MAPPING AND MOLECULAR CHARACTERIZATION OF DWARF *(OsGAI/Sd)* INSERTIONAL MUTANT IN BASMATI

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree

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DOCTOR OF PHILOSOPHY

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by

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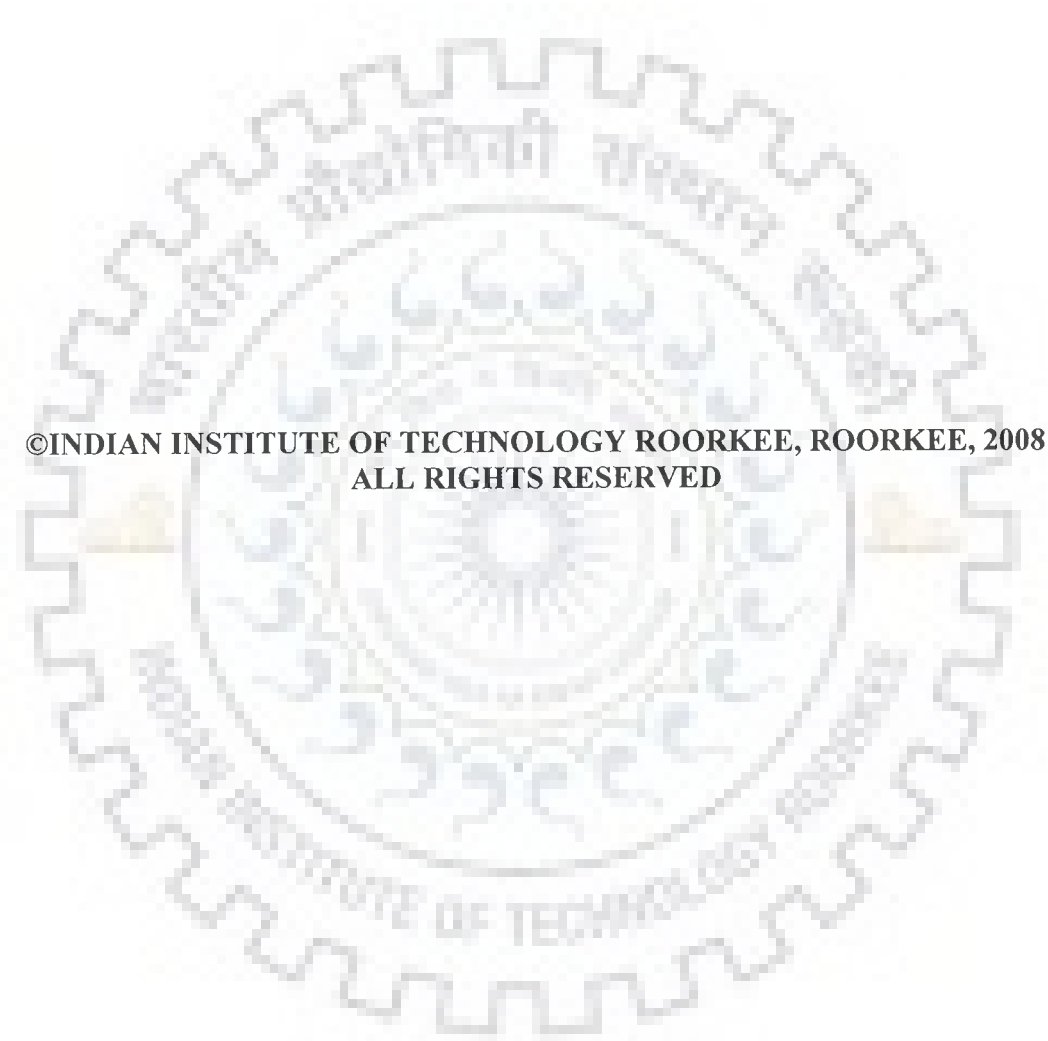

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in this thesis entitled **MAPPING AND MOLECULAR CHARACTERIZATION OF DWARF (*OsGAI/Sd*) INSERTIONAL MUTANT 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 a period of January 2004 to May 2008 under the supervision of Dr. H. S. Dhaliwal and Dr. G. S. Randhawa, Professors, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

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

Mankesh Kumar.

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

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The PhD Viva-Voce Examination of **Mr. Mankesh Kumar**, Research Scholar has been held on .....

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ABSTRACT

Rice (*Oryza sativa* L.) is a staple food for half of the world population and its production was almost doubled due to cultivation of semi-dwarf cultivars containing *sd1* gene form Dee-Geo-Woo-Gen (DGWG) source. Besides its commercial importance, rice is a model monocot for plant biotechnologists due to the availability of its complete genome sequence, its small genome size (~389 Mb) and its efficient tissue culture response. Basmati 370 is the first wonder Basmati variety which was released during pre-independence era and continues to reign till today. But it lodges due to its tall stature, resulting in its poor grain yield. In the present study, a T-DNA insertional dwarf mutant (*OsGAI/Sd*) of Basmati 370 with approximately half the plant height as compared to Basmati 370 was isolated by T-DNA transformation containing Hm^R*Ds* cassette. It was found to be insensitive to exogenous Gibberellic acid (GA_3) application. About 55-60% of the plants carrying the *hpt* gene showed dwarf and GA-insensitive phenotype which can be due the presence of incomplete penetrance and variable expressivity of the mutant. Incomplete penetrance in this context refers to the presence of dwarf phenotype in 55-60% of the plants carrying the *hpt* gene in homozygous condition while variable plant height from severe-dwarf, dwarf, semi-dwarf to tall individual plants in the selfed progenies and GA_3 sensitivity from 2-30% in between mutant and Basmati 370 indicated variable expressivity. The dwarfing gene *OsGAI/Sd* had pleiotropic effects for reduced seed size, tillering, panicle length and fertility. Another insertional semi-dwarf mutant *OsGAS/Sd* was found to be sensitive to exogenous GA_3 application at 120 ppm concentration. Presence of T-DNA in the dwarf, semi-dwarf and another insertional mutant B-3-1 was confirmed by PCR amplification of *hpt* gene used as the selectable marker in Hm^R*Ds* construct of the T-DNA. The seeds of mutants were also resistant to

hygromycin at 80 ppm concentration during germination in petri-plates. Single copy insertion of T-DNA in *OsGAI/Sd* was confirmed by Southern hybridization and 3:1 segregation of *hpt* gene in 164 F_2 plants obtained from *OsGAI/Sd* X PR106 in PCR amplification and germination on hygromycin at 80 ppm. Total leaf chlorophyll content was found to be more in the dwarf mutant. In histological studies, the cell size of the *OsGAI/Sd* was found to be reduced due to loss-of-function of the gene in the mutant. 164 F_2 progenies of *OsGAI/Sd* X PR106 cross segregated in 3:1 ratio for GA-insensitive *vs.* GA-sensitive phenotype, whereas 107 F_2 plants of *OsGAI/Sd* X Basmati 370 segregated in 3:1 ratio for dwarf *vs.* tall phenotype. Out of 213 rice mapped SSR markers, 95 showed parental polymorphism between Basmati 370 and PR106. Based on the data of recombination frequency in the mapping population of *OsGAI/Sd* X PR106 cross, the anchored SSR markers RM14645 and RM14667 of chromosome 3 mapped at distance of 1.21cM and 6.49cM, respectively from the T-DNA insertion containing *hpt* gene. The T-DNA flanking region isolated through TAIL-PCR showed a single hit on chromosome 3 in BLASTN with the total *japonica* cv. Nipponbare genome sequence present in NCBI database. Using PCR genome walking, the T-DNA flanking region of the *OsGAI/Sd* could not be amplified due to lack of appropriate restriction sites near the T-DNA insertion. The T-DNA insertion was found at the second exonic region of a gene which encodes for sixth subunit of Anaphase Promoting Complex/Cyclosome (APC/C). The APC/C plays role in the protein degradation of several proteins through ubiquitin-proteasome mediated proteolysis pathway. The 8.6 kb of the candidate gene encodes 728 amino acid protein containing a conserved Tetra Tricopeptide Repeat (TPR) domain. The only paralog of APC/C in rice, isopencillin N-synthase family protein on chromosome 3, was without the TPR domain. The ortholog of Anaphase Promoting Complex (APC)

component 6 in *Arabidopsis thaliana* showed highest identity with the candidate gene. In the different models of GA-signaling proposed so far in rice, the SCF^{GID2} complex mediates the degradation of a DELLA protein SLR1 which in turn switches on the GA-dependent growth. The present study reports a novel function of APC/C holo-enzyme in Basmati rice which may degrade the SLR1 protein via ubiquitin mediated proteolysis pathway. Through RT-PCR, the expression of wild type gene was found in both roots and shoots. Four Flanking Sequence Tags (FSTs) of the candidate gene *OsGAI/Sd* were found in OryGeneDB, whereas two FSTs were found for *OsGAS/Sd* in RiceGE database. FST along with RNAi mediated gene silencing approach can be used for the functional validation of the target gene in both the GA-insensitive and sensitive mutants of Basmati 370.

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relatives and well wishers, whose names may not have been mentioned here, but whose good wishes and support have enabled me to complete this work. I dedicate this dissertation to my father, who dreamt of me getting a doctorate and to instill into me, the value of hard work.

Mankesh Kumar.

Date: 11/5/2008 **MANKESH KUMAR**

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ABBREVIATIONS USED

%	Percentage
µ	Micron
µg	Microgram
µm	Micrometer
AD primers	Arbitrary Degenerate primers
APC/C	Anaphase Promoting Complex/Cyclosome
BLAST	Basic Local Alignment and Search Tool
BLASTN	Nucleotide BLAST
BLASTX	Search protein database using a translated nucleotide query
bp	Base pairs
BSA	Bulk Segregant Analysis
Cdc genes	Cell division cycle genes
cDNA	Complementary DNA
cM	Centi Morgan
cm	Centimeter
cv.	Cultivated Variety
Cys	Cystine
α-P^{32} dCTP	Radiolabelled P^{32} Cytosine Triphosphate
DNase	Deoxyrinuclease
DEPC	Diethyl Pyrocarbonate
dNTPs	Nucleotide Triphosphates
Ds	Dissociation Element
EDTA	Ethylenediaminetetraacetic acid
EDTA	Ethylene Diamine Tetraacetic Acid
F_1	First Filial Generation
F_2	Second Filial Generation
F_3	Third Filial Generation
Fig.	Figure

FST	Flanking Sequence Tags
G	Gram
GA	Gibberellic Acid
H	Hour
hpt	Hygromycin Phosphotransferase
IRGSP	International Rice Genome Sequencing Project, 2005
Kb	Kilobase pairs (10^3 bp)
kDa	Kilo Dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
L	Litre
LB	Left Border
LS	Longitudinal Section
Lys	Lysine
M	Molarity
Mb	Megabase pairs (10^6 bp)
Mg	Milligram
min	Minute
Ml	Millilitre
Mm	Millimeter
mM	Millimole
NCBI	National Centre for Biotechnology Infirmation
NPT	Neomycin Phosphotransferase
°C	°Centigrade
ORF	Open Reading Frame
OsGAI/Sd	*Oryza sativa* Gibberellic Acid Insensitive/ Semi-Dwarf
OsGAS/Sd	*Oryza sativa* Gibberellic Acid Sensitive/ Semi-Dwarf
PCR	Polymerase Chain Reaction
POSTECH	Pohang University of Science and Technology
ppm	Parts per million
RB	Right Border
Ri plasmid	Root inducing plasmid
RNAi	RNA interference

RT-PCR	Reverse transcriptase-PCR
RiceGE	Rice Functional Genomic Express Database
SDS	Sodium dodecyl sulfate
Sec	Second
SEM	Scanning Electron Microscopy
SPT	Streptomycin Phosphotransferase
SSC	Sodium Citrate
SSRs	Simple Sequence Repeats
TAE	Tris Acetate
TAIL-PCR	Thermal asymmetric interlace -PCR
TBE	Tris Borate
T-DNA	Transfer-DNA
Ti plasmid	Tumour inducing
TE	Tris EDTA
TPase	Transposase
TPR	Tetratrico Peptide repeat
TS	Transverse Section
v/v	Volume/Volume
w/v	Weight/Volume

CHAPTER 1

INTRODUCTION

1. Introduction

Rice is the staple crop for more than 70% of Indians which is grown on 44 million hectares throughout the country. The production of rough rice (paddy) reached 92.76 million tones in 2006-07 from a base year production of 32.3 million tonnes in 1950-52. This was possible mainly due to the adoption of high yielding semi-dwarf rice cultivars during green revolution in 1960s and decades after that. But with the fast growing scenario of Indian population, India's rice production needs to be increased to 140 million tonnes by the year 2025 AD. Besides its economical importance, rice has become a model system for genomics because of its relatively small genome of 389Mb, which is one of the smallest in the Grammineae family. Moreover, it is closely related to major cereals like maize, sorghum and wheat. It has a compact genome size, which is one-sixth the size of maize genome and 40 times smaller than the wheat genome (Jung *et al.*, 2008). It can be more efficiently and routinely transformed as compared to other cereals, which provides a powerful tool for gene discovery through reverse genetics and insertional mutagenesis. After the complete genome sequencing of rice genome, the information about its genetic and molecular resources has become very much enriched. The current map-based rice genome sequence assembly (372.1Mb) covers over 95% of the *japonica* genome. The remaining 5% includes 38 physical gaps within the 12 pseudomolecules and gaps at 10 centromeres and 10 telomeres (IRGSP, 2005).

