal-RB1, Seedling lethal-RB2/ Seedling lethal-LB1, and Seedling lethal-LB2) (Fig. 4.26). Expected PCR product size is given in Table 4.5.

PCR was performed in combination with the T-DNA (T-RB1, T-RB2 and T-RB3) specific primers and rice genome-specific primers (Seedling lethal-RB1 and Seedling lethal-RB2). Amplification of expected size was obtained in mutant when T-DNA and genome specific primer combinations were used and no amplification was observed in Basmati 370.

Expected size of PCR amplification was obtained with genome-specific primer combinations (Seedling lethal-RB1, Seedling lethal-RB2, Seedling lethal-LB1 and Seedling lethal-LB2) in Basmati 370, but not in Seedling lethal because of the insertion of approximately 10 Kb fragments which cannot be amplified by the Taq used for the PCR (Fig. 4.27).

```
>Oss1
ATTACACACTATTGCGGGCCTAACTTTTGGTGTGATAGATGCTGACTGGGCAACTTTTTTTTTAATCCA
CAGGCCCATTCTATCCAATGCCTTAAGAACCATTCCCAACCCAATGTCTAAGAATAGTGTCCATAGCAT
TAAAATAAGTGGCCACATAGGAATGAAAAAGTGATGTGGCAAGTGAAATAAATGAAGAAAAGAGAAGGA
AACCATGTATGGGCTATGAAGNACTTGTTTTACTGACACAACATTCCAAGGACACCCAGCCCCGGGCCC
GTACAACCACGCCGTGACCCTAATAATTAAAGCCTATTGGGGGCCCCGGCGTTGGGTAAAGGGTTCTAA
CCAAGGGGCCAAAAATATAGCGACCCAAACTGGGGTGGGGGGGGGCCCCCCAACACAAAAAAAAAAAAA
AAAGGGTGCCTGTGCGGGAGGGGAACCCAACAATTTTTTAAAAAAACAAAAAATTGGATGAAAGAAGAA
GGAAAACGAGAAAAATTACAAGAAAAGGAAAGAGAGAAAAAAAAGAGAGGAAGGACACCACACAAAGAA
AAAAGTAACTAAAAAAAAGAAAANANAANAAGAAANAAAANAAAGAAAAGTANACAAAAAA

Identities = 191/213 (89%), Gaps = 18/213 (8%), Strand=Plus/Plus

Query  50        GCAACttttttttttAATCCACAGGCCCATTCTATCCAATGCCTTAAGAAccattcccaac  109
                  ||||||||||||||||||||||||||||||||||||||||||||| || ||||||||||
Sbjct  15588099  GCAACTTTTTTTTTAATCCACAGGCCCATTCTATCCAATGCCTTA-GA-CCATTCCCAAC
15588156

Query  110       ccaatgtctaagaatagtgtccatagcattaaaataagtggccacataggaatgaaaaag  169
                  |||||||||||||| ||||||||||||||||||| ||||| |||||||||||| |||||| |
Sbjct  15588157  CCAATGTCTAAGA-TAGTGTCCATAGCATTAAA-TAAGTTGCCACATAGGA-TGAAAA-G
15588212

Query  170       tgatgtggcaagtgaaataaatgaagaaaagagaaggaaaccatgtatgggctatgaagn  229
                  |||||||||||||||| ||||||| ||||||||||||||||||| | | |||| |
Sbjct  15588213  TGATGTGGCAAGTGAA-TAAATGA-GAAAAGAGAAGGAAACCATGTCTTG-G-ATGA-G-
15588266

Query  230       ac-ttgttttactgacacaacattccaagGACA  261
                  || || ||| || ||||||||| |||| |||
Sbjct  15588267  ACATTATTT--GT-ACACAACAT-CCAAG-ACA  15588294
```

Fig. 4.25: T-DNA flanking genome sequence in Seedling lethal mutant through Genome walking and its BLAST alignment with Nipponbare sequence.

Table 4.5: Primer combinations and expected size of PCR product in wild type and Seedling lethal mutant.

Primer combination	Expected size Basmati370 (bp)	Expected size in Oligo culm mutant(bp)
Seedling Lethal-RB1 + T-RB1	-	876
Seedling Lethal-RB1 + T-RB2	-	836
Seedling Lethal-RB1 + T-RB4	-	826
Seedling Lethal-RB2 + T-RB1	-	1405
Seedling Lethal- RB2 + T-RB2	-	1365
Seedling Lethal-RB2 + T-RB4	-	893
Seedling Lethal-RB1 + Seedling Lethal-LB1	1933	-
Seedling Lethal-RB1 + Seedling Lethal-LB2	1697	-
Seedling Lethal-RB2 + Seedling Lethal-LB1	1355	-
Seedling Lethal-RB2 + Seedling Lethal-LB2	1168	-

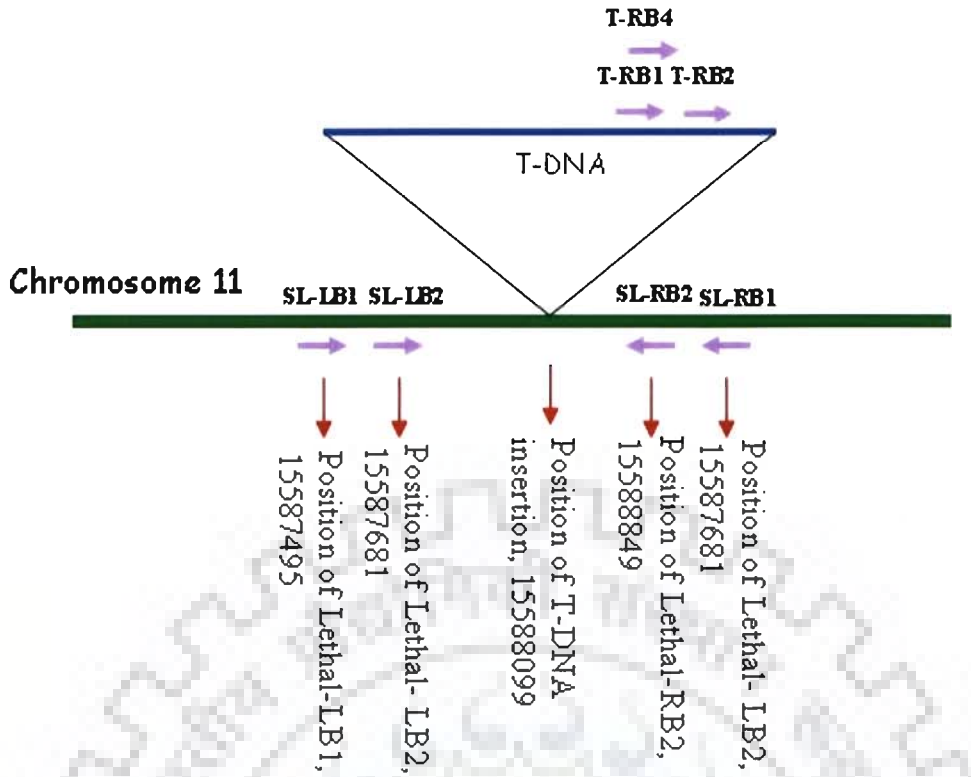

Fig. 4.26: Diagrammatic presentation of designing rice genome specific primers to confirm the position of T-DNA insertion in Seedling lethal.

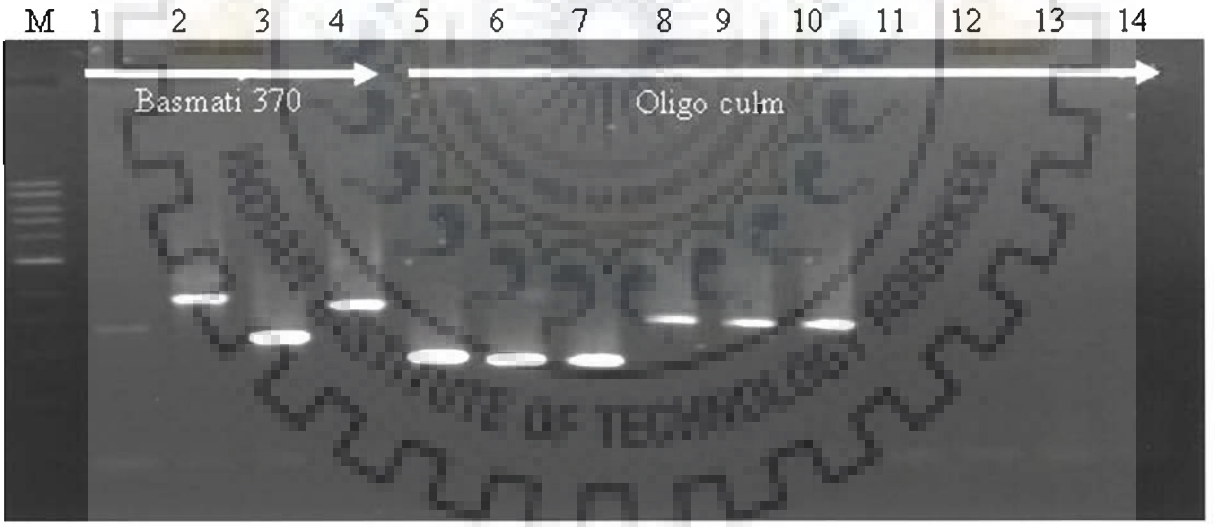

Fig. 4.27: Confirmation of T-DNA insertion position on chromosome 11 of Seedling lethal mutant.