The high quality, full length sequence of the 12 chromosomes of rice has been recently completed by the International Rice Genomics Sequencing Consortium (IRGSP, 2005). Independent automated annotations from several research groups have revealed an unsuspected wealth of predicted genes, about half of which have clearly identified

homologs in the *Arabidopsis* genome. Now there is a challenge of determining the function of most of the rice genes in the coming decade. Reverse genetics provides a link between a candidate gene and a phenotype, represents the most straight forward experimental strategy to assign a biochemical, cellular, developmental or adaptive role to these sequences (Droc *et al.*, 2006).

The Indian aromatic rice, often called 'Basmati' is nature's gift to the sub-continent and human kind at large. Basmati rice is highly priced in the domestic as well as international markets. India has become a leading exporter of aromatic rice exporting 941.05 thousand tonnes of Basmati rice which contributed Rs. 2477.56 crores to the Indian economy during 2006-07. With the growing demand for aromatic rice in international market, concerted efforts are made on the improvement of basmati types (Joshi and Behra, 2006). Because of its long and slender grains and pleasant aroma, Basmati 370 is popular variety and commonly grown in India. However, the plants of Basmati 370 are tall, with weak culms, and are highly susceptible to damage by wind and rain. This causes considerable yield losses and a reduction in grain quality. Previous attempts using conventional breeding methods to reduce the height of Basmati 370 while retaining its good qualities was not successful owing to the loss of the unique characters for which it is valued (Peng *et al.*, 1999).

Dwarf stature is one of the most valuable traits in rice breeding because semi-dwarf cultivars are not only more-resistant to damage by wind and rain (lodging-resistant), but enable an increase in grain yield rather than straw biomass in response to fertilizer application. Gibberellic acid (GA)-mutants are related to dwarfness in plants and can be classified into GA-sensitive and GA-insensitive types, depending on their responsiveness

to exogenously applied GAs. Mutants of the first class are characterized by defects in the synthesis of GAs, while in the latter the response to GAs is affected, either at the level of signal perception or signal transduction ('GA-response' mutants). Mutants belonging to the latter class, therefore, show the symptoms of GA deficiency, although their GA levels are normal, or even higher than those found in wild-type controls (Martin *et al.*, 1996). Some dwarf mutants, like the GA-insensitive *Rht* (Reduced height) and the GA-sensitive *sd1* (semi-dwarf1), were exploited by breeders during the "green revolution" in the 1960s and 70s for the production of semi-dwarf, high-yielding varieties of wheat and rice, respectively. The most widely utilized semi-dwarf wheat cultivars contain the *Rht-B1b* or *Rht-D1b* alleles which encode a mutant form of a DELLA protein, which is a GA signaling repressor (Peng *et al.*, 1999; Hedden, 2003). DELLA proteins are encoded by *Rht* and its orthologs in *Arabidopsis* (*Arabidopsis thaliana*; GAI, RGA, RGL1, and RGL2), maize (*Zea mays*; D8), grape (*Vitis vinifera*; VvGAI), barley (*Hordeum vulgare*; SLN1), and rice (SLR1). These proteins have conserved function as repressors (negative regulator) of GA signaling (Sun and Gubler, 2004). Five DELLA protein genes (GAI, RGA, RGL1, RGL2, and RGL3) are present in *Arabidopsis*, and with the exception of RGL3, these genes have been shown to share partially overlapping roles in repressing GA-regulated plant growth and development (Thomas and Sun, 2004). On the other hand, rice and barley contain only one DELLA protein gene namely, SLR1 and SLN1, respectively.

Recent studies on GA-signaling pathway in rice show that GA derepresses its signaling pathway by inducing degradation of the DELLA protein SLR1 and this proteolysis event is targeted by an ubiquitin E3 ligase complex SCF^{GID2} which binds to

26S proteasome. In wild-type plants, DELLA repressor SLR1 is degraded in the presence of GA, and GA-promoted growth occurs. GID2 is an F-box subunit of the SCF E3 complex which interacts with phosphorylated SLR1 protein and regulates the gibberellin-dependent, ubiquitin-mediated degradation of SLR1 in rice. SCF complexes are E3 ubiquitin-protein ligases which are similar to the anaphase-promoting complex or cyclosome (APC/C) in yeast and animals. The APC/C is an essential ubiquitin protein ligase that regulates mitotic progression and exit by controlling the stability of cell cycle regulatory proteins, like securin and the mitotic cyclins. In plants, the function, regulation, and substrates of the APC/C are poorly understood (Capron *et al.*, 2003). Cdc16 (or APC6), a subunit of APC/C creates linkage with an APC subunit Doc1 to cause the holo-enzyme to partially close around a substrate.

Insertional mutagenesis in rice is a powerful tool for generating knock out mutations for reverse genetics. It facilitates the assigning of biological functions to as yet uncharacterized open reading frames (ORFs) identified in genomic sequencing or EST databases. Insertional mutagenesis using maize transposons elements *Activator/Dissociation* (*Ac/Ds*) and *Enhancer/Suppressor-mutator* (*En/Spm*) (Martienssen, 1998) and *Agrobacterium* T-DNAs (Azpiroz-Leehan and Feldmann, 1997) have been widely used for revealing gene function in rice (Chin *et al.*, 1999). The main advantage of transposon-based approaches is their relative efficiency in generating large populations of insertions and their ability to transpose to linked sites. This makes it possible to remobilize the element for insertion in the vicinity of the initial insertion site. There are several strategies for recovering the unknown sequences flanking insertions of known sequences, like T-DNAs and transposons (Hui *et al.*, 1998). Among them, thermal

asymmetric interlaced PCR (TAIL-PCR) has the advantage that it minimizes DNA manipulations and is therefore readily adaptable to multiplexing (Liu *et al.*, 1995; Liu and Whittier, 1995). The amplified fragments from TAIL-PCR can be directly sequenced and the sequences can be used to search genomic and EST databases for identities and similarities to the already sequenced and annotated genes. In some cases, it leads to the immediate identification of the candidate gene containing the insertion (Tsugeki *et al.*, 1996). With the completion of the sequencing of *Oryza sativa* cv. Nipponbare genome, essentially all insertion site sequences can be traced to an exact chromosomal position.

More than 40,000 putative genes were identified throughout the rice genome (IRGSP, 2005; Sasaki *et al.*, 2002; Feng *et al.*, 2002). To assign the functions of these putative genes, several approaches have been developed. Among these, random insertional mutagenesis by T-DNA or transposon has been used widely to generate Flanking Sequence Tags (FSTs) from insertional mutant pools in *Arabidopsis* and rice. Since the genomes of both the plant species are not too large, and transformation efficiency is well established, T-DNA can be used as mutagen for achieving near saturation mutagenesis. Currently, 172,500 FSTs have been generated through the efforts of researchers around the world (11 institutes in 7 countries) who have created insertional mutations in 27,551 rice genes corresponding to 57,142 gene models. All rice FSTs are publicly available at the Rice Functional Genomic Express (RiceGE) (developed by the Salk Institute) or OryGeneDB Database. The seeds of the mutant lines are provided by individual suppliers like POSTECH, South Korea (Jung *et al.*, 2008). OryGeneDB (http://orygenesdb.cirad.fr/), a database dedicated to rice reverse genetics contains 44166 FSTs generated by most of the rice insertional mutagenesis projects.

In gain- or loss-of-function approaches of insertional mutagenesis, it is difficult to find all the knockout mutants or tagged lines for all members of a gene family. In contrast, the RNA interference (RNAi)-induced gene silencing approach can possibly silence multigene families and homoeologous genes in polyploids. It is based on sequence specific RNA degradation thereby silence one, several or all members of a multigene family in plants like rice by targeting the unique sequences or the sequences shared by several genes (Miki *et al.*, 2005).

For the rapid identification of linkage between different PCR based molecular markers and the gene of interest, Bulk segregant analysis (BSA) method was suggested by Michelmore *et al.* (1991). In BSA, two bulked DNA samples are developed from a segregating population of a single cross. The bulks are then screened for DNA polymorphism and compared against a randomized genetic background of unlinked loci. A marker that differs between the two bulks is expected to be linked to the particular trait. Simple sequence repeats (SSR) or microsatellites are ubiquitous in eukaryotes which are widely used PCR based markers in BSA.

Keeping in view the various aspects of insertional mutagenesis for functional genomics in rice, the present investigation was carried out to clone and map a dwarfing gene present in a T-DNA insertional dwarf mutant (*OsGAI/Sd*) of Basmati 370. Along with the dwarf mutant, two independent insertional mutants viz; B-3-1 and B-2-2-3 (*OsGAS/Sd*) were also included in the present study. All the three mutants were isolated by T-DNA transformation of superfine quality *indica* rice cultivar Basmati 370 through *Agrobacterium tumefaciens* strain EHA101 containing plasmid Hm^{R}*Ds* (Dhaliwal *et al.*, 2001). The present investigation was carried out with following broad objectives:

- To study the inheritance pattern and mapping of the dwarfing mutants.
- Histological characterization of the dwarf mutant *OsGAI/Sd.*
- Isolation and molecular characterization of the dwarfing genes.
- Annotation of the dwarfing gene through public databases.

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CHAPTER 2

REVIEW OF LITERATURE

2. Review of Literature

Rice is central to the lives of billions of people around the world. Possibly the oldest domesticated grain (~10,000 years), rice is the staple food for 2.5 billion people and growing rice is the largest single use of land for producing food, covering 9% of the earth's arable land. Rice provides 21% of global human per capita energy and 15% of per capita protein. Asia accounts for over 90% of the world's production of rice, with China, India and Indonesia producing the most. Rice in India accounts for more than 42% of the total foodgrain production of the country and is cultivated in over 40 million hectare area with a production of around 90 million tones. In world, India stands first in rice area and second in production which almost tripled from 30.4 million tonnes (milled rice) in 1966 to record production of 93.3 million tonnes, average productivity being 2.08 t/ha in 2001-02. But for the past five years since 2001, the production and productivity of rice has become stagnated, in contrast to ever increasing growth of the Indian population.

Rice belongs to the genus *Oryza* and has two cultivated and 22 wild species. The cultivated species are *Oryza sativa* and *Oryza glaberrima*. *Oryza sativa* is grown all over the world while *Oryza glaberrima* has been cultivated in West Africa. Basic chromosome number (x) of rice is 12 and both the cultivated species are diploid. The two subspecies of cultivated rice viz., *japonica* and *indica* are believed to have evolved from one of the wild species *(Oryza ruffipogon)* through long-term domestication (Khush, 1997). *Indica* rice is prevalently grown in the lowlands of tropical Asia, whereas *japonica* rice is cultivated in the upland hills of southern China, Japan, northeast and southeast Asia, Indonesia and regions outside Asia e.g. Africa, North America, Europe and South

America (Khush, 1997; Cheema *et al.*, 2007). The hybrids between *japonica* and *indica* subspecies are partially fertile with a high degree of DNA polymorphism which helped in constructing high density molecular maps of the two subspecies.

The relevant literature pertaining to the present study has been reviewed under the following heads:

2.1. Rice as a model crop

2.2. Basmati rice

2.3. Role of dwarfing gene in green revolution

2.4. Mechanism of gibberellic acid biosynthesis and signaling in plants

2.5. Gibberellic acid biosynthetic and signaling mutants in plants

2.6. Role of Ubiquitin-proteasome mediated proteolysis in GA signaling

2.7. Model of gibberellic acid signaling in rice

2.8. *Agrobacterium tumefaciens* mediated transformation

2.9. Bulk Segregant Analysis (BSA)

2.10. TAIL-PCR and Genome Walking in T-DNA insertional mutagenesis

2.11. Validation of gene function in rice

2.1. Rice as a model crop:

Rice has been a model monocot for functional genomics research due to its small genome size (389Mb) relative to other cereals, its ease of transformation, and its economic importance. Rice was the choice for the first genome sequencing of a crop plant for the following reasons. Rice has the smallest genome size (389Mb) among the major cereal crops. It is 16 and 40 times smaller than the maize and wheat (Gill *et al.*, 2004; Hossain *et al.*, 2004; Kalavacharla *et al.*, 2006; Li *et al.*, 2004) genomes, respectively. Rice can be transformed on a large scale on a routine basis. Isolation of genes from rice could facilitate isolation of orthologs from other cereal crops (Conley *et al.*, 2004). Much molecular and genetic information (ESTs, markers, genetic and physical maps, etc.) about rice is available (Hsing *et al.*, 2006). Rice seems to encode more genes that have a redundant function as compared with *A. thaliana* because the duplicated genomic segments estimated to cover 27-65.7% of its genome (Ouyang *et al.*, 2007; Yu *et al.*, 2005; Singh *et al.*, 2007). This high level of redundancy in the rice genome complicates the analysis of mutant phenotypes (Shiu *et al.*, 2004; Tian *et al.*, 2004).

2.2. Basmati rice:

Basmati is known as the "crown jewel" of South Asian rice. Prized for its exquisite aroma and taste, it commands a premium price in both domestic and international markets. Scientists describe it as tall, low-yielding variety with beautiful, long slender and lustrous grains, possessing exquisite scent, sweet ambrosial taste, soft texture, high kernel elongation with the least breadth wise swelling, intact and non-sticky kernels (Singh *et al.*, 2006a). India being one of the centers of origin of rice plant, there exist a lot of diversity in rice including the scented ones. Basmati rice cultivation in India

is confined largely to Punjab, Haryana, Uttarakhand and Western Uttar Pradesh. Haryana, among the four states, has the largest area under Basmati, followed by Uttar Pradesh, Punjab and Uttarakhand. In Uttarakhand, Basmati is grown in about 17% of the total rice area. Basmati exports in India in 2004-05 touched an all time high of 11.2 lakh tonnes which was approximately Rs. 2,741 crore in value terms. Basmati 370, a traditional Basmati variety, was selected from a land race in united Punjab in 1933 at Kala Shah Kaku, now in Pakistan. It commonly suffers from the problem of lodging due to its very tall stature. Attempts using conventional breeding methods to reduce its height by crossing Basmati 370 with the varieties having semi-dwarfing (*sd1*) gene from DGWG (Dee-Geo-Woo-Gen) source were not successful due to failure of recovery of its quality characteristics. Isolation and exploitation of a dwarf mutant in Basmati 370 capable of enhancing its harvest index without deterioration of its exquisite quality will help in increasing area, production and return under its cultivation.

2.3. Role of dwarfing gene in green revolution:

The term "Green Revolution" refers to the huge increases in grain yields after 1960s. It was the outcome of the introduction of new dwarf varieties of wheat and rice for cultivation in the developing world. It was recognized in 1978 by the award of Nobel Peace prize to Dr. Norman E. Borlaug of the International Maize and Wheat Improvement Center (CIMMYT), Mexico that increase in foodgrain production was a major factor in maintaining per capita food supplies worldwide in the late-twentieth century, despite a doubling in the world population during this time (Evans, 1998). Prior to the green revolution, scientists developed high yielding wheat and rice varieties suitable for growing in subtropical and tropical climates. These varieties were higher

grain yielder under increased use of fertilizers and pesticides. However, the heavier grain caused the plants to become unstable and prone to lodging (falling over) during high winds and rain. Borlaug introduced dwarfing genes into wheat, giving the plants a stronger and shorter stem that resisted lodging. These genes have resulted in the increase in grain yield of both wheat and rice through an improvement in the "harvest index" (the proportion of plant weight in the grain) because a greater proportion of the products of photosynthesis accumulated in the grains rather than in the leaves.

2.4. Mechanism of gibberellic acid biosynthesis and signaling in plants:

2.4.1. Gibberellic acid biosynthesis:

The endogenous phytohormone gibberellins (GA) are a group of the several compounds, which form a large family of tetracyclic diterpenoid phytohormones. GA plays role in the regulation of various growth and developmental processes which are associated with dwarf phenotype in higher plants. Bioactive GAs, such as GA_1 and GA_4, are synthesized from trans-geranylgeranyl diphosphate (GGDP) as shown in Fig. 2.1 (Hedden and Kamiya, 1997; Hedden and Phillips, 2000). GGDP is converted to the tetracyclic hydrocarbon *ent*-kaurene via *ent*-copalyl diphosphate (CDP) by two kinds of diterpene cyclases in plastids, CDP synthase (CPS) and *ent*-kaurene synthase (KS). *ent*-kaurene is then modified by sequential oxidations to produce GA_{12} via ent-kaurenoic acid. These steps are catalyzed by two membrane-associated Cyt P450 monooxygenases, *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO). The final stage of bioactive GA synthesis, from GA_{13}/GA_{12} to GA_1/GA_4, is catalyzed through two parallel pathways (i.e. early-13-hydroxylation and non-13-hydroxylation pathways) by two soluble 2-oxoglutarate-dependent dioxygenases (2ODDs) in the cytosol, GA20-oxidase

(GA20ox) and GA3-oxidase (GA3ox). The bioactive GA_1/GA_4 and their immediate precursors GA_{20}/GA_9 are inactivated by a third 2ODD, GA 2-oxidase (GA2ox). In some species, GA_9 and GA_{20} are also converted to GA_7 and GA_3, respectively via 2, 3-didehydroGA_9 and GA_5 (Albone *et al.*, 1990). The genes encoding the seven GA metabolic enzymes (CPS, KS, KO, KAO, GA20ox, GA3ox, and GA2ox) have been isolated from various plants. Any mutation in the genes, except GA2ox, causes dwarfism, and the dwarfing state can be restored by the application of exogenous GA_3 (Hedden and Phillips, 2000; Sakamoto *et al.*, 2004). Singh (2003) studied the biochemical effects of exogenous GA_3 on morphologically GA_3 responding and non-responding rice genotypes by the foliar spray of GA_3 at 120 ppm on 20 and 45 days old rice plants. He found that GA_3 responding dwarf genotypes showed marked growth, morphological and biochemical response to GA_3 as compared to non-responding GA_3 genotypes.

2.4.2. Regulation of genes by GA-signaling:

Regulation of genes responsible for GA biosynthesis is a complex process which is governed by GA signal transduction. Hedden and Kamiya (1997) defined GA signal transduction as "the series of biochemical events leading from the perception of the active GA molecule for the final response." The mechanism by which plants use to maintain GA homeostasis (i.e. keeping the concentrations of bioactive GAs within certain limits) involves feedback regulation of GA biosynthesis, as illustrated in Fig. 2.2. A certain GA-receptor present in the cell transfers GA-signal to two different genes A and B. This causes the genes to either switch-off or on so that several GA-dependant responses are carried out.

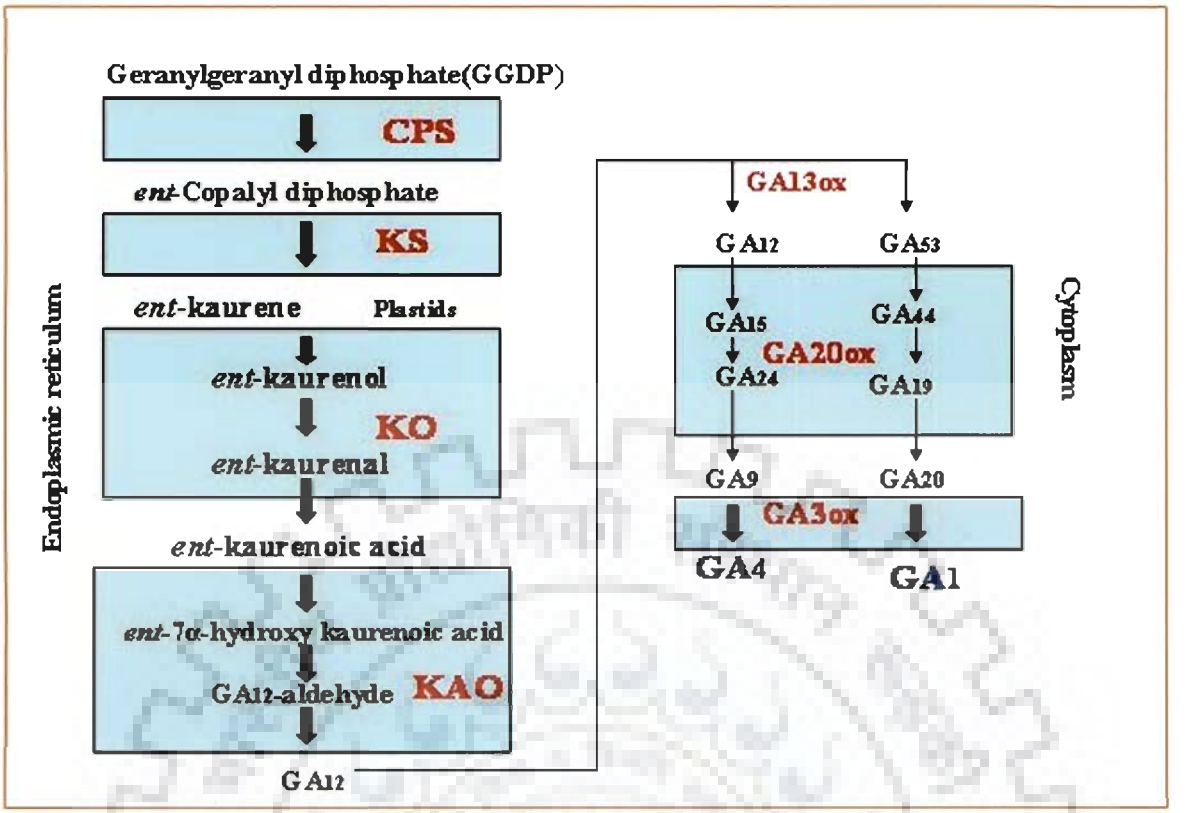

Figure 2.1 GA-biosynthetic pathway in plants

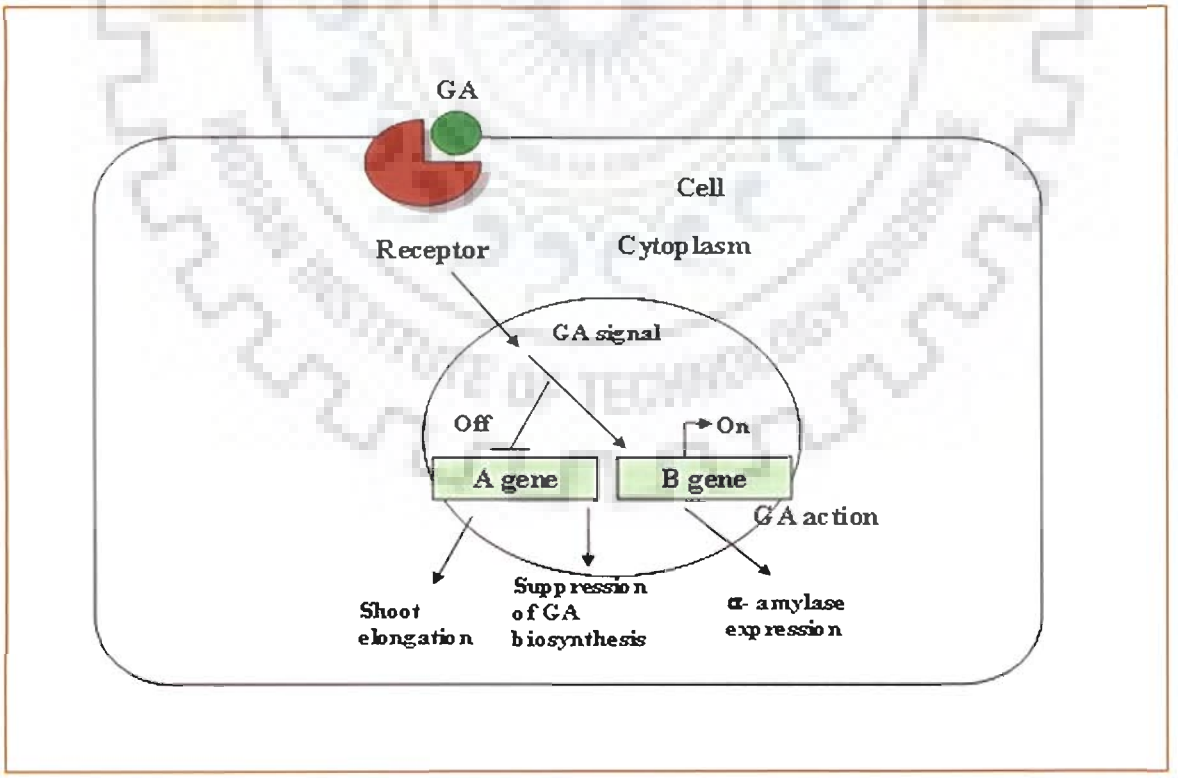

Figure 2.2 Gibberellic acid signaling pathway in plants

2.5. Gibberellic acid biosynthetic and signaling mutants in plants:

The GA-related mutants may be divided into two groups: those that influence GA-hormone levels (hence called GA-synthesis mutants) and those that influence GA-hormone response (hence called GA-response mutants). The synthesis mutants respond to the exogenous application of GA with stem elongation and are called GA-sensitive mutants. Most of the GA-sensitive mutants are recessive and involve in the loss of wild-type function (Herskowitz, 1987). GA response mutants do not respond to the exogenous application of GA and participate in GA-signaling in plants. The factors responsible for GA-signaling can be broadly divided into positive and negative regulators, depending upon their effects on the final GA-induced responses. Several positive and negative regulators of GA-signal transduction have been identified in several plant species. List of positive and negative regulators of GA-signaling along with their function in different plant species have been summarized in Table 2.1 and Table 2.2.