Rice genome specific primers combination: Line 1&11- Seedling Lethal-RB1/Seedling Lethal- LB2, Line 2&12- Seedling Lethal-RB1/Seedling Lethal-LB1, 3&13- Seedling Lethal-RB2/Seedling Lethal-LB2, 4&14- Seedling Lethal-RB2/Seedling Lethal-LB1.

Rice genome specific and T-DNA specific primer combination: 5, 6&7- Seedling Lethal-RB1 + T-RB1/T-RB2/T-RB4, 8, 9&10- Seedling Lethal -RB2 + T-RB1/T-RB2/T-RB4.

4.6.7 Chromosomal location of T-DNA insertion in Small grain (*Ossg*) mutant

Small grain mutant T-DNA flanking sequence obtained with TAIL-PCR was used for BLAST alignment, which showed significant alignment with the *Oryza sativa* (*japonica* cultivar Nipponbare) with just a single hit at chromosome 11 (Fig. 4.28). The T-DNA insertion position was identified at 26784188bp on chromosome 11 which is in the intronic position of the pectin methyl esterase gene (Os11g0683800).

4.7 Details of ORF, mRNA and protein sequence of the Oligo culm (*Osoc*) and Small grain (*Ossg*) genes in *Oryza sativa japonica* cultivar

The T-DNA insertion in *Osoc* mutant was present within the exon region of the transcription elongation factor gene (http://www.ncbi.nlm.nih.gov). The function of this protein was predicted to be like that of transcription elongation factor (Elf1) in *Oryza sativa*. This family of short proteins contains a putative zinc binding domain with four conserved cysteines (Marchler-Bauer *et al.*, 2004). The gene starts at 1819090bp position and stops at 1816334bp (complementary sequence) on chromosome 2 of *Japonica* rice. The length of the gene was 2,757bp with UTR region, 4 exons and 4 introns (Table 4.6, Fig. 4.29, Fig. 4.32). The length of the mRNA was 875bp (Fig. 4.30) (http://www.ncbi.nlm.nih.gov/sites/entrez) which coded for 105 amino acid long protein (Fig. 4.31). An internal promoter was found in the gene region by using the Promoter 2.0 (Knudsen *et al.*, 1999) and TSSP-TCM (Shahmuradov *et al.*, 2005).

The orthologs and paralogs of the identified gene were searched by using NCBI and KEGG (http://www.genome.jp/dbget-bin). One paralog was identified on chromosome 7. Paralogs are defined as duplication of gene occupying two different

Query= 9_RB3-1 >9_RB3-1

GAAAGAAAAAAACGAAAAAAAAGGGCTGGTCCAAATTAAAATTTTGCTCGGGTTGGTGTAACATCAAATAGCGGGACATCAACGCC
ATCTAGCTCAGCTTCAACGTCGAGCAACTCAACTGTTTAAGAACCGACGGGGAGTAGTAACTCAACTGCATCGGCTAATAACAGGG
AAAGCAATCCACACTGCAGAAGCGCGGACGGCAACTAAGAAACTCAACTGCGTCAGGAACCGACAAGTAATTCACTTGCTGCAGCA
AAGGCGACTAAATACTCCAGGTCCAAGTCCACCGCCGAAAAGAAGTGGTTAATACAAGTTCCCCGGCTTACCACAGACACAAACCC
GTAAACTCGCTTCATTTGGGCCCAACACAATTCTTGTTTGAGAAGCCCCGTAAAATTGTAGCTGTCTGTGGTGAAATGGGGGCCGG
GAATAATCAAATTCGCCGGTCCCTTGTAGATGTCCCACGTCCGGGTAACAGGTTGTTTTTTTTTTTTGAGTGTGGT

Identities = 81%, Strand=Plus/Minus

Fig. 4.28: T-DNA flanking genome sequence in Small grain (*Ossg*) mutant through TAIL-PCR and its BLAST alignment with Nipponbare sequence.

Table 4.6: Details of exon and intron positions of Oligo culm gene

1816334-1819014	Exonic/Intronic region	Exonic region	Size
1816334-1816790	exon	UTR	457 bp
1816791-1816902	exon	CDS	112 bp
1816903-1817001	intron		99 bp
1817002-1817023	exon	CDS	22 bp
1817024-1817770	intron		747 bp
1817771-1817841	exon	CDS	71 bp
1817842-1817950	intron		109 bp
1817951-1818063	exon	CDS	113 bp
1818064-1818068	exon	UTR	23 bp
1818087-1819013	intron		927 bp
1819014-1819090	exon	UTR	77 bp

>ref|NC_008395.1|:c1819090-1816334 Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 2

GGTAACCCAAGCCACCCGCCTCCTCTCCCTCCCGCGTAGCTAGGATTTGCTCTCCTGCTCGC
TATCTATCTCGTTTACTCTTCGGTGATGTGGGGTGGAGTGCTTGGATGCTGCTGCGCTAGTGAGCTGAG
TGTTTCGCTTTGATCGATCGATTGTCCTGGGGAATTTTGTTTTTTAAGGGGTTTAGGTTTTTAGGTTAG
GTATGCTTGTAGATGCGGATGAGTCGCGAGGTAGTATTTGTGATTTTGCTGTTCGTTTGTGCTTAGGCT
GCTAGAATTTTGTAGTGATTTTACTCACGGTGCATGAAATCGCTGGGGTTGATTAGGGAATTTCAGAGC
AGAGGGTTTTTGGGGGTTTTGGGTACGGTCAGAGCTATTTTAAGTGCAACCGCTTGATGGAATTTTAGT
GAATTCTTTAAGAAAAAAAAAAGAGAAGAAGTTGATAATCGAAGCTAAGCAGATAGGCTGTTGTTAGAT
GCTTAGATTGTGAGGTTTCTTAAAAGGTGACTTCGGTTGTGATTTTGAGGGGGTTTGTATAGATGCTTA
ATTGCCGCTTCTATTATCATTTGGCTGGGCATATGCTGTGCATTTGAAATTTTGAGCCAACAACTTAGA
TTGTGACTTTAGGAGGCCTGTATAGATGCTAAATTGTTGCATTGAACCAACAACACCTTGTGATTTGAT
TGAAAAATATTGCAAATCTATAGGATGATGTTTGTTCTGCATATTAGTGCTCAATGTTGAGATATCTCA
AATTGCATGTGCTGATTTGCATCTCTATTGTGTGCTCAATGTTGAGTATCATTTACTTCTATGTGTTTC
CTTCATGCTGTAACTTTTGAGAGTGGAGACTTTGTCTCTTTGTGGCGCAAAAATATGACAGCATCAATA
TAATTCAATTGTTACTATTTTTGAACTACGTGCAAAA**ATG**

GTAAGTATGTTTTGTGACCATTCACATACCTG
GCTTTTTGTTTCTCTTGAATTTCTCTGTCCGTGCTAGTTTTTGGCAGTGCAGTGACACCTTCTTCTGTG
TTTCGCAG

GGTAATATGCTCTGGAACCATGTGGCCATTCGTTTTGGATCAGTGGTTTAGTATTTGAAG
TGACCCCTTTTCCTGTTGTGAACTTTGCAATCGAGCATAATTGTGCTTTGTGTTCTCTCTTTAGGTTAT
TTGGTTAGTTACTGTTGGCGTCATGCTTACATTTCTTTATAATAAATTGGAATAAGATTTAATCATCTG
TAGATAACTTATGCCTCTGCAAAACCAAAATGAAAAGGTTGGGGAATCAGGTCTTAACTTTCAGCATCT
GTGCTTCCAAGTTCCAACACCTTTTGATTATCTTAGCCATTTATAGTTGATAACTGTTTTTCATGCTTT
GTACTAGTATTGCACTGCTGCATTGGGGCAGGAGTATAAATTTGCCTACGTGTCGTTAGTGCTTTCTTT
GTTCAGTTAGCTGGGCCTGCAGGCTAGATTTTGTGGGCCCTCCAAACCTGTCATTTGAGTTCATGTCTC
CCTTCCTAGTTAATGATAGGCAACATCCTCGCTAGTCCTTTTGGAAAAGAAATAACCATAATATAAGAA
CTCCACAAGACTATGTAGGCAGTATGTATAACGCAATTCCCTCTTAGTTGCTTACTAAAATTGCAGAAA
TTTCCTTCTGTTTGTCCAAGATTAGGTTTGTTTAGGAAGACAATACGCCTGATGCAATTGCCTCTGTAA
ATGCTTACCATTTGATATCTGAACAAAATAATGCTGACTTGACCATTTTTTTTCCTCCTTTCTGCA

GTAAGTTTGCTCTTGAGTCTTGAGAACCATCTATTTAGGCTCGTGCAGA
TGAACTGTTGGTGGACACACCCCGAAACTCATCGAACTTCTTCATCGCAG

Fig. 4.29: Genomic DNA sequence of Oligo culm gene of *japonica* cultivar Nipponbare. Highlighted (Coloured) region show exonic region of gene. Blue: Translation start point.