The dwarf mutants containing negative regulator are either semi-dominant or recessive. The semi-dominant mutation in *Arabidopsis* (*gai* and *rga1-3*), maize (*d8*), wheat (*Rht1* and *Rht*2) and barley (*Hrt*) cause dwarf phenotype whereas recessive loss-of-function mutations in rice (*slr1*) and barley (*sln1*) cause increased growth. The wild type proteins of semidominant mutants have a highly conserved DELLA domain which is the target for proteolysis by the ubiquitin-proteasome mediated pathway. The *gai-1* mutant in *Arabidopsis* contains a 51 bp in-frame deletion that results in the loss of 17 amino acids within the DELLA domain. The *gai* was cloned via *Ds*-transposon insertional mutagenesis (Peng *et al.*, 1997). SPY shows sequence similarity to Ser (Thr)-*O*-linked N-acetyl glucosamine (O-GlcNAc) which regulates target protein function by glycosylation of serine or threonine residues (Hart, 1997).

Table 2.1 Positive regulators of GA-signaling in different plants

Crop	Gene	Symbol	Function	Reference
Rice	*DWARF1*	*D1*	Encodes a heterotrimeric G protein α-subunit	Ashikari *et al.*, 1999; Fujisawa *et al.*, 1999
Rice	*GIBBERELLIN INSENSITIVE DWARF1*	*GID1*	GA-receptor	Sasaki *et al.*, 2001
Rice	*GIBBERELLIN INSENSITIVE DWARF2*	*GID2*	F-box protein	Sasaki *et al.*, 2003
Potato	*PHOTOPERIOD RESPONSIVE*	*PHOR1*	Growth under short days	Amador *et al.*, 2001
Barley	*GA-INDUCED MYB TRANCRIPTION FACTOR*	*GAMYB*	Activates α-amylase promoter	Gocal *et al.*, 2001
Arabidopsis	*GA-INDUCED MYB TRANCRIPTION FACTOR (GLABROUS)*	*GL1*	Initiation and branching of trichomes	Perazza *et al.*, 1998
Arabidopsis	*SLEEPY*	*SLY*	Seed germination, F-box protein	Mc Ginnis *et al.*, 2003; Sasaki *et al.*, 2003; Dill *et al.*, 2004
Arabidopsis	*PICKLE*	*PKL*	Formation of ectopic stipules and meristems in leaves, recessive mutation affects GA-induced differentiation of the seedling primary root.	Oogas *et al.*,1997;Ori *et al.*, 2000

Table 2.2 Negative regulators of GA-signaling in different plants

Crop	Gene	Symbol	Mutant phenotype	Reference
Rice	*SLENDER RICE1*	*SLR1*	Recessive, increased growth	Itoh *et al.*, 2002; Ikeda *et al.*, 2001
Arabidopsis	*REPRESSOR OF GA1-3, GIBBERELLIC ACID INSENSITIVE*	*RGA 1-3, GAI*	Semidominant, dwarf	Peng *et al.*,1997; Peng *et al.*, 1999; Silverstone *et al.*, 1998
Barely	*Slender1*	*SLN1*	Recessive, increased growth	Chandler *et al.*, 2002; Gubler *et al.*, 2002
Maize	*Dwarf 8*	*D8*	Semidominant, dwarf	Peng *et al.*, 1999
Wheat	*Reduced height1 and Reduced height 2*	*RHT1, RHT2*	Semidominant, dwarf	Peng *et al.*, 1999
Arabidopsis	*SPINDLY*	*AtSPY*	Recessive, Seed germination reduced	Jacobson and Olszewski, 1993;
Barley	*SPINDLY*	*HvSPY*	Recessive slender, resembles wild-type plants that have been treated with GA	Jacobson *et al.*,1996; Robertson *et al.*, 1998
Petunia	*SPINDLY*	*PhSPY*	-Do-	Izhaki *et al.*, 2001
Arabidopsis	*SHORT INTERNODE*	*SHI*	Semidominant, Zinc-finger transcription factor	Fridborg *et al.*, 1999

2.5.1. The Dwarfing genes of Wheat:

Many genes are associated with a semi-dwarf growth habit of wheat (Ellis *et al.*, 2005) which is known as *R*educed *h*eight (*Rht*) genes. Many of the *Rht* genes are dominant

or semi-dominant which actively inhibits growth through a gain-of-function mutation. *Rht-B1b* and *Rht-D1b*, having an additive effect on growth, were formerly called *Rht1* and *Rht2* and are being used in many commercial wheat varieties. Their corresponding homologues are present on the B and D genomes of wheat, respectively. The *Rht-B1b* and *Rht-D1b* have a series of multiple alleles which cause semi-dwarfing to extreme-dwarfing phenotypic expressions (Ivandic *et al.*, 1999). The *Rht-B1b* and *Rht-D1b* dwarfing genes were derived from Norin 10, which was a semi-dwarf variety bred in Japan and released in 1935 (Gale and Youssefian, 1985). Norin 10 was used in United States wheat breeding programs during 1950s for improving lodging resistance in winter wheat under high rates of nitrogenous fertilizer application. A selection from a cross of Norin 10 with the United States variety, Brevor, was high grain yielder and used by Dr. Borlaug in the development of the "Green Revolution" wheat varieties. The wild-type (non mutant) alleles of *Rht-B1b* and *Rht-D1b* were isolated a few years ago (Peng *et al.*, 1999) and shown to encode DELLA proteins, which are the components of the GA signal transduction pathway. Peng *et al.* (1999) found that both *Rht-B1b* and *Rht-D1b* dwarfing alleles contained a point mutation which introduced a stop codon into a conserved region known as the DELLA domain, present near the N-terminus of the proteins.

2.5.2. The Dwarfing genes of Rice:

2.5.2.1. semi-dwarf1 *(sd1)* mutant:

The semi-dwarf1 (*sd1*) is a single most widely used dwarfing gene in rice involved in Green revolution in 1960s. Many alleles have been reported for this gene which unlike *Rht* genes in wheat, responds to exogenously applied GA. This indicates that the *sd1* gene containing plants are deficient in GA which is due to defect in one of the GA20-

oxidase genes (*GA20ox2*) that catalysis the penultimate steps in GA-biosynthesis. In rice, there are four GA20-oxidase genes (Sakamoto *et al.*, 2004). The rice dwarfing gene *sd1* arose as a spontaneous mutation in the Taiwanese *indica* strain Woo-Gen. The resulting strain Dee-Geo-Woo-Gen was used in breeding programs in eastern Asia to produce many of the high-yielding semi-dwarf cultivars grown today. Nagano *et al.* (2005) reported that the *sd1* allele in DGWG contains a 383-base-pair deletion in the first and second exon of *GA20ox2* gene. This deletion introduces a stop codon in such a manner that a truncated, inactive enzyme is produced.

In rice, there are two kinds of dwarf genes, semi-dwarf and dwarf. Dwarf genes that have been named in '*d*' system refer to the genes with strong dwarfing effect. The height of the dwarf plant is usually <50% that of the normal tall plant; while semi-dwarf genes that have been named in '*sd*' system refer to those with weak dwarfing effect, and the plant height is about 50%-100% of the normal tall plant (usually about 100 cm) (Guohua *et al.*, 2004). There are few mutants reported which are associated with the signaling mechanism of the gibberellic acid in rice. The details of these mutants are as follows:

2.5.2.2. Slender Rice 1 (*SLR1*) or *OsGAI* mutant:

The *slr1* mutants have a slender phenotype with an elongated stem and leaf and reduced root number and length. Its phenotype is similar to that of rice plants having treated with GA_3 (Ikeda *et al.*, 2001 and Itoh *et al.*, 2002). The *slr1* mutant is saturated with endogenous GAs and GA-inducible α-amylase (*Ramy1A*) is produced in the aleurone cells of mutant seeds even in the absence of GA application. The GA-saturation phenotype of

slr1 is not affected by treatment with uniconazole which is a GA biosynthesis inhibitor (Ikeda *et al.*, 2001; Itoh *et al.*, 2002). The SLR1 protein shares high amino acid identity with *Arabidopsis* GAI (47.2%), RGA (41.2%), wheat RHT-D1a (77.2%) and maize D8 (80.3%). The SLR1 gene is located on the short arm of rice chromosome 3. This region in the third chromosome shows the genome synteny with the wheat *Rht* locus of chromosome 4 and maize D8 locus of chromosome 1 which confirms that these genes are orthologous among these grass species (Peng *et al.*, 1999; Ikeda *et al.*, 2001).

The deduced SLR1 protein has 625 amino acid residues and contains the DELLA, TVHYNP domain in the N-terminal region which is conserved among *Arabidopsis* GAI, maize D8 and wheat RHT (Peng *et al.*, 1999). SLR1 also contains other consensus domains at the C-terminal region, such as leucine heptad repeat, NLS, VHIID, PFYRF and SAW, which belong to the GRAS family (Pysh *et al.*, 1999) (Fig. 2.3). The SLR1 protein functions as a negative regulator of GA signaling (Itoh *et al.*, 2002) and is localised in the nucleus. The SLR1 protein disappears after treatment with GA_3 which has been confirmed by immunoblot analysis using anti-SLR1 antibody (Itoh *et al.*, 2002). Thus, in the absence of a GA signal, the SLR1 protein is localised in the nucleus and suppresses GA activity as transcriptional negative regulator. But SLR1 is rapidly degraded in the presence of a GA signal and release the suppression of GA action (Itoh *et al.*, 2002). Similar findings have also been reported for SLR1 homologous proteins: the *Arabidopsis* RGA and barely SLN which are localised in nuclei but disappear following the application of GA_3 (Dill and Sun 2001; Silverstone *et al.*, 2001). On the other hand the GAI and RGL1 (RGA- like1) proteins in *Arabidopsis* are stable in the presence of GA in nuclei and not degraded by the GA treatment (Fleck and Harberd 2002; Weng and Chang, 2002).

2.5.2.3. Rice dwarf mutant *d1* :

In addition to the dwarfism, the Daikoku *d1* mutant in rice displays pleiotropic phenotypes, such as dark-green leaves, compact panicles and small-round grains. *d1* also lacks the ability to induce α-amylase in cereal aleurone (Mitsunaga *et al.*, 1994; Ueguchi *et al.*, 2000). The *D1* gene has been cloned by positional cloning and found to encode α subunit of heterotrimeric G-protein (Ashikari *et al.*, 1999; Fujisawa *et al.*, 1999). Heterotrimeric G proteins are associated with the cytoplasmic face of the plasma membrane of eukaryotic cells and mediate signaling from receptors present on the cell surface. The alfa-subunits of heterotrimeric G (Gα) proteins transduce signals from G protein-coupled receptors to effector proteins, accompanied by the GTPase-catalyzed hydrolysis of GTP. However, α-amylase induction occurs even in *d1* in the presence of high gibberellic acid concentrations.

The responsiveness of internode elongation to GA is much lower in *d1* than in wild-type plants. The expression of *OsGA20ox*, which encodes GA20 oxidase, is up-regulated and GA levels are elevated in the stunted internodes of *d1* (Ueguchi *et al.*, 2000). In addition, analysis of a double mutant between *d1* and *slr1* has revealed that *SLR1* is epistatic to *D1* which supports the idea of Gα proteins to be involved in GA signaling.

2.5.2.4. Gibberellin-insensitive dwarf 1 (*Gid1*) mutant:

The *gid1* mutant show very severe dwarf phenotype in which *GA20ox* gene is highly expressed and consequently the level of GA_1 is 100 times higher than in wild-type plants. *GID1* encodes a positive regulator of GA signal transduction. A *gid1-1/slr1-1* double mutant exhibits the *slr1* phenotype, indicating that *SLR1* is epistatic to *GID1* (Ueguchi-Tanaka *et al.*, 2005). Based on protein-protein interaction between the GID1

and SLR1 in yeast-two-hybrid assay, it was observed that GID1 may modify the stability or suppressive action of the SLR1 protein. The *Arabidopsis* genome has at least three *GID1* homologous genes, which may function in a redundant manner. *GID1* gene has been mapped to chromosome 5 through map based cloning of rice mutant (Ueguchi-Tanaka *et al.*, 2005). The second leaf sheath of *gid1-1* does not elongate in response to treatment with large amounts of GA_3 and over accumulates bioactive GA because GA signaling inhibits biosynthesis and promotes catabolism of these GAs. GA_1, a bioactive GA of rice, accumulates in *gid1* mutants up to 100-fold level as compared to the concentration in wild-type plants. In a yeast two hybrid assay, GID1 interacts with the rice DELLA protein SLENDER RICE1 (SLR1) in a GA-dependent manner which provides evidence that GID1 is a GA receptor. The GID1–SLR1 interaction was shown to be dependent on bioactive GA_3 (Ueguchi-Tanaka *et al.*, 2005). The *GID1* gene was cloned by chromosome walking which encodes a protein with similarity to hormone-sensitive lipases (HSLs), including the conserved HSL motifs HGG and GXSXG (Osterlund, 2001).