>gi|115444062|ref|NM_001052346.1| Oryza sativa (japonica cultivar-group) Os02g0134300 (Os02g0134300) mRNA, complete cds

GGTCGCGATCATTCTGGACTCCGGTTTCGTCGTCCGTTTTGCCTCCGTCTCCGTCTC
CGTCTCCGCCGCTCGCCGAGGCGGGAAAAATAAGCGTTGAATCATGGGCAAGAGGAA
GTCCAAAGCAAAACCACCTCCCAAGAAGAGAATGGACAAGCTTGATACTGTCTTTTG
CTGTCCATTTTGCAACCACGGAAGTAGTGTTGAGTGTCGCATTGATCTAAAGAACTT
AATCGGCGAGGCCTCATGCAGAATTTGTTTGGAAACTTTTAGCACATCTGTTAATGC
TCTGACTGAACCCATCGACATATACAGCGAATGGATCGACGAATGCGAGCGCGTGAA
CAACCCTGAGGAGGACAACGCCAACCACTATGAAGATGACAATGCCAGCCGCTACCG
CTACGACGAAGAAGAATAGTGGTCCGTTGGCGCTTGTTCCATAAAACGAGTTGCCTG
AATGCTGTGCAAAACAGTGTTGATGTAGAGTGATGTGGTACTGTTACCCGTGTCAGT
GAACCTTACCTTACAATGGGGCATACCTGGTAGTCTAAAATAGCATCTGAGAGTAGT
GACACAGTTTTAGCTTTGTGAGTTGGATAGTGGTGCATCAGCTATTCAACTTGTCCT
GTACATTTAACATGCCGTGGTCGTGCCTGAACAAGAACTCTAGTATCTTTAGTATTT
TGTTCCGTCGTTTATAAGTCATGGCATGTACAAATACAACAATGCTATTGTAGTGTG
GATTTGCGTGATGCAACTAGGCTGTAATCGATGGCACTGTACTGTACATGTGAGAGT
AAAGCTTTGGAAGTTGGATAGTGGTGCAGTATAACAGAGTAGGATCATGATGCATCA
GCTATTCAACTGTACTGTCC

Fig. 4.30: mRNA sequence of Oligo culm gene.

>gi|115444063|ref|NP_001045811.1| Os02g0134300 [Oryza sativa (japonica cultivar-group)]

MGKRKSKAKPPPKKRMDKLDTVFCCPFCNHGSSVECRIDLKNLIGEASCRICL
ETFSTSVNALTEPIDIYSEWIDECERVNNPEEDNANHYEDDNASRYRYDEEE

Fig. 4.31: Protein sequence of Oligo culm gene.

positions in the genome. The two paralogs which were aligned using CLUSTALW program, showed high conservation (Fig 4.33). Highly conserved Orthologs were found throughout the living kingdom. Multiple alignment of the plant proteins (transcription elongation factor) were aligned using ClustalW program (Fig. 4.34). The phylogenetic tree (Fig. 4.35) based on the amino acid sequence of transcription elongation factor shows that the Elf1 coded by *Osoc* is highly conserved in *Oryza sativa* spp *japonica* and *indica* and is closely related to that of *Vitis vinefera* followed by *Arabidopsis thaliana*. The fungal and animal Elf1 sequences cluster with different groups, with fungal cluster closer to that of the plants.

The Small grain (*Ossg*) mutant flanking sequencing results with TAIL-PCR showed homology on chromosome 3 of rice. The T-DNA insertion was present in the intronic region of the pectin esterase gene. The length of the gene was 2,185bp with five exons and two introns. The length of mRNA was 1749bp which coded for 423 amino acid length protein (http://www.ncbi.nlm.nih.gov/sites/entrez). The protein Pectin esterase contains putative pectin esterase domain. The gene expression profile of EST count was retrieved from database (Os.87674). The gene expression was more in panicles and flowers only (Table 4.7).

Table 4.7: Expression profile suggested by analysis of EST counts of Small gain (*Ossg*) gene.

Pool Name	Transcripts per million	Gene EST/Total EST in pool
Callus	0	0/164803
Flower	139	19/136501
Leaf	0	0/171750
Panicle	333	46/138119
Root	0	0/68198
Seed	0	0/32358
Stem	0	0/126877
Vegetative meristem	0	0/4594

4.8 BLAST of *Osoc* gene with FSTs

Insertional mutant database containing 172,000 FSTs have been developed by the effort of 11 institutes in 7 countries (TIGR; Jung *et al.*, 2008). All the flanking sequence was available at Rice functional genomic Express database (Rice GE) developed by SALK institute as well as NCBI (http://signal.salk.edu/cgi-bin/RiceGE). FSTs for Oligo culm gene were searched and five hits were found (Fig. 4.36). Out of five FSTs seeds were not available for 4 FSTs. No phenotypic descriptions were available for these FSTs.

4.9 Expression analysis studies

RT-PCR (Reverse transcription polymerase chain reaction) technique was used as part of the functional analysis of the knockout gene. Oligo culm gene specific and actin I (reference gene) primers were used for analyzing the gene expression studies in Oligo culm and wild type Basmati 370.

4.9.1 RNA isolation, control of genomic DNA contamination

Total RNA was extracted with TRIzol®Plus RNA purification kit (Invitrogen) from shoot and root tissues as described in the methods. The protocol gives high quality and quantity of RNA (Fig. 4.37). RNA isolation protocols usually show contamination with low amounts of genomic DNA. RNase-free DNase (Promega) treatment was given to remove genomic DNA contamination (Sambrook *et al.*, 1989). Resulting DNA free RNA was further purified by using RNeasy MiniElute clean up kit to avoid all possible interference at the time of performing RT-PCR. Actin I and Oligo culm gene specific primers were used to amplify total RNA as the template and

no amplification was observed suggesting that the total RNA was free from DNA contamination.

4.9.2 cDNA synthesis and analysis of gene expression

Total RNA was used to synthesize cDNA by using Super Script™II RT (Invitrozen Cat no. 10777-019) and random primers. The cDNA obtained was used as the template for analysing gene expression. Oligo culm gene specific and reference gene (actin I) primers were used on both shoot and root cDNA. For gene specific primers expected size (350bp) of the amplification was obtained in wild type Basmati 370 shoot/root cDNA and no amplification was observed in Oligo culm. In case of actin I expected size of 118bp was observed in both Basmati 370 and the mutant (Fig. 4.38). The results of RT-PCR showed that the gene was knocked out in the mutant Oligo culm and was expressed in Basmati 370.

Actin1 gene (Os03g50890) was selected as the reference gene for RT-PCR. Because of the availability of predicted gene sequences of *Oryza sativa* (*japonica*) at NCBI (http://www.ncbi.nlm.nih.gov/mapview/maps), it is possible to design primers for targeted gene. Primers were designed based on the japonica cultivar gene sequence. To nullify the risk of amplifying contaminated genomic DNA, primers were designed at intron/exon junctions and outer sides of the insertion position. Further the specificity of each primer was confirmed by 'BLAST' search of the primer sequence using NCBI (http://www.ncbi.nlm.nih.gov) to ensure that the primer pair targeted at unique site. Gene specific primers showed amplification in the wild type but not in the mutant while actin I showed amplification in both the wild type and mutant which ensured that the transcription elongation factor gene was knocked out in the Oligo

culm mutant and was expressed in Basmati 370. The transcription elongation factor expressed more in roots followed by callus panicle and stem (Table 4.8).

Table 4.8: Expression profile suggested by analysis of EST counts of Oligo culm gene.

Pool Name	Transcripts per million	Gene EST/Total EST in pool
Callus	127	21/164802
Flower	21	3/136502
Leaf	34	6/171750
Panicle	45	6/132789
Root	249	17/68202
Seed	30	1/32357
Stem	39	5/126881
Vegetative meristem	0	0/4594

4.10 Sequencing of Basmati 370 Oligo culm (*Osoc*) gene

Nested and overlapping primers were designed based on *japonica* sequence and amplified using Basmati 370 genomic DNA as the template. The amplified products were sequenced using specific forward and reverse primers. Sequencing errors were eliminated by aligning both strands. Alignment of *japonica* and Basmati 370 Oligo culm gene sequence showed 23 SNPs (1% dissimilarity). Gene and protein sequences were predicted using GeneScan software (http://genes.mit.edu/GENSCAN.html). Protein alignment results of Basmati 370 and japonica *Osoc* showed 3 amino acid differences with the same function predicted as transcription elongation factor. The sequence of Oligo culm gene (*Osoc*) of Basmati 370 is given in Fig 4.39.

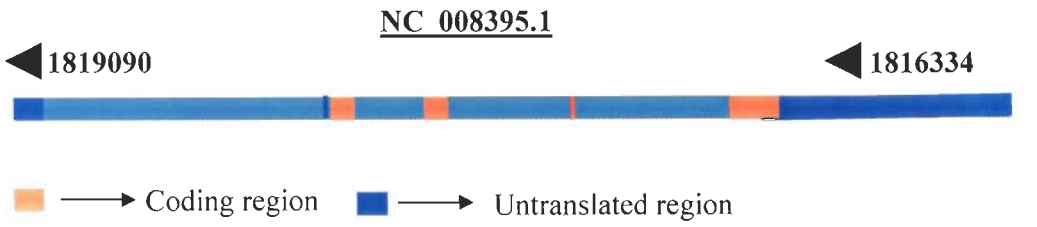

Fig 4.32: Schematic presentation of Oligo culm gene Introns and Exons (Source : NCBI).

```
Osoc-chromosome-2       MGKRKSKAKPPPKKRMDKLDTVFCCPFCNHGSSVECRIDLKNLIGEASCRICLETFSTSV
Paralogs-chromosome-7   MGKRKSAAKPPPKKRMDKLDTVFSCPFCNHGSSVECRIDMKNLIGEASCRICQENFSTTV
                        ****** ***************.***************:************ *.***:*

Osoc-chromosome-2       NALTEPIDIYSEWIDECERVNNPEEDNANHYEDDNASRYRYDEEE
Paralogs-chromosome-7   NALTEPIDIYSEWIDECERVNNVEDDDGA----------------
                        ********************** *:*:.
```

Fig. 4.33: Paralog of Oligo culm gene and the alignment using CLUSTALW.

```
Oryza           MGKRKSKAKPPPKKRMDKLDTVFCCPFCNHGSSVECRIDLKNLIGEASCRICLETFSTSV 60
Vitis           MGKRKSRAKPPPKKRMDKLDTVFSCPFCNHGTSVECRIDMKNLIGEAVCRICQESFSMTV 60
Populus         MGKRKSTAKPPPKKRMDKLDTVFSCPFCNHGTSVECRIDMKNLIGEAVCGICQESFSMTI 60
Arabidopsi      MGKRKSRAKPAPTKRMDKLDTIFSCPFCNHGSSVECIIDMKHLIGKAACRICEESFSTTI 60
                ****** ***.*.********:*.*******:**** **:*:***:* * ** *:** ::

Oryza           NALTEPIDIYSEWIDECERVNNPEEDNANHYEDDNASRYRYDEEE---------------- 105
Vitis           NALSEPIDVYSEWIDECERVNTLDEDAAK------------DED----------------- 92
Populus         TALTEPIDIYSEWIDECERVNSLEDDGA-------------------------------- 88
Arabidopsi      TALTEAIDIYSEWIDECERVNTAEDDVVQEEEEEVEEEEEEEEEEDDEDDHVSVKRKYNF 120
                .**:*.**:************. ::* .
```

Fig. 4.34: Plant orthologs of Oligo culm gene and the alignment using CLUSTALW.

'*' indicates positions which have a single, fully conserved residue, ':' indicates that one of the following 'strong' groups is fully conserved:- (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW), '.' indicates that one of the following 'weaker' groups is fully conserved:- (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY)

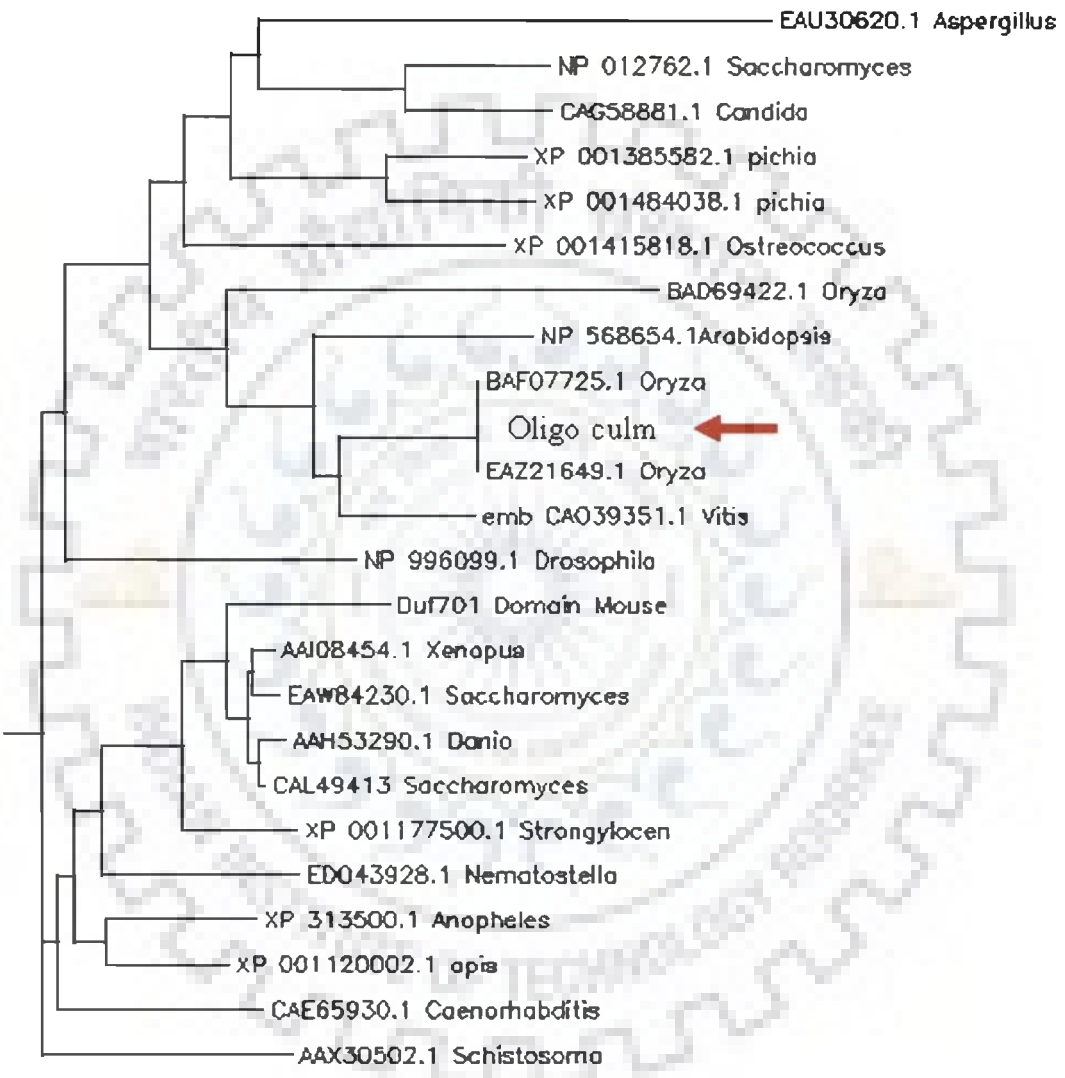

Fig. 4.35: Unrooted phylogenetic tree of a multiple alignment of Oligo culm (*Osoc*) and related proteins from various organisms.