2.5.2.5. Gibberellin-insensitive dwarf 2 (*Gid2*) mutant:

Similar to other GA-related mutants in rice such as *d1* and *d18*, the *gid2* mutant lines show a severe dwarf phenotype with wide leaf blades and dark green leaves (Ashikari *et al.* 1999; Itoh *et al.*, 2001). The *gid2* mutant shows three signs of GA-insensitivity viz. second leaf sheath elongation, α-amylase induction in aleurone, and feedback expression of *GA20 oxidase*. Even though the *gid2* mutants have severe dwarfism, they accumulate more than 150 times the level of bioactive GA_1 than that in wild type plants. The GA-insensitive phenotype of *gid2* is similar to *gid1* mutants, but there are some differences, like *gid1* dwarfism is more severe than that of *gid2*, and the

amount of accumulated SLR1 in *gid1* is lower than in *gid2*. *GID1* functions upstream from *SLR1* in the GA signaling pathway, but not in SLR1 degradation. The *GID2* gene encodes a 636bp open reading frame, capable of producing a polypeptide of 212 amino acid residues. The deduced amino acid sequence of *GID2* contains an F-box domain, which is a conserved motif of F-box proteins that form a component of an E3 ubiquitin-ligase complex. The F-box sequence in *GID2* is well conserved in other F-box proteins from *Arabidopsis (SLY1)*, yeast, mold, and humans. Many F-box proteins contain a protein-protein interaction domain, such as leucine-rich repeat (LRR) or WD-40 repeat sequences outside the F-box (Dashaies, 1999; Li and Jonston, 1997; Skowyra *et al.*, 1997; Winston *et al.*, 1999).

2.5.3. DELLA protein is a key regulator in gibberellin signaling:

Localised in the nucleus, the DELLA proteins are proposed to be a transcription factor for GA-signaling in plants (Silverstone *et al.*, 2001; Itoh *et al.*, 2002). These proteins function as a key repressor of GA-signaling by inhibiting the GA-regulated gene expression (Sun and Gubler, 2004). These repressors accumulate in the nucleus and are rapidly degraded in response to GA (Silverstone *et al.*, 2001, Fu *et al.*, 2002). The GA-signals seems to be perceived by N-terminal region of the protein containing the DELLA domain. GA induces degradation of DELLA proteins via the ubiquitin/proteasome pathway (Itoh *et al.*, 2003). But light promotes accumulation of DELLA proteins by reducing GA levels (Achard *et al.*, 2007). Mutations within the DELLA domain render these proteins resistant to degradation which results in a GA-insensitive dwarf phenotype (Dill *et al.*, 2001; Peng *et al.*, 1999). This domain binds to the GA receptor GID1 in a GA dependent manner in rice, and promotes interaction with the F-box protein GID2. Polyubiquitination of DELLA proteins by the SCF^{GID2} complex, signals the degradation

of DELLA proteins by the 26S proteasome pathway. The functional mechanism of DELLA proteins regulation of gene expression and promoting photomorphogenesis was studied by Lucas *et al.* (2008). They found that the *Arabidopsis* nuclear transcription factor PIF4 (encoded by *PHYTOCHROME INTERACTING FACTOR4*) is negatively regulated by DELLA proteins and light photoreceptor phyB.

2.6. Role of Ubiquitin-proteasome mediated proteolysis in GA signaling:

Avaram Hersko, Aaron Ciechanover and Irwin Rose were awarded Nobel Prize in 2004 for their discovery of ubiquitin-mediated protein degradation. In *Arabidopsis thaliana,* more than 1400 genes (about 5% of the proteome) encode components of the ubiquitin/26S proteasome (Ub/26S) pathway (Smalle and Vierstra, 2004). Approximately 90% of these genes encode subunits of the E3 ubiquitin ligases, which confer substrate specificity to the pathway. The general function of ubiquitination pathway is to conjugate ubiquitin to Lys residues with substrate proteins, thereby targeting the degradation by proteasome (Smalle and Vierstra, 2004).

The 76 amino acid ubiquitin protein is attached to a substrate through the action of three enzymes-The ubiquitin activating enzyme (E1), Ubiquitin conjugating enzyme (E2) and Ubiquitin protein ligase (E3). The E1 forms a thioester bond with the C terminus of ubiquitin (Ub) in an ATP-dependent manner and transfers the activated Ub to an E2 enzyme (Fig. 2.4 and Fig. 2.6a). The E2 either transfers ubiquitin directly to the E3 in case of HECT E3s or binds to the E3 and transfers the ubiquitin to the substrate. In either case E3 specifies the substrate. This process is then repeated several times to attach several ubiquitin molecules to the substrate. This polyubiquitylation is necessary for degradation of the substrate by the 26S proteasome (Wilkinson, 2000; Doherty *et al.*, 2002; Smalle and Vierstra, 2004).

2.6.1. 26S proteasome:

The 26S proteasome is a multisubunit complex which consists of a cylindrical 26S core protease capped on each end by a 19S regulatory particle (Groll and Hubber, 2003). The 19S regulatory particle can be further divided into lid and base components. The lid contains 9 subunits that plays role in recognizing ubiquitinated substrate and in removing the Ub chains. The base contains several subunits that work to unfold the substrate. The 19S regulatory particle serves as the gate into the interior of core protease. The core is made up of a stack of proteolytic α and β subunits surrounding a narrow chamber, where the substrate proteins are finally degraded into the constituent amino acids that can be recycled for further metabolic activities (Voges *et al.*, 1999; Yang *et al.*, 2004).

2.6.2. Ubiquitin protein ligases (E3s):

Ubiquitin ligases (E3s) are critical regulators of metabolism, the cell cycle, DNA damage response, stress response, and receptor signaling. These enzymes catalyze the transfer of ubiquitin from an ubiquitin conjugase (E2) to a substrate that results in substrate degradation via the 26S proteasome. Ubiquitin ligases fall into three categories: the HECT domain ligases, the U-box ligases, and RING-finger ligases (Pickart and Eddins, 2004). The RING-finger ligases can be further divided into those that act as single subunits and those that act as part of a multisubunit complex, classified by an associated cullin subunit (cullin-RING ligases) (Fig. 2.5). Two of the most widely studied cullin-RING ligases are the anaphase-promoting complex (APC) and SKP1-Cullin-F-box (SCF) protein, both of which are composed of multiple subunits and serve as important regulators of the cell cycle (Vodermaier, 2004).

2.6.2.1. The SCF complex:

The SCF classes of E3 ligases are most widely studied in plants. The name is derived from the first letter of three of its four subunits: SKP1, Cullin, and the F-box protein (Fig. 2.5). The fourth subunit is the RING finger protein RBX1. Known targets of SCF include transcription factors, cell cycle regulators, and factors involved in development and signal transduction (Hershko and Ceichanover, 1998; Dharamasiri and Estelle, 2002; Itoh *et al.*, 2003).

The SCFs plays various roles in development processes such as hormone response, photo morphogenesis circadian rhythms, floral development, and senescence. It degrade repressors (negative regulators) of hormone responses (Auxin, Gibberellic acid and Jasmonic Acid), whereas in response to ethylene, the SCF degrades positive regulators in the absence of the hormone. GA promotes the degradation of both GAI and RGA via the E3 SCF^{SLY} in *Arabidopsis*. Mutations in the F-box gene *SLEEPY1 (SLY1)* results in the stabilization of RGA and GAI even in the presence of GA which indicates that SCF^{SLY} targets the DELLA proteins for degradation and alleviates DELLA mediated inhibition of GA-regulated growth. SLR1 in rice is ortholog of *Arabidopsis* RGA whereas the rice F-box gene GID2 is a putative ortholog of *Arabidopsis* SLY (Sasaki *et al.*, 2003). SLR1 levels are found to be more abundant in *gid2* mutants than in wild type. GA-induced phosphorylation of SLR1 is shown to increase the affinity between SLR1 and SCF^{GID2} (Sasaki *et al.*, 2003; Gomi *et al.*, 2004). Phosphorylation is the most common type of post transcriptional substrate modification for interaction with F-box subunit of an SCF complex. Fig. 2.6 shows the detailed view of polyubiquitinylation of substrate protein by SCF E3 ligase. Ubiquitin (Ub) is linked via a thioester bond to the ubiquitin activating enzyme (E1).