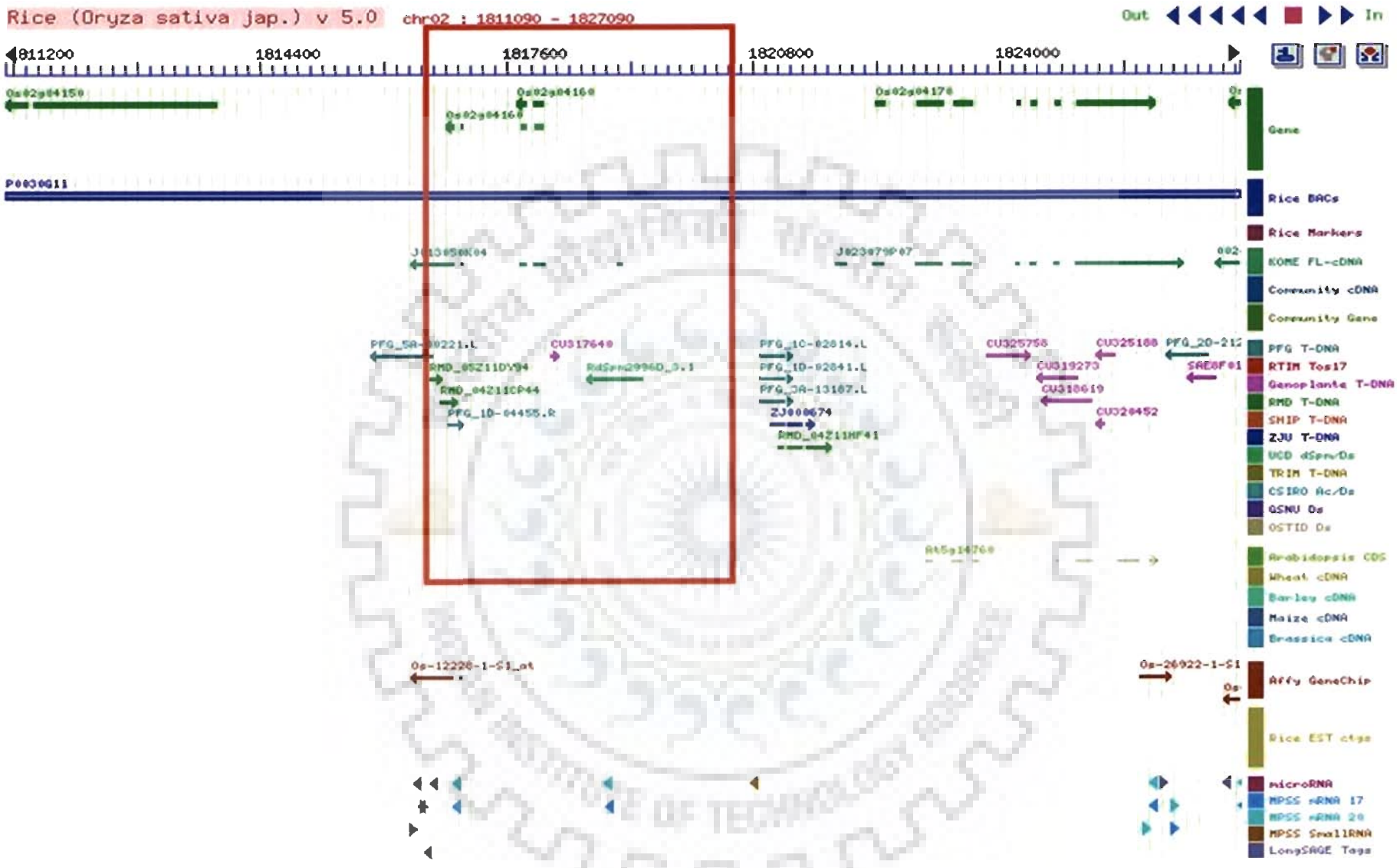

Fig. 4.36: FSTs in *Osoc* region on rice chromosome 2

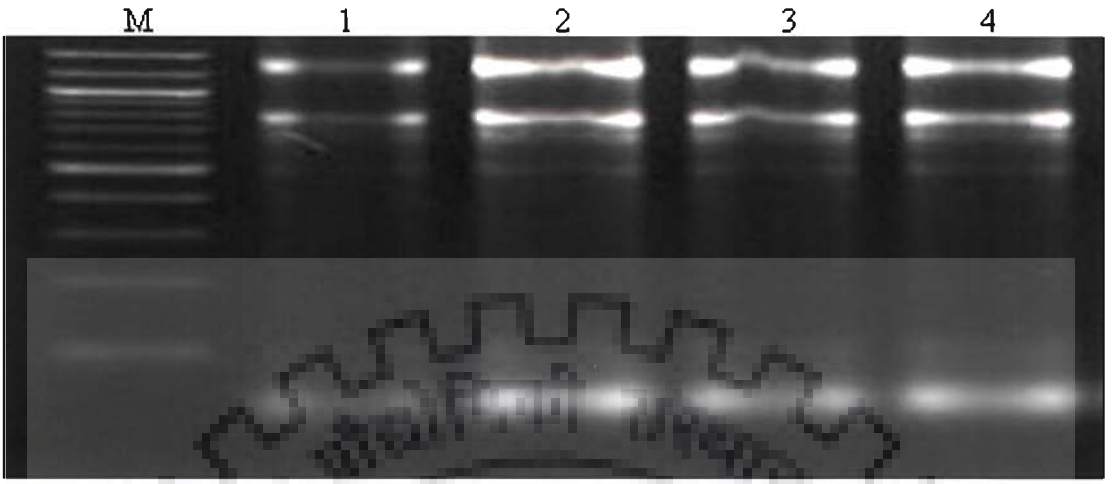

Fig 4.37: Isolation of total RNA. M- 100bp Ladder, 1- Basmati 370 shoots, 2- Basmati 370 roots, 3- Oligo culm shoots, 4- Oligo culm roots.

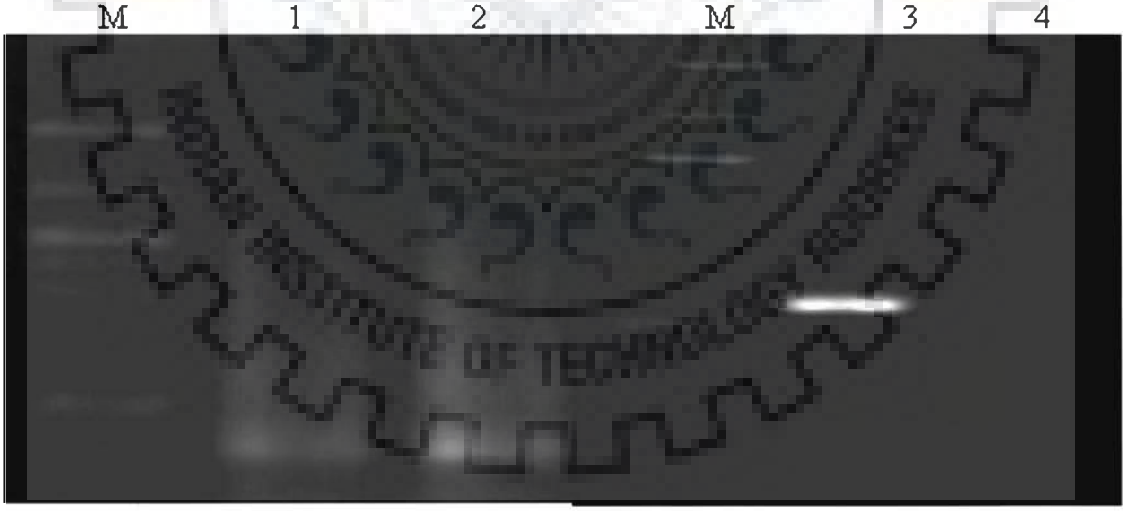

Fig 4.38: Expression analysis studies. M- 100bp ladder, 1&3- Basmati 370 root cDNA, 2&4- Oligo culm root cDNA.

>Oligo culm (*Osoc*)

Fig. 4.39: Oligo culm (*Osoc*) gene sequence of Basmati 370. Highlighted (red coloured) region showed exonic region of gene. Highlighted (yellow coloured) bases showed SNP difference between Basmati 370 and *japonica* sequence. Blue: Translation start point.

CHAPTER 5

DISCUSSION

Rice is one of the most important staple food crops for the mankind. The rice genome sequence (370 Mb out of 389 Mb) has been completed (IRGSP 2005). *Agrobacterium* mediated transformation is being routinely used for insertional mutagenesis and isolation of genes in rice. The generation of rice T-DNA insertional lines is no longer a limiting factor because of the establishment of efficient transformation protocol (Sallaud *et al.*, 2003, 2004). The T-DNA insertional mutagenesis has been used in rice (Suyoung *et al.*, 2003; Jeon *et al.*, 2000; Shuangyan *et al.*, 2003), *Arabidopsis* (Parinov *et al.*, 1999; Sessions *et al.*, 2002) and other plant species. Preliminary screening of these transgenic lines has revealed mutations affecting many different traits (Feldmann *et al.*, 1991). In the present report the T-DNA insertional transformants of Basmati 370 (Dhaliwal *et al.*, 2001) were used for their phenotypic and molecular characterization. These insertional mutants were screened for phenotypic mutants, Oligo culm (*Osoc*) and Seedling lethal (*Ossl*) mutants were identified and used for molecular characterization. Another insertional mutant Small grain (*Ossg*) with 10-15% smaller grains was also characterized.

The construct used to generate these mutants contained hygromycin phosphotransferase (*hpt*) as the selectable marker. The presence of T-DNA insertion in these mutants was confirmed through the selectable marker. The mutants germinated at 80ppm hygromycin where as Basmati 370 seeds did not germinate. The hygromycin resistance was further confirmed by PCR amplification of the *hpt* fragment using *hpt* specific primers.

Basmati 370 insertional mutant, Oligo culm (*Osoc*) showed loss of activity of one of the important genes involved in axillary meristem development. The Oligo culm (*Osoc*) mutant had fewer tillers numbers, retarded growth of seminal roots, reduced plant height and smaller panicles. Mutants defective in branching have been

characterized in various species such as monoculm (Liu *et al.*, 2003b) in rice, Lateral suppressor (*Ls*) (Schumacher *et al.*, 1999) in tomato, Lateral suppressor (*LAS*) (Greb *et al.*, 2003) in *Arabidopsis* and several rice reduced culm number (*rcn*) mutants (Tang *et al.*, 2001; Dong *et al.*, 2006). All the genes responsible for reduced culm number mutant phenotypes were involved in axillary meristem development and their subsequent growth. The seminal root system anchors the young seedlings and absorbs required amount of water for the first three weeks and hence is important under drought conditions (Grando *et al.*, 1995).

In the Seedling lethal mutant (*Ossl*), with a variable penetrance and expressivity, a small proportion of albino plants die after a few days of germination while others with highly retarded growth usually with single tiller and pale yellowish colour die within 30 to 50 days after transplantation in soil. The penetrance is a qualitative and expressivity is a quantitative measure of expression of a mutant. The penetrance relates to whether a phenotype is expressed for a particular genotype whereas the expressivity deals with the degree to which a phenotype is expressed after the penetrance. Expressivity is thus dependent on penetrance (Kral, 1999). The homozygous Seedling lethal (*Ossl*) insertional mutant, confirmed on the basis of hygromycin resistance and *hpt* amplification had 20-30% penetrance. There was high expressivity of the mutant with 5-15% of albino seedlings at the time of germination and 10-20% lethality of the green seedlings within a month of transplantation in soil without any tillering. Survival of high proportion of the homozygous mutant plants to maturity suggests the presence of a paralog of the candidate gene in Basmati 370 or high genotype x environment interaction for the expression of *Ossl*. Yang *et al.* (1999) characterized the *Arabidopsis* mutant defective in plant development (cytokinesis defective 1). Mutants defective in carotenoid biosynthetic path way leading to albino

plants in rice (Wurtz *et al.*, 2001). Lin *et al.* (2006), reported differential expression studies between albino and green plants of *Bambusa edulis*.

Basmati 370 T-DNA insertional mutant Small grain (*Ossg*) had 10-15% smaller grain length and weight. Song *et al.*, (2007) reported that the loss of Grain weight 2 (*GW2*) gene function resulted in a larger hull, subsequently enhanced grain width, weight and yield. *GW2* coded for RING-type protein with E3 ubiquitin ligase activity involved in degradation of cells by the ubiquitin-proteasome pathway.

The F_2 mapping population was developed by crossing Oligo culm (*Osoc*) mutant with distantly related fine rice variety PR106. The F_1 of *Osoc*/PR106 and *Osoc*/Basmati 370 crosses had tiller number nearer to that of PR106 and Basmati 370 parents indicating that Oligo culm (*Osoc*) was recessive. The histogram for tiller number in F_2 plants had skewed distribution towards low tillering indicating that *Osoc* may be partially dominant. Germination data of F_2 seeds on 80ppm hygromycin and *hpt* specific amplification of F_2 mapping population showed a good fit to 3:1 ratio suggesting a single copy T-DNA insertion in the Oligo culm mutant. The result of single copy insertion was supported and confirmed by Southern hybridization.

The Oligo culm (*Osoc*) phenotype is due to T-DNA insertion. In F_2 population, all the Oligo culm plants showed *hpt* amplification. The SSR markers were used to map Oligo culm mutant. Bulk Segregation Analysis and mapping of Oligo culm (*Osoc*) mutant located it on chromosome 2 and linkage with markers RM236 (2.45), RM279 (6.37) and RM12413 (1.47cM). BSA has been applied by different workers for molecular tagging using different marker series in several studies on rice (Huang *et al.*, 2001; Yang *et al.*, 2003; Chen *et al.*, 2007; Wang *et al.*, 1995). BSA was also applied in different species using RAPD (Poulsen *et al.*, 1995, Tabor *et al.*, 2000) and SSRs (Sandhu *et al.*, 2005) markers to identify markers linked to the genes of interest.

Yan *et al.* (1998) and Wu *et al.* (1999) reported that some QTLs for tillering also effected plant height. In rice, several reduced culm mutants namely *rcn*1, *rcn*2, *rcn*5, *rcn*8 and *rcn*9 have been mapped on chromosome 6, 4, 6, 6 and 1, respectively (Dong *et al.*, 2006). In addition, Tang *et al.* (2001) mapped rice few tillering 1 mutant (*FT*1) on chromosome 2 linked with RM263, C424 and S13984 markers far away from the marker linked to *Osoc*. The Oligo culm (*Osoc*) mutant being characterized in the study has not been reported or mapped.

The Oligo culm (*Osoc*) mapping results were further confirmed by identifying T-DNA flanking genome sequence. Several techniques are available to identify flanking genome sequence like TAIL-PCR (Liu *et al.*, 1995; Liu and Whittier 1995), iPCR (Ochman *et al.*, 1988) and Genome walking (Siebert *et al.*, 1995). In the present study TAIL-PCR and Genome walking techniques have been used for identification of flanking genome sequence.

The techniques TAIL-PCR and Genome Walking enable researchers to identify flanking genomic sequence starting from the known sequence (Liu *et al.*, 1995; Smith *et al.*, 1996; Tsugeki *et al.*, 1996; Xiao-Dong *et al.*, 2002; Rishi *et al.*, 2004). In Genome Walking method, choice of restriction enzyme is an important feature that has an effect on the size of flanking sequence to be identified. Five Genome Walker libraries were constructed for Oligo culm (*Osoc*) mutant. *Dra*I, *Rsa*III and *Eco*RV libraries resulted in amplification of 150bp, 200bp and 700bp fragments and no amplification was observed with the *Hae*III and *Hpa*1 libraries. The amplification with *Dra*I, *Rsa*III and *Eco*RV, was due to the presence of restriction sites nearer the T-DNA flanking region and no amplification with *Hae*III and *Hpa*I was observed because of absence of restriction sites near the T-DNA flanking region.

*EcoR*V and *Dra*I Genome Walker libraries were constructed for Seedling lethal (*Ossl*) mutant and a 300bp PCR product was observed with *Eco*RV library.

PCR amplified product of ~250bp was obtained for three mutants (Oligo culm, Seedling lethal and Small grain) by TAIL-PCR. Selection of an optimal T-DNA specific primer for the primary TAIL-PCR is important for successful amplification (Liu and Whittier, 1995). This high throughput technique has been used for isolation of flanking region from a large number of samples because it is very simple, efficient and highly specific. No other manipulations are required apart from PCR (Liu *et al.*, 1995).

Flanking genome sequence results were not obtained while using T-DNA left border primers. This may be because of the modifications or loss of the T-DNA left border. According to McClean (1998) the only structural requirement for T-DNA transfer is the TR direct repeat border. The T-DNA transfer occurs in the right to left direction. The genes required for oncogene functions are linked with the right border. Thus, when right border was used for T-DNA transfer, the genes required for the oncogenic functions were transferred, but when the left border was used in lieu of the right border, transfer did not include these genes and no transformation was detected. Many a times left border was found to have deletions during transformation. In *Ascochyta rabei* transformation, the T-DNA left border region contained deletion of upto 72bp upon integration (White and Chen, 2006).

Oligo culm mutant (*Osoc*) flanking genome sequence results, with Genome Walking and TAIL-PCR, showed its alignment on rice chromosome number 2. The T-DNA was inserted in the exonic region of the predicted transcription elongation factor. The length of the gene was 2,757bp with 4 exons, 4 introns and 5`UTR. The length of the mRNA was 875bp which coded for protein with 105 amino acids

(http://www.ncbi.nlm.nih.gov/sites/entrez). The protein contains putative zinc binding domain with four conserved cysteine residues (Marchler-Bauer *et al.*, 2004).

The upstream region from the site of 5` UTR was searched for promoter region using Promoter 2.0 (Knudsen *et al.*, 1999) and TSSP-TCM (Shahmuradov *et al.*, 2003). *In silico* promoter prediction analysis did not predict any promoter sequence. When 900bp upstream sequence and 5` UTR sequence were used for searching promoter sequence, TATA less promoter was identified in UTR region at 103bp with Promoter 2.0 and at 124bp with TSSP-TCM Program (monocot promoter database). Internal promoters have short consensus sequence located within the transcription unit and cause initiation to occur at fixed distance upstream. RNA pol III enzyme generally transcribes genes with internal promoters (Lewin, 2005). Genes with internal promoters are more commonly involved in gene duplications (Huang *et al.*, 2000).

RT-PCR analysis studies confirmed the absence of expression of *Osoc* gene in Oligo culm mutant. Highly conserved orthologs of *Osoc* were found through out the living kingdom indicating the importance of the gene in development. The loss of function of the ortholog gene in *Saccharomyces cerevisiae* (Prather *et al.*, 2005) causes lethality. But in Basmati 370, loss of function of this gene caused only reduced tillering, retarded growth of seminal roots and reduced plant height. The presence of a paralog with high similarity on chromosome 7, is probably buffering against lethality due to loss of function of the Oligo culm (*Osoc*) gene in Basmati 370.

The complete sequencing of Oligo culm (*Osoc*) gene in Basmati 370 and its alignment search with *japonica* cultivar sequence found 23 SNPs which makes 1% nucleotide difference between the two sequences. The protein sequence of *Osoc* of Basmati 370 was predicted from nucleotide sequence using GeneScan

(http://genes.mit.edu/GENSCAN.html). The Basmati 370 Oligo culm (*Osoc*) gene also coded for transcription elongation factor protein (105 amino acids) which differed from the *japonica* cultivar only for three amino acids. There are several reports of single nucleotide polymorphism (SNP) diversity between the two rice groups. Yu *et al.* (2005) predicted 3.0 SNP/Kb in the coding regions and 27.6 SNP/Kb in the transposable elements, with an average rate of 6.1 SNP/Kb and 1.3 indel/Kb. Feltus *et al.* (2004) identified 408,898 candidate SNP/indels after filtering multiple copy and low quality sequence based on the two draft sequences of *indica* and *japonica* cultivars.

BLAST alignment of T-DNA flanking genome sequence of Seedling lethal mutant (*Ossl*) with Genome Walking and TAIL-PCR showed it on rice chromosome 11. The T-DNA insertion position on chromosome 11 was further confirmed using genome specific primers. The T-DNA insertion was found in the junk region where no gene has been identified in *japonica* and *indica* cultivars. Basmati 370 might have an important gene at the insertion site on chromosome 11 where no gene could be present or predicted in the Nipponbare sequence. There is a difference between the two subspecies *indica* and *japonica* cultivars in gene content. Yu *et al.*, (2005) reported that out of the estimated gene count of 38,000-40,000 only 2-3% of the genes are unique to the subspecies.

In the insertional mutant Small grain (*Ossg*), flanking sequencing results using TAIL-PCR showed homology on chromosome 11 of rice. The T-DNA was inserted in an intronic region of the Pectin methyl esterase gene. The total length of the gene was 2,185bp with five exons and two introns. The length of mRNA was 1749bp which coded for a protein with 423 amino acid residues (http://www.ncbi.nlm.nih.gov/sites/entrez). The gene Pectin methyl esterase contains

putative pectinesterase domain. Pectin methyl esterase (PME, EC 3.1.1.11) is a cell wall bound enzyme which catalyses the hydrolysis of methyl ester group from galacturonosyl residues (Fisher and Bennett, 1991). EST count profile retrieved from the database suggested that expression of the identified knockout gene (Small grain size) was more in panicles and flowers. Several Pectin methyl esterase isoforms with different pH, isoelectric points and molecular weight are present in a genome (Warrilow and Jones, 1995). Few genes are expressed in all the plant organs and others are expressed only at particular organs or developmental stages. Mutations in *Arabidopsis* gene mucilage modified 4 (*mum4*) results in a significant decrease in seed coat mucilage. Mucilage is composed primarily of pectin, the major component of which is rhamnogalacturon 1(Western *et al.*, 2000, 2001 and 2004) The orthologs of Pectin methyl esterase involved in fruit ripening have been identified and cloned in grapes, strawberry and from several plant species (Laurent *et al.*, 2001; Castillejo *et al.*, 2004). From the EST database and cloned ortholog genes it is clear that the knockout Pectin esterase gene (*Ossg*) was being expressed only in panicle and flower. The mechanism of Pectin methylesterase gene in determining grain size can be established through further studies using this T-DNA knockout Small grain (*Ossg*) mutant.