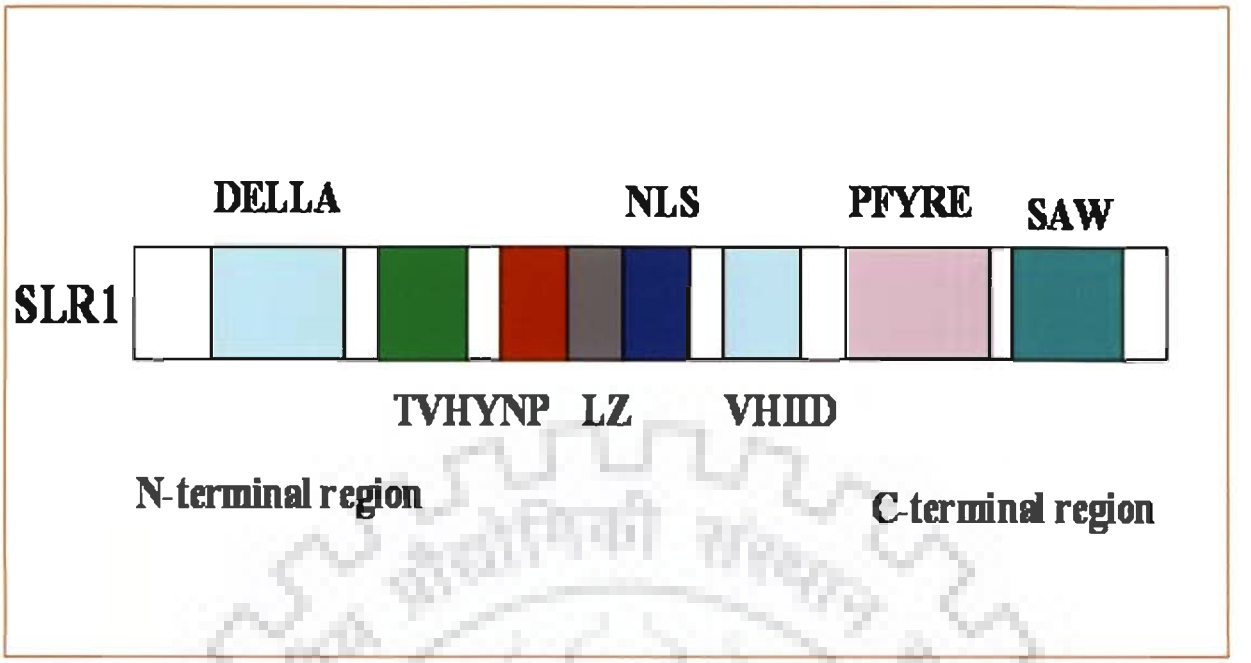

Figure 2.3 Domain structure of SLR1 in rice

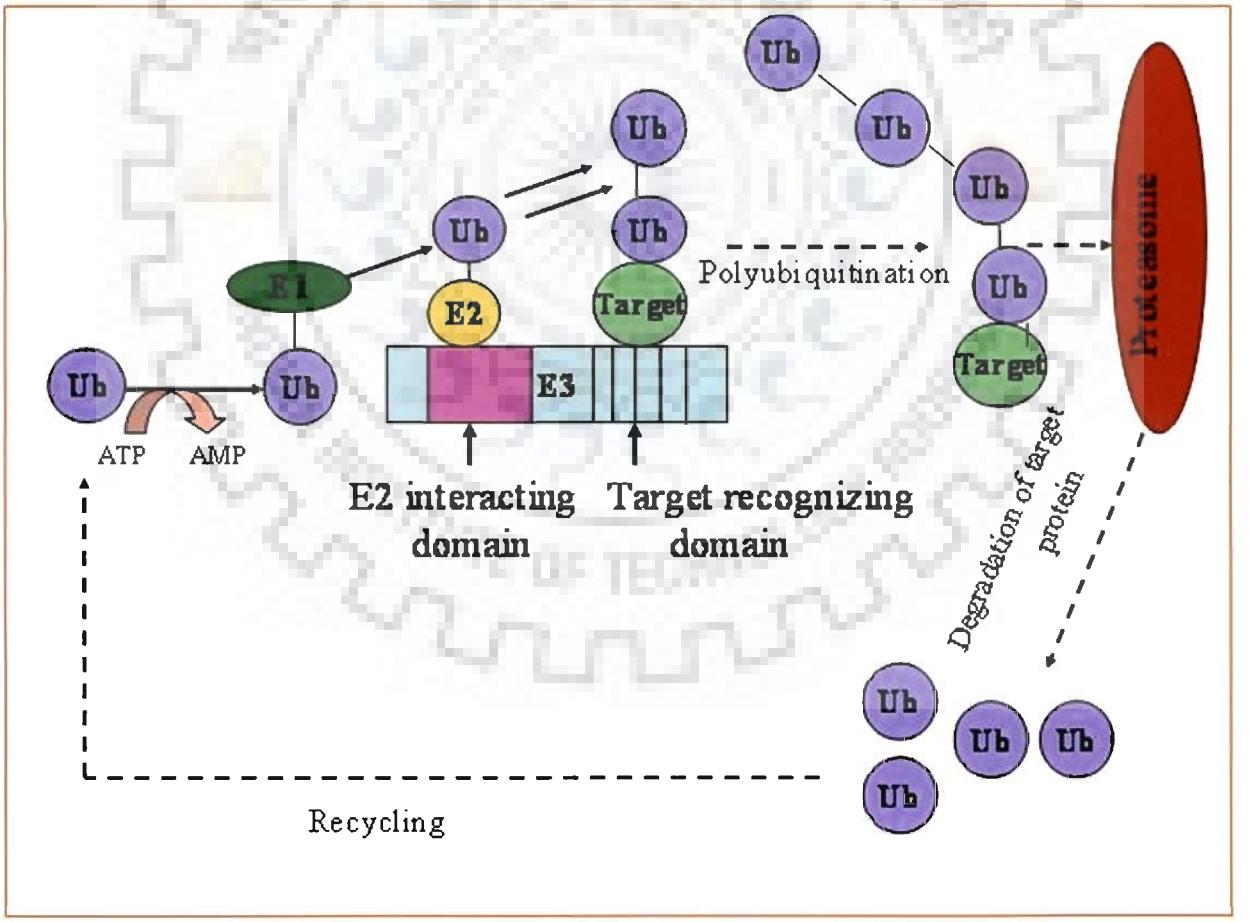

Figure 2.4 Ubiquitin mediated proteolysis pathway in plants (Source: KEGG pathway - http://www.ncbi.nlm.nih.gov).

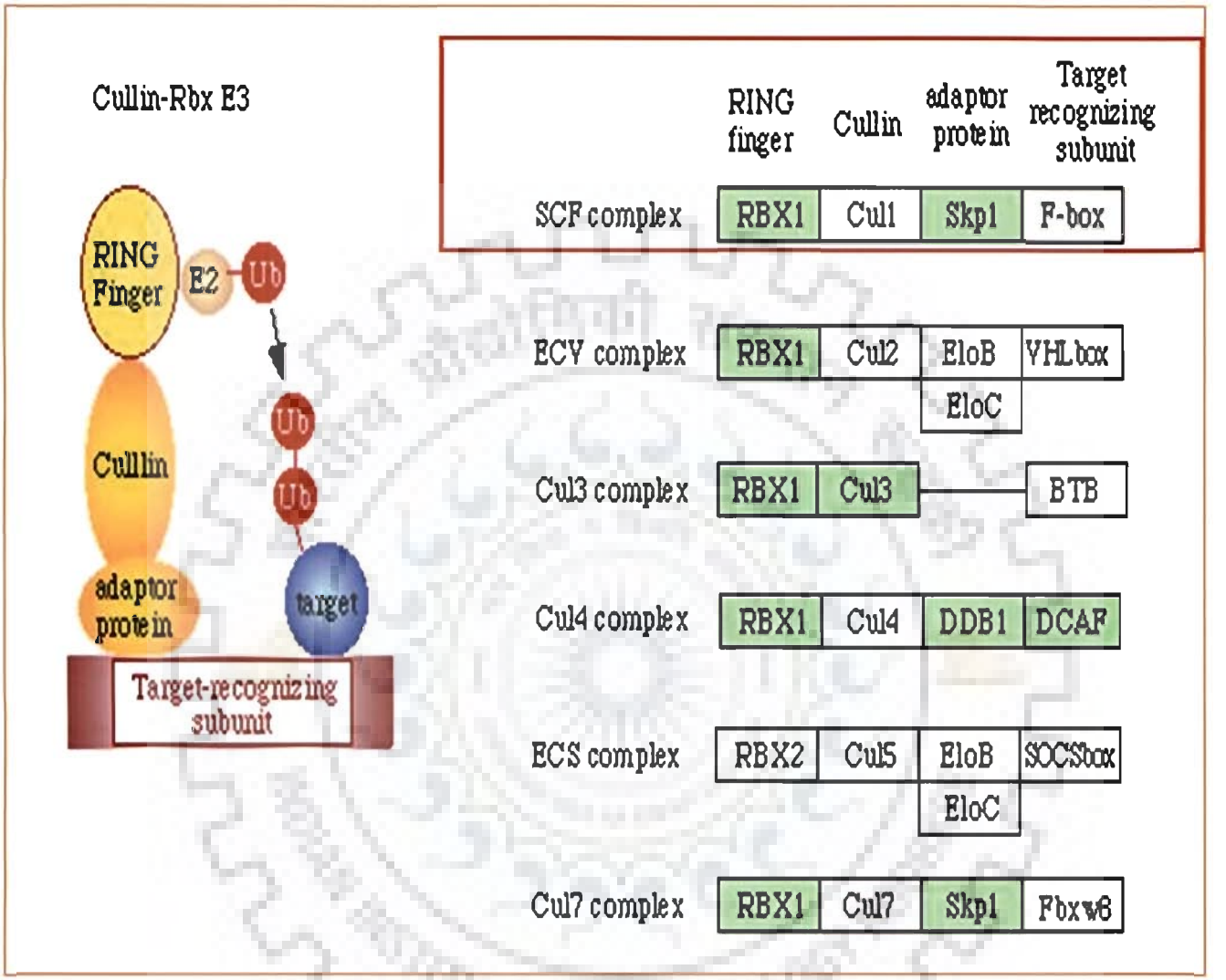

Figure 2.5 Diagrammatic structure of cullin-Rbx E3 which is a type of multisubunit RING-finger type E3s. The different subunits involved in ubiquitin mediated proteolysis pathway have been shown in the boxes. The SCF complex is marked in red box due to its involvement in GA-dependant proteolysis of DELLA protein (SLR1) in rice. Source: KEGG pathway (http://www.ncbi.nlm.nih.gov).

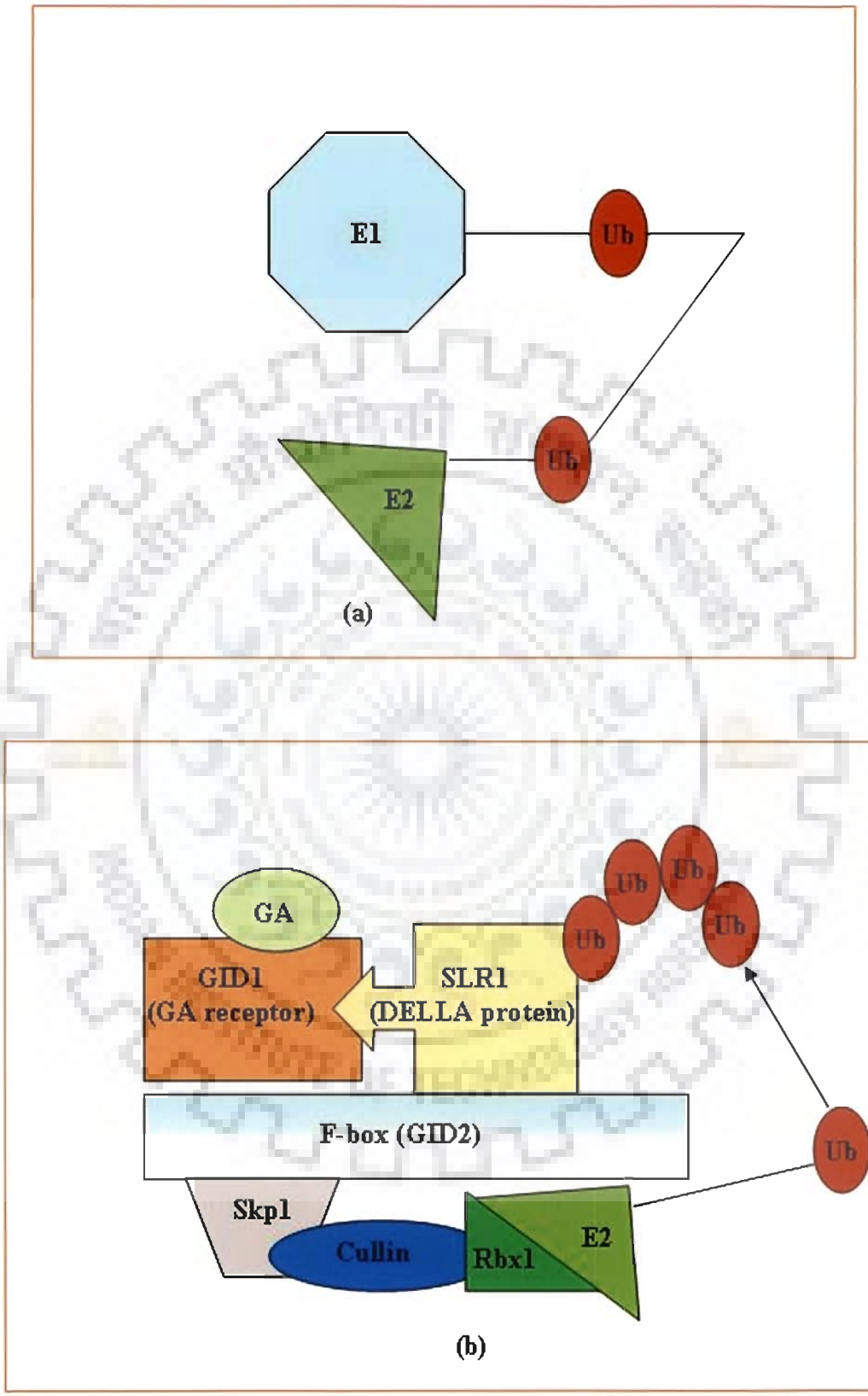

Figure 2.6 Substrate polyubiquitylation by SCF E3 ligase

Ub is transferred from E1 to the cysteine of the ubiquitin-conjugating enzyme (E2) Fig. 2.6.a. The SCF E3 ubiquitin ligase (Skp1, cullin, F-box and Rbx1) catalyze the transfer of Ub from E2 to a lysine residue on the substrate protein (the substrate protein might be SLR1 in case of rice). Formation of a polyubiquitin chain on the substrate protein targets it for degradation by the 26S proteasome (Fig. 2.6.b).

2.6.2.2. Anaphase promoting complex (APC):

The APC is a type of cullin-RING ligase that ubiquitinates key regulators of mitosis, such as the mitotic and S-phase cyclins and the anaphase inhibitor securin (Peters, 2002; Passmore, 2004). The research on APC has lagged behind SCF because of the large size of the complex and its inability to reconstitute from purified components. The APC in *Saccharomyces cerevisiae* is composed of 13 core subunits (Yoon *et al.*, 2002) and is activated by either of two weakly associating subunits Cdc20 or Cdh1 (Schwab *et al.*, 1997; Visintin *et al.*, 1997). Eight out of thirteen subunits (APC1, APC2, APC3, APC4, APC5, APC6, APC8 and APC11) are essential for viability. All subunits are conserved in eukaryotes and remain highly associated throughout the cell cycle (Passmore, 2004). Like SCF, the APC contains a conserved RING-finger subunit (APC11) and cullin-domain subunit (APC2) (Gmachl *et al.*, 2000; Leverson *et al.*, 2000; Tang *et al.*, 2001). APC activity is primarily regulated by the binding of the activating subunits, Cdc20 and Cdh1 (Rudner and Murray, 2000). Two domains are thought to play roles in the binding of these subunits to the APC: a short internal motif called the C-box (Schwab *et al.*, 2001) and a C-terminal IR dipeptide (Vodermaier e*t al.*, 2003). TPR (Tetra Tricopeptide Repeat) domain containing protein Cdc27 (or APC3) has been implicated as an important binding site for the IR dipeptide motif (Thornton and Toczyski, 2003). Using cryo-electron microscopy and angular reconstitution, Gieffers *et*

al. (2001) obtained a three-dimensional model (Fig. 2.7) of the human APC at a resolution of 24 A^0. The APC has a complex asymmetric structure 140 A^0 X 140 A^0 X 135 A^0 in size, in which outer protein wall surrounds a larger inner cavity. They hypothesized that this cavity may be the site for ubiquitination reaction.