The Rice Annotation Project Database (RAP-DB) has integrated the information of all the insertional mutations generated by various independent groups (http://signal.salk.edu/cgi-bin/RiceGE and http://orygenesdb.cines.fr/). The *Osoc* gene BLAST against rice FST database showed five hits. No phenotypic descriptions are available for these FSTs. Only one FST insertional mutant seed are available. These seeds can be obtained and grown for phenotypic comparison. The FST database can help to validate the function of the identified genes.

The available material and knowledge can help in further characterization of the candidate genes. The T-DNA cassette has Hm^{R}*Ds* construct in the insertional mutants (Dean *et al.*, 1992). In the two-element transposon tagging system, the whole genome can be saturated with *Ds* element with few transformations. The *Ds* element in the insertional mutants can be mobilised to other positions by crossing them with a transgenic plant carrying the *Ac* element. The *Ds* element will preferentially transpose to other sites closely linked to the donor sites (Smith *et al.*, 1996; Machida *et al.*, 1997). The two element system has been used successfully to establish large collections of insertional mutants in rice and *Arabidopsis* (Kolesnik *et al.*, 2004; Alonso and Ecker, 2006; Kolesnik *et al.*, 2004; Greco *et al.*, 2003). The final validation of the Oligo culm (*Osoc*) gene and Small grain size (*Ossg*) can be done by genetic complementation or RNAi techniques.

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APPENDIX

Appendix 1: Phenotypic data of Oligo culm/PR106 F_2 mapping population

Plant no.	Plant height(cm)	Tiller number	Days to flowering
Osoc 1	107	10	104
Osoc 2	139	7	107
Osoc 3	147	11	126
Osoc 5	121	10	122
Osoc 6	161	10	126
Osoc 7	93	8	117
Osoc 8	102	12	137
Osoc 9	127	3	135
Osoc 10	121	7	124
Osoc 11	154	9	135
Osoc 12	109	4	135
Osoc 13	113	16	138
Osoc 14	79	7	135
Osoc 15	118	9	137
Osoc 16	130	10	112
Osoc 17	130	7	128
Osoc 18	100	11	143
Osoc 19	104	4	112
Osoc 20	125	7	106
Osoc 21	119	9	120
Osoc 22	96	10	106
Osoc 23	91	2	150
Osoc 24	108	9	102
Osoc 25	85	2	117
Osoc 26	116	10	128
Osoc 27	124	12	137
Osoc 28	106	13	136
Osoc 29	135	7	137
Osoc 30	100	8	140
Osoc 31	112	6	116
Osoc 32	89	3	123
Osoc 33	92	6	137
Osoc 34	43	1	153
Osoc 35	60	2	121
Osoc 36	154	10	134
Osoc 37	113	4	121
Osoc 38	96	3	123
Osoc 40	93	6	109
Osoc 41	78	4	135
Osoc 42	109	4	127
Osoc 43	126	9	136
Osoc 44	141	3	121
Osoc 45	122	3	102
Osoc 46	135	10	137

Osoc 47	126	9	137
Osoc 48	125	5	112
Osoc 49	74	6	122
Osoc 50	108	3	135
Osoc 51	99	6	137
Osoc 52	42	1	124
Osoc 53	102	6	138
Osoc 54	73	1	143
Osoc 55	129	9	137
Osoc 56	136	7	135
Osoc 57	85	4	141
Osoc 58	96	3	137
Osoc 59	114	9	137
Osoc 60	80	2	138
Osoc 61	111	8	126
Osoc 62	86	4	126
Osoc 63	113	9	135
Osoc 64	76	6	137
Osoc 66	123	6	123
Osoc 67	92	6	138
Osoc 68	120	14	138
Osoc 69	163	11	153
Osoc 70	120	7	118
Osoc 71	139	9	137
Osoc 72	148	11	125
Osoc 73	130	5	128
Osoc 74	147	9	126
Osoc 75	93	3	128
Osoc 76	152	19	113
Osoc 77	85	1	128
Osoc 78	123	11	136
Osoc 79	147	8	115
Osoc 80	137	6	113
Osoc 81	57	1	141
Osoc 82	94	2	137
Osoc 83	146	8	136
Osoc 84	123	12	106
Osoc 85	83	5	137
Osoc 86	100	7	128
Osoc 87	90	2	128
Osoc 88	122	5	116
Osoc 89	111	8	136
Osoc 90	74	7	134
Osoc 91	108	6	106
Osoc 92	86	5	118
Osoc 93	119	4	117
Osoc 94	128	13	109
Osoc 95	170	5	135

Osoc 96	135	8	114
Osoc 97	115	5	135
Osoc 98	110	3	111
Osoc 100	122	5	111
Osoc 101	101	2	103
Osoc 102	126	6	136
Osoc 103	154	8	137
Osoc 104	142	6	116
Osoc 105	96	9	135
Osoc 106	122	11	104
Osoc 107	109	3	108
Osoc 109	115	6	137
Osoc 110	126	9	137
Osoc 112	104	7	120
Osoc 113	81	2	135
Osoc 114	115	10	135
Osoc 115	100	6	138
Osoc 116	131	6	117
Osoc 117	131	5	106
Osoc 118	110	7	105
Osoc 119	102	8	137
Osoc 120	102	11	141
Osoc 121	140	17	128
Osoc 122	106	1	135
Osoc 123	136	6	134
Osoc 124	135	8	137
Osoc 125	130	9	106
Osoc 126	117	7	138
Osoc 127	113	9	135
Osoc 128	67	1	135
Osoc 129	111	7	134
Osoc 130	147	13	135
Osoc 131	80	6	137
Osoc 132	120	11	135
Osoc 133	108	2	137
Osoc 134	110	7	136
Osoc 135	108	11	135
Osoc 136	141	6	125
Osoc 137	77	4	137
Osoc 138	101	5	136
Osoc 139	121	4	115
Osoc 140	75	7	155
Osoc 141	107	5	142
Osoc 142	115	10	116
Osoc 143	112	13	136
Osoc 144	105	8	136
Osoc 145	146	5	135
Osoc 146	133	8	118

Osoc 147	115	2	102
Osoc 148	132	7	137
Osoc 149	109	9	140
Osoc 150	100	5	135
Osoc 151	147	10	124
Osoc 152	148	6	124
Osoc 153	166	6	135
Osoc 154	73	1	122
Osoc 155	86	6	124
Osoc 156	135	5	136
Osoc 157	62	1	135
Osoc 158	106	5	119
Osoc 159	120	11	112
Osoc 160	60	3	150
Osoc 161	93	4	137
Osoc 162	100	5	120
Osoc 163	102	8	129
Osoc 164	99	4	135
Osoc 165	115	9	135
Osoc 166	115	8	138
Osoc 167	89	2	143
Osoc 168	151	16	135
Osoc 169	123	11	124
Osoc 170	63	2	137
Osoc 171	106	5	118
Osoc 172	84	9	140
Osoc 173	132	7	137
Osoc 174	124	7	136
Osoc 175	113	9	143
Osoc 176	95	4	135
Osoc 177	78	7	137
Osoc 178	107	6	135
Osoc 179	124	7	124
Osoc 180	114	11	137
Osoc 181	121	4	117
Osoc 182	52	1	151
Osoc 183	126	4	121
Osoc 184	45	1	145
Osoc 185	102	7	137
Osoc 186	117	5	128
Osoc 187	94	2	135
Osoc 188	68	2	137
Osoc 189	102	5	137
Osoc 190	85	2	138
Osoc 191	104	9	128
Osoc 192	100	7	135
Osoc 193	53	1	141
Osoc 194	58	1	121

Osoc 195	121	11	137
Osoc 197	112	11	135
Osoc 198	106	8	119
Osoc 200	86	3	141
Osoc 201	131	11	138
Osoc 202	93	3	122
Osoc 203	120	3	124
Osoc 204	98	4	116
Osoc 205	126	7	135
Osoc 206	95	6	135
Osoc 207	102	4	124
Osoc 208	73	2	144
Osoc 209	72	1	124
Osoc 210	104	12	136
Osoc 211	74	3	119
Osoc 212	129	9	135

Appendix 2: Polymorphic SSR markers between Basmati 370 and PR106

Rice Microsatellite	Forward primer	Reverse primer
RM3	acactgtagcggccactg	cctccactgctccacatctt
RM4	ttgacgaggtcagcactgac	agggtgtatccgactcatcg
RM5	tgcaacttctagctgctcga	gcatccgatcttgatggg
RM11	tctcctcttcccccgatc	atagcgggcgaggcttag
RM 17	tgccctgttattttcttctctc	ggtgatcctttcccatttca
RM 18	ttccctctcatgagctccat	gagtgcctggcgctgtac
RM 25	ggaaagaatgatcttttcatgg	ctaccatcaaaaccaatgttc
RM 30	ggttaggcatcgtcacgg	tcacctcaccacacgacacg
RM 38	acgagctctcgatcagccta	tcggtctccatgtcccac
RM 42	atcctaccgctgaccatgag	tttggtctacgtggcgtaca
RM 51	tctcgattcaatgtcctcgg	ctacgtcatcatcgtcttccc
RM 55	ccgtcgccgtagtagagaag	tcccggttattttaaggcg
RM 84	taagggtccatccacaagatg	ttgcaaatgcagctagagtac
RM 85	ccaaagatgaaacctggattg	gcacaaggtgagcagtcc
RM 104	ggaagaggagagaaagatgtgtgtcg	tcaacagacacaccgccaccgc
RM 107	agatcgaagcatcgcgcccgag	actgcgtcctctgggttcccgg
RM 127	gtgggatagctgcgtcgcgtcg	aggccagggtgttggcatgctg
RM 128	agcttgggtgatttcttggaagcg	acgacgaggagtcgccgtgcag
RM 130	tgttgcttgccctcacgcgaag	ggtcgcgtgcttggtttggttc
RM 131	tcctccctcccttcgcccactg	cgatgttcgccatggctgctcc
RM 141	caccaccaccaccacgcctctc	tcttggagaggaggaggcgcgg
RM 154	accctctccgcctcgcctcctc	ctcctcctcctgcgaccgctcc
RM 156	gccgcaccctcactccctcctc	tcttgccggagcgcttgaggtg
RM 160	agctagcagctatagcttagctggagatcg	tctcatcgccatgcgaggcctc
RM 161	tgcagatgagaagcggcgcctc	tgtgtcatcagacggcgctccg
RM 163	atccatgtgcgcctttatgagga	cgctacctccttcacttactagt
RM 169	tggctggctccgtgggtagctg	tcccgttgccgttcatccctcc
RM 174	agcgacgccaagacaagtcggg	tccacgtcgatcgacacgacgg
RM 186	tcctccatctcctccgctcccg	gggcgtggtggccttcttcgtc
RM 197	gatccgtttttgctgtgccc	cctcctctccgccgatcctg
RM 201	ctcgtttattacctacagtacc	ctacctcctttctagaccgata
RM 205	ctggttctgtatgggagcag	ctggcccttcacgtttcagtg
RM 208	tctgcaagccttgtctgatg	taagtcgatcattgtgtggacc
RM 210	tcacattcggtggcattg	cgaggatggttgttcacttg
RM 212	ccactttcagctactaccag	cacccatttgtctctcattatg
RM 216	gcatggccgatggtaaag	tgtataaaaccacacggcca
RM 222	cttaaatgggccacatgcg	caaagcttccggccaaaag
RM 233	ccaaatgaacctacatgttg	gcattgcagacagctattga
RM 235	agaagctagggctaacgaac	tcacctggtcagcctctttc

RM 236	gcgctggtggaaaatgag	ggcatccctctttgattcctc
RM 242	ggccaacgtgtgtatgtctc	tatatgccaagacggatggg
RM 247	tagtgccgatcgatgtaacg	catatggttttgacaaagcg
RM 252	ttcgctgacgtgataggttg	atgacttgatcccgagaacg
RM 253	tccttcaagagtgcaaaacc	gcattgtcatgtcgaagcc
RM 254	agccccgaataaatccacct	ctggaggagcatttggtagc
RM 256	gacagggagtgattgaaggc	gttgatttcgccaagggc
RM 263	cccaggctagctcatgaacc	gctacgtttgagctaccacg
RM 264	gttgcgtcctactgctacttc	gatccgtgtcgatgattagc
RM 273	gaagccgtcgtgaagttacc	gtttcctacctgatcgcgac
RM 279	gcgggagagggatctcct	ggctaggagttaacctcgcg
RM 280	acacgatccactttgcgc	tgtgtcttgagcagccagg
RM 286	ggcttcatctttggcgac	ccggattcacgagataaactc
RM 289	ttccatggcacacaagcc	ctgtgcacgaacttccaaag
RM 295	cgagacgagcatcggataag	gatctggtggaggggagg
RM 296	cacatggcaccaacctcc	gccaagtcattcactactctgg
RM 302	tcatgtcatctaccatcacac	atggagaagatggaatacttgc
RM 306	caaggtcaagaatgcaatgg	gccactttaatcattgcatc
RM 309	gtagatcacgcacctttctgg	agaaggcctccggtgaag
RM 328	catagtggagtatgcagctgc	ccttctcccagtcgtatctg
RM 330	caatgaagtggatctcggag	catcaatcagcgaaggtcc
RM 334	gttcagtgttcagtgccacc	gactttgatctttggtggacg
RM 335	gtacacacccacatcgagaag	gctctatgcgagtatccatgg
RM 338	cacaggagcaggagaagagc	ggcaaaccgatcactcagtc
RM 341	caagaaacctcaatccgagc	ctcctcccgatcccaatc
RM 346	cgagagagcccataactacg	acaagacgacgaggagggac
RM 348	ccgctactaatagcagagag	ggagctttgttcttgcgaac
RM 413	ggcgattcttggatgaagag	tccccaccaatcttgtcttc
RM415	cttcgatccatcatccatgg	attgctgtacgcagtttcgg
RM 421	agctcaggtgaaacatccac	atccagaatccattgacccc
RM 424	tttgtggctcaccagttgag	tggcgcattcatgtcatc
RM 427	tcactagctctgccctgacc	tgatgagagttggttgcgag
RM 445	cgtaacatgcatatcacgcc	atatgccgatatgcgtagcc
RM 451	gatcccctccgtcaaacac	cccttctcctttcctcaacc
RM 465	gtgcctccatcatcatcatc	taggacaagcgaagaaaccg
RM 466	tccatcaccacattcccc	accettctctcgctctctcc
RM 470	tcctcatcggcttcttcttc	agaacccgttctacgtcacg
RM 486	ccccctctctctctctctc	tagccacatcaacagcttgc
RM 488	cagctagggttttgaggctg	tagcaacaaccagcgtatgc
RM 490	atctgcacactgcaaacacc	agcaagcagtgctttcagag
RM 492	ccaaaaatagcgcgagagag	aagacgtacatgggtcaggc
RM 496	gacatgcgaacaacgacatc	gctgcggcgctgttatac

RM 502	gcgatcgatggctacgac	acaacccaacaagaaggacg
RM 511	cttcgatccggtgacgac	aacgaaagcgaagctgtctc
RM 517	ggcttactggcttcgatttg	cgtctcctttggttagtgcc
RM 519	agagagcccctaaatttccg	aggtacgctcacctgtggac
RM 520	aggagcaagaaaagttcccc	gccaatgtgtgacgcaatag
RM 527	ggctcgatctagaaaatccg	ttgcacaggttgcgatagag
RM 528	ggcatccaattttacccctc	aaatggagcatggaggtcac
RM 536	tctctcctcttgtttggctc	acacaccaacacgaccacac
RM 545	caatggcagagacccaaaag	ctggcatgtaacgacagtgg
RM 553	aactccacatgattccaccc	gagaaggtggttgcagaagc
RM 555	ttggatcagccaaaggagac	cagcattgtggcatggatac
RM 560	gcaggaggaacagaatcagc	agcccgtgatacggtgatag
RM 562	cacaacccacaaacagcaag	cttccccaaagttttagcc
RM 570	gttcttcaactcccagtgcg	tgacgatgtggaagagcaag
RM12413	gaccgaggtgggtacatcatcg	gacatcccaatgcagccaatcc

RESEARCH PUBLICATIONS

Research Publication:

1. Routray P, **Basha O**, Garg M, Singh N. K, Dhaliwal H. S. (2007). Genetic diversity of landraces of wheat (*Triticum aestivum* L.) from hilly areas of Uttaranchal, India. Genet Resour Crop Evol. 54: 1315–1326.

2. Rao H. S, **Basha O. P**, Singh N. K, Sato K, Dhaliwal H. S. (2007). Frequency distributions and composite interval mapping for QTL analysis in 'Steptoe' x 'Morex' barley mapping population, Barley Genetics Newsletter 37: 5-20.

Paper Presentations:

1. **P. Osman Basha** and H.S. Dhaliwal "Characterization of *Ds* insertional Oligo culm (*Osoc*) and seedling lethal (*Ossl*) mutant in Basmati 370" paper presentation at National symposium on Basmati Rice Research: Current Trends and Future Prospects, 6th -7th September 2005, Department of Genetics and Plant Breeding SVBP University of Agriculture and Technology, Meerut.

2. **Osman Basha**, Anju Bhalla, Mankesh Kumar, Deepak Rajpurohit, G.S. Randhawa and H.S. Dhaliwal. "Molecular characterization of Oligo culm (*Osoc*) mutant in Basmati 370" Poster presentation at International Rice Congress 2006, October 9- 13, 2006, New Delhi.

3. Anju Bhalla, **P. Osman Basha**, Mankesh Kumar, Deepak Rajpurohit, G.S. Randhawa and H.S. Dhaliwal. "Characterization of *Ds* insertional Polyembryonic, Oligo culm, and Dwarf mutants in Basmati 370" at National biotechnology Conference-2006, Current Trends and Future Perspectives. Department of Biotechnology, IIT Roorkee and Indian Federation of Biotechnologists (IFB-India).

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