2.7. Model of gibberellic acid signaling in rice:

SLR1 is a member of DELLA proteins family which negatively regulates response of rice plant to GA. In rice, barley, and *Arabidopsis*, GA signaling causes the rapid destruction of DELLA proteins by the proteasome pathway. Destruction of a protein by the proteasome is initiated when the DELLA protein interacts specifically with an F-box protein component of the SCF ubiquitin ligase complex. The protein is then ubiquitinated and degraded by the proteasome pathway. GID2 is the rice (Sasaki *et al.*, 2003) and SLY and SNE are the *Arabidopsis* (McGinnis *et al.*, 2003; Dill *et al.*, 2004; Strader *et al.*, 2004) F-box proteins involved in this process. The interaction of DELLAs with the F-box protein was initially unclear. It was proposed that phosphorylation of DELLA proteins in a GA-dependent manner is essential to interact with the F-box proteins (Fu *et al.*, 2002; Sasaki *et al.*, 2003; Gomi *et al.*, 2004), but recent studies by yeast two-hybrid assay shows (Hussain *et al.*, 2005; Itoh *et al.*, 2005) that in yeast cells GID1 interacts with SLR1 in a GA-dependent manner. This raised the possibility that the interaction with GID1 rather than phosphorylation causes SLR1 to interact with the F-box protein (Fig. 2.8) (Ueguchi-Tanaka *et al.*, 2005). Consistent with this hypothesis, *slr1* is epistatic to *gid1*, and SLR1 protein accumulates in *gid1* plants.

This indicates that the GA-GID1 complex interacts directly with SLR1 and probably transduces the GA signal to SLR1. The GA-binding activity of GID1 is

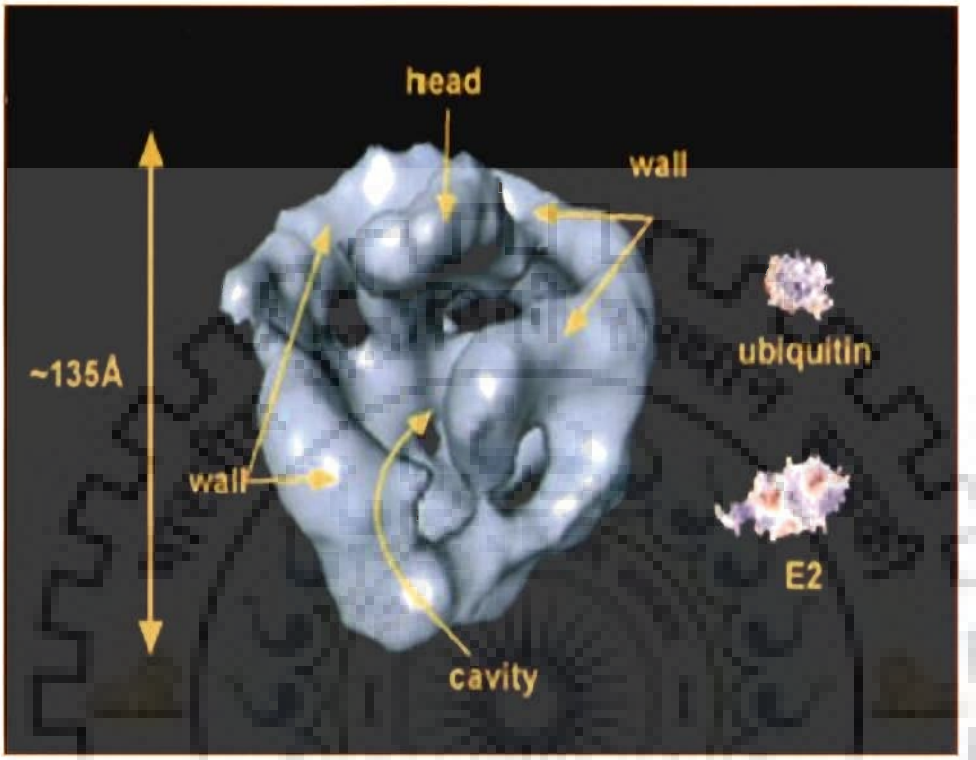

Figure 2.7 A 3D model of the human APC obtained by cryo-electron microscopy. It is the front view of the APC at 24 A0 resolution. Comparison of the sizes of ubiquitin (Vijay-Kumar *et al.*, 1987) and E2-complex (Jiang and Basavappa, 1999) are shown in comparison to APC.

Source: Geifers *et al.*, 2001

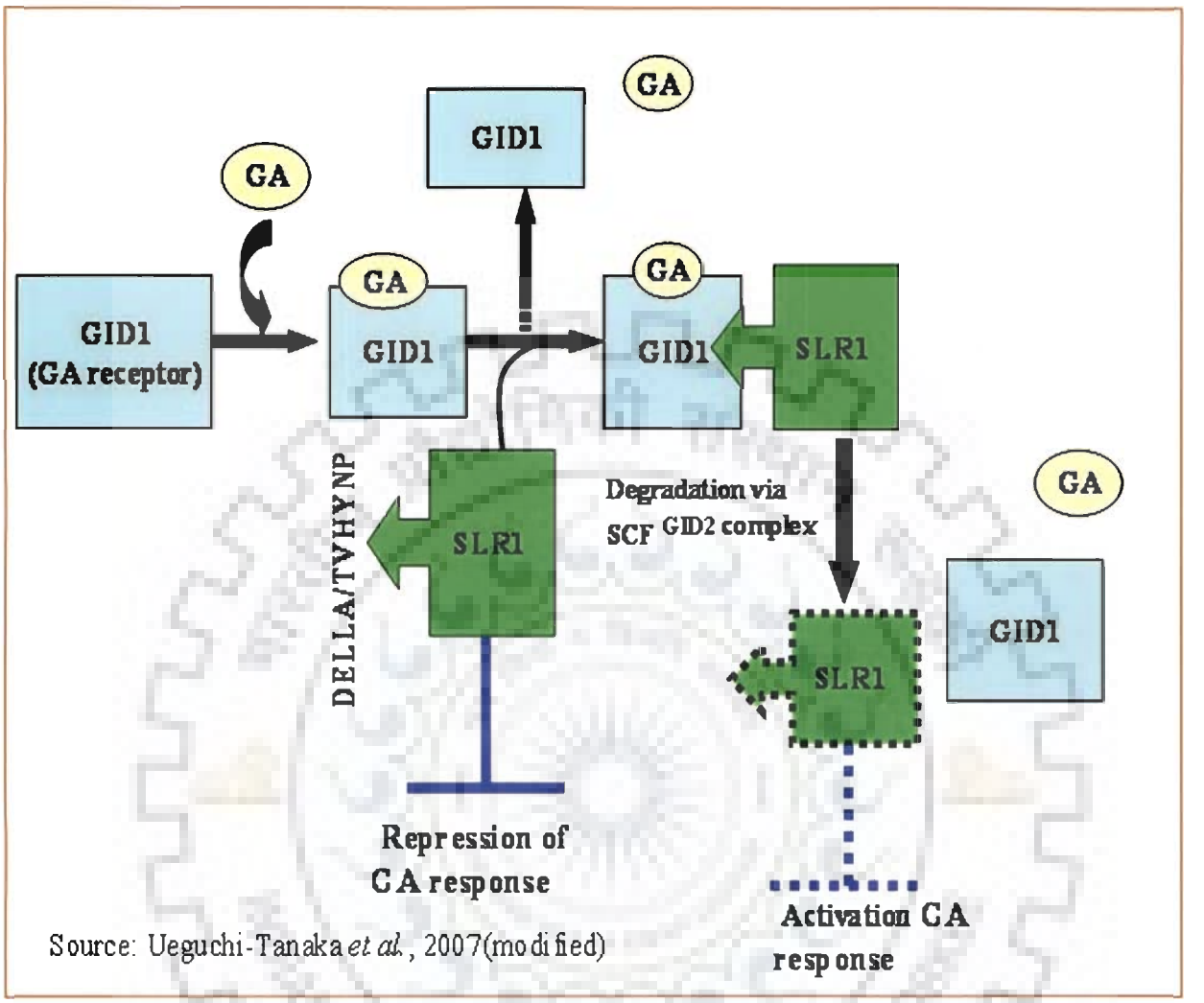

Figure 2.8 Model of gibberellin signaling in rice. Under low GA concentrations, SLR1 repress the GA responses. Under high GA concentrations, a soluble receptor, GID1, binds to GA; however, the binding is unstable and easily dissociates from the other. The GID1-GA complex specifically interacts with SLR1 at the site of DELLA and TVHYNP domains. The triple complex of GID1-GA-SLR1 is stable and does not easily dissociate. The triple complex is targeted by SCF^{GID2} complex and the SLR1 protein is degraded by the 26S proteasome, which releases the repressive state of GA response.

increased about three fold in the presence of SLR1. Thus, SLR1 stabilizes the interaction between GID1 and GA. When GID1 binds to GA, the GA-GID1 complex can interact with SLR1 probably by some conformational change. The region containing the DELLA/TVHYNP domains of SLR1 and the conserved HSL regions of GID1 are essential for the interaction between GID1 and SLR1. The association and dissociation of GID1 and the GA molecule occur rapidly in the absence of SLR1, but when the GID1-GA complexes interacts with the SLR1, GID1-GA complex is greatly stabilized. The stabilized trio-complex consisting of GA, GID1, and SLR1 might be a target of GID2, leading to the degradation of SLR1 by 26S proteasome through ubiquitination of the SCF^{GID2} complex (Ueguchi-Tanaka *et al.*, 2007) (Fig. 2.8).

2.8. *Agrobacterium tumefaciens* mediated transformation:

Agrobacterium is widely used bacterial genus which is capable of transferring genes to plants. *Agrobacterium tumefaciens* containing a Ti (***tumor inducing***) plasmid is a ubiquitous soil bacterium that induces crown galls on plants. Similarly, *A. **rhizogenes*** containing Ri (***root inducing***) plasmid causes the hairy root disease on several plants. The discovery that this crown gall formation is due to integration of bacterial DNA (T-DNA) into the plant genome laid the foundations of vector-mediated plant transformation. The tumor inducing principle (Ti-principle) of the bacterium has been fairly well described and has been developed as an effective vector for introduction of foreign genes into plants (Chilton, 1983; Klee and Rogers, 1989).

2.8.1. Ti plasmid:

The Ti-plasmid of *A. tumefaciens* is made up of 200 Kb of which only a 23 Kb fragment is introduced into the host cell. The transformed DNA (T-DNA) carries gene for

the synthesis of opines and phytohormones. The T-DNA is bordered with 25 base pair direct repeated sequences of nucleotides called right border (RB) and left border (LB) which are required in *cis* for the T-DNA transfer to the plant genome. Any DNA piece inserted between the border sequences is introduced into the genome of plants.

Just outside the RB segment of T-DNA, is a 24 bp 'overdrive' (*ode*) region which contributes to the efficiency of the T-DNA transfer (Peralta *et al.*, 1986). A 35 Kb region of the plasmid, outside the T-DNA region is termed virulence region (*vir* region) which is required to transfer T-DNA into plant cells. The *vir* region of the octopine Ti plasmid consists of eight distinct loci, *vir* A to *vir* H while the nopaline Ti-plasmid lacks the *vir* F and *vir* H loci (Hooykaas and Beijerbergen, 1994). Only the *vir* A gene is expressed constitutively. Other genes are induced by phenolic compounds produced by dicotyledonous plants on wounding. Certain phenolic compounds, such as acetosyringone, have been purified from plant exudates, which act as inducers of the *vir* genes. The *vir* D1 and *vir* D2 proteins recognize the border repeat sequences located the 5' end of the T-DNA thus producing a single stranded nick. The *vir* D2 protein guides single-stranded DNA into the plant nucleus which integrates to the plant genome by illegitimate recombination (Kim *et al.*, 2003).

Disarming (removal of oncogenes) of the Ti-plasmid is a common practice nowadays in order to check its tumorigenic properties in the transformed cells of plants. The Ti-plasmid based vectors are classified according to whether the DNA to be introduced is physically linked to the *vir* genes of the Ti plasmid or is separate. The former type of vectors are known as 'co-integrating vectors' or '*cis* vectors' and the latter type are referred to as 'binary vectors' or '*trans* vectors'.

2.8.2. *Agrobacterium*-mediated transformation in rice:

The monocots, including rice (*Oryza sativa* L.), are not amenable to *Agrobacterium* mediated transformation because these plants do not serve as natural host for the bacterium (Davey *et al.*, 1989). The monocots, particularly cereals, lack wound response and are, therefore, not susceptible to infection by the bacterium. A protocol for efficient transformation of rice mediated by *Agrobacterium* was developed (Hiei *et al.*, 1994; Rashid *et al.*, 1996) by the use of a super-binary vector and a transformation stimulator, namely acetosyringone, during co-cultivation. Chan *et al.* (1993) and Hiei *et al.* (1994) reported heritable transformation of rice mediated by *A. tumefaciens.* Hiei *et al.* (1994) substituted the wound response by adding acetosyringone to the co-cultivation medium and found that the temperature (22-28^{0}C) during co-cultivation was critical for rice transformation. Since the first production of transgenic rice plants 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.

There is a wide difference between the tissue culturability of *indica* and *japonica* rice, the former being less responsive than the latter (Visarada *et al.*, 2002). Thus, it is important to establish parameters for the transformation of popular *indica* rice varieties. Tyagi *et al.* (2007) developed *Agrobacterium* mediated transformation protocols for a semi-dwarf *indica* rice cultivar ADT39. Forkan *et al.* (2004) successfully transformed Bangladeshi *indica* rice cultivars BR26 and Binni by *Agrobacterium tumefaciens* strain LBA 4404 (pTOK 233). They reported that 100μM acetosyringone was optimum concentration for stable transformation. Mohanty *et al.* (1997) reported *Agrobacterium* mediated transformation of Pusa Basmati 1. Rashid *et al.* (1996) reported 22%

transformation efficiency of Basmati 370 which was as high as reported in *japonica* rice and dicots by using *Agrobacterium tumefaciens* strain EHA101 (pIG121Hm).

2.8.3. T-DNA transposon tagging as a tool for functional genomics:

Functional genomics can be defined as the "development and application of global genome-wide or system-wide experimental approaches for assessing the gene function by making use of the information and reagents provided by structural genomics" (Hieter and Boguski, 1997). Forward and reverse genetics approaches are used to discover the biological functions of genes. Forward genetics begins with a mutant phenotype and goes towards the genotype i.e., it deals with the sequence of the mutant gene that caused the altered phenotype. Reverse genetics begins with a mutant gene sequence and goes to the resulting change in phenotype (Krysan *et al.*, 1999, Fig. 2.9). In reverse genetics, the different mutagens like ethylmethyl sulphonate (EMS), fast neutron treatment, or insertion of an element such as a transposable element or T-DNA (Koorneef *et al.*, 1982; Sundersan, 1996; Krysan *et al.*, 1999) are used to disrupt the gene.

It is difficult to establish linkage between visible phenotypes caused by mutation and the T-DNA transposon insertion using conventional forward genetics approach. Transposon and T-DNA have been used most widely as insertional mutagens for reverse genetics (Mathur *et al.*, 1998; Wissman *et al.*, 1998). Insertional mutagenesis in principle provides a more rapid way to clone a mutated gene. Loss-of-function mutations in plants can be created by using either transposons DNA elements that are able to insert at random within chromosomes (Martienssen, 1998) or the T-DNA of *Agrobacterium tumefaciens* (Azpiroz-Leehan and Feldmann, 1997). Because the sequence of the inserted element is known, the gene in which it is inserted can be easily recovered using various cloning or PCR-based strategies (Bauchez and Hofte, 1998).

T-DNA as the insertional mutagen has an advantage over transposons because the T-DNA insertions do not transpose subsequent to integration within the genome and are therefore chemically and physically stable through multiple generations. On the other hand, transposon mutagenesis has one advantage due to its ability to transpose to nearby locations which provide a convenient method for mutations within all of the members of the gene family within a single gene (Krysan *et al.*, 1999).

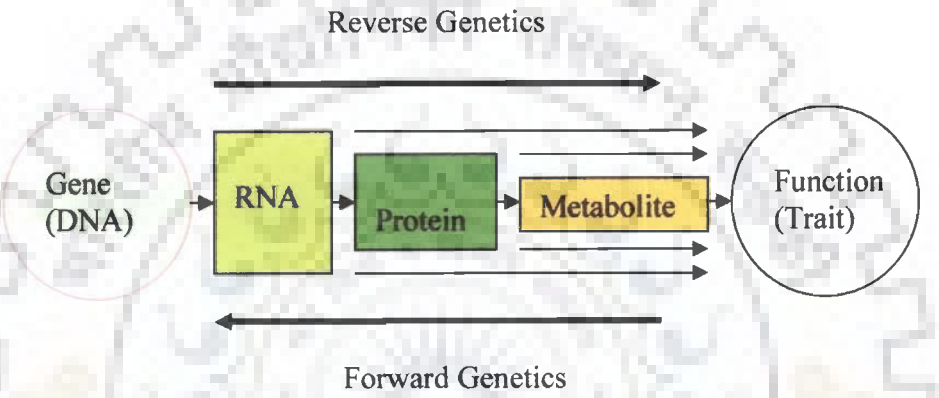

Figure 2.9 Functional genomics approaches

In insertional mutagenesis, it is difficult to identify the function of redundant genes, or of genes required in early embryogenesis or gametophyte development. One of various modified designs of the insertional mutagenesis, the gene trap system involves creation of fusions between the tagged genes and a reporter gene, such as β-*glucuronidase* (*Gus*) or green fluorescent protein (*Gfp*; Sundaresan *et al.*, 1995; Springer, 2000). This system identifies a novel gene based on its expression pattern. The inserted promoterless reporter not only suppresses the normal gene function but also activates expression of the reporter gene. As the expression levels of the inserted gene can be monitored even in heterozygote plants, the gene trap system is thus useful for the

functional study of several plant genes, including essential genes that cause lethal mutations. In activation tagging, the T-DNA or a transposable element containing multimerized cauliflower mosaic virus (CaMV) 35S enhancers (Hayashi *et al.*, 1992; Suzuki *et al.*, 2001; Jung *et al.*, 2008) is inserted in the gene which can function in either orientation and at a considerable distance from the coding regions. This results in dominant gain-of-function mutations or transcriptional activation of the genes or the nearby genes. Gene activations by such method can produce novel phenotypes which can identify important genes that are either redundant members of a gene family or are necessary for survival.

However, in T-DNA insertions, the integration of the T-DNA is complex, due to its tandem direct and inverted repeats and deletions in one or more borders. This makes difficulties in the further molecular analysis of large scale flanking sequence database strategies. Moreover, the T-DNA approach is not useful and feasible in those plants where the transformation is difficult or labour intensive. These drawbacks of T-DNA insertional mutagenesis can be overcome by insertional mutagenesis using transposable elements (Ramachandran and Sudaresan, 2001).

To isolate genes from several plant species like maize and Snapdragon, mobile genetic elements *Ac* and *Ds* have been widely used (Fedoroff *et al.*, 1984) in which an engineered transposable transformed element from maize i.e., *Ac* and *Ds*, acts as insertional mutagens. The autonomous *Ac* element is 4565 bp in length, which encodes an 807 amino acid "transposase" protein that requires about 200bp terminal regions for mobility (Pohlman *et al.*, 1984; Kunze *et al.*, 1987). The non-autonomous *Ds* elements do not have the ability to produce transposase, but carry all the sequences necessary for transposition if transposase is supplied in *trans* (Mc Clintock, 1948) from an *Ac* element.

In two-element *Ac/Ds* system, the TPase source (from *Ac*) is stabilized by clipping its border sequences and the clipped *Ac* cannot transpose on their own but would induce transposition of *Ds* in *trans*. In this system, a *Ds* element inserted in parental line can be moved to another position by crossing with a line carrying the *Ac*-transposase gene. This remobilization property of *Ds* elements can be useful for confirming the mutational effects of insertions. Moreover, *Ds* elements transpose preferentially to sites located in the vicinity of the donor sites (Smith *et al.*, 1996; Machida *et al.*, 1997), thus can be used to saturate the genome with random events of transposon insertions. This system can be improved by using resistance conferring genes (e.g. *iaaH, hpt* etc.) as markers so as to allow efficient field selection of plants in which transpositions have occurred (Phogat *et al.*, 2000).

2.9. Bulk Segregant Analysis (BSA):

The technique of BSA was first described by Michelmore *et al.* (1991). It has been widely used tool to find DNA based markers, linked to target genes or to other DNA based markers. In BSA, two pools or bulks of DNA samples are contributed equally from 10-20 individual plants from a segregating population. These two bulks should differ for a trait of interest (e.g. tall vs. dwarf for plant height). By making DNA bulks, all loci are randomized, except for the gene of interest. Markers are screened across the two bulks. Polymorphic markers may represent markers that are linked to a gene or QTL of interest. The entire population is then genotyped with these polymorphic markers and a localised linkage map may be generated (Collard *et al.*, 2005).

The BSA method is effective method for rapidly mapping major loci and has been used for mapping quantitative (Chalmers *et al.*, 1993) as well as qualitative traits (Michelmore *et al.*, 1991). Gottwald *et al.* (2004) used BSA for the fine mapping of a

gibberellic acid-insensitive gene *sdw3* in barely on 2HS chromosome. BSA approach has been used in oat to identify random amplified polymorphic DNA (RAPD) marker linked to genes for oat stem rust resistance (Penner *et al.*, 1993). Muangprom and Osborn (2004) used BSA to identify molecular markers linked to the dwarf genes by using 18 plants of each phenotypic classes (short and tall or intermediate) from BC_1F_1 population and 92 *Brassica* RFLP probes. Milach *et al.* (1997) mapped the three dwarfing loci available in oat using RFLP markers and identified regions of the genome which contribute to plant height, and verified the utility of BSA and the hexaploid oat RFLP map for mapping dwarfing genes in cultivated oat.

For mapping a gene, polymorphism in the nucleotide sequence is usually required for a molecular marker. This polymorphism is revealed by molecular techniques such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), microsatellite or simple sequence repeat polymorphism (SSRP), random amplified polymorphic DNA (RAPD), cleavable amplified polymorphic sequences (CAPS) and single strand conformation polymorphism (SSCP). Genetic maps have been constructed in many crop plants using these markers on a single segregating population. Simple sequence repeats (SSR) or microsatellites are ubiquitous in eukaryotes. SSR polymorphism (SSRP) reflects polymorphisms based on the number of repeated units in a defined region of the genome being investigated. The number and composition of microsatellite repeats differ in plants and animals. The frequency of repeats longer than 20 bp has been estimated to occur every 33 kb in plants unlike mammals where it is found to occur every 6 Kb. In humans, AC or TC is a very common repeat unit, but in plants AT is more common followed by AG or TC. But in general, plants have about 10 times less SSRs than humans. Nucleotide sequence flanking the

repeat is used to design primers to amplify the different number of repeat units in different varieties. This type of polymorphism is highly reproducible. These primers are very useful for rapid and accurate detection of polymorphic loci and the information could be used for developing a physical map based on these sequence tags.

Several groups of scientists (Temnykh *et al.*, 2001; McCouch *et al.*, 2002) throughout the world have been engaged in developing a high density molecular map of SSRs in rice based on genetic, physical and sequence-based maps of rice. Till the completion of IRGSP in 2005, a total of 18,828 class I (more than 20 nucleotides in length) SSRs were identified and annotated uniformly over the 12 rice chromosomes.

2.10. Thermal asymmetric interlaced (TAIL)-PCR and PCR genome walking in T-DNA insertional mutagenesis:

2.10.1. TAIL-PCR:

Thermal asymmetric interlaced (TAIL)-PCR is a technique to effectively isolate a DNA segment adjacent to known sequence (Liu *et al.*, 1995). TAIL-PCR is very simple, efficient, and highly specific in which no other manipulations apart from PCR are required. TAIL-PCR is especially suitable for isolation of targeted unknown sequences from a large number of samples. This technique has been used to recover insert ends from rice BAC clones for chromosome walking and mapping (Yang *et al.*, 1997). Briefly, TAIL-PCR utilizes three nested gene specific primers in three consecutive reactions together with an arbitrary degenerate (AD) primer having a lower T_m (melting temperature), so that the relative amplification efficiencies of specific and non-specific products can be thermally controlled. In the primary reaction, one low-stringency PCR cycle is conducted to create one or more annealing sites for the AD primer in the targeted

sequence. Specific product is then preferentially amplified over non-specific ones by interspersion of two high-stringency PCR cycles with one reduced-stringency PCR cycle (Fig. 2.10). The nested PCR amplifications help to achieve higher specificity in consecutive secondary and tertiary PCR reactions. Three PCR reactions are carried out sequentially to amplify target sequences using nested T-DNA specific primers on one side and an AD primer on the other. One or more sites within the flanking sequences are adapted for annealing to the AD primer through a special low-stringency cycle. Even after creation of sites adapted for the AD primer, however, high temperature annealing still favours the specific primer, resulting in a linear amplification of target molecules. To achieve adequate thermal asymmetry, the specific and AD primers were designed to have T_ms of 57^0-62^0C and 44^0-46^0C, respectively. By interspersing reduced-stringency cycles to allow AD priming, double-stranded molecules can be formed, and the preferential linear amplification of target molecules becomes logarithmic. In the secondary and tertiary reactions non-specific products fail to be reamplified.

2.10.2. PCR genome walking:

Genome walking is an efficient and reliable procedure that enables the identification of unknown regions flanking a known DNA sequence based on PCR amplification (Fig. 2.11). It needs to construct DNA libraries and little information about the restriction sites in the flanking sequences to be amplified is required. Several different genome walking strategies, based on restriction digestion and PCR, have been reported such as inverse PCR (Ochman *et al.*, 1988), and adapter-specific PCR (Universal Genome Walking Kit, CLONTECH, Palo Alto, CA, USA).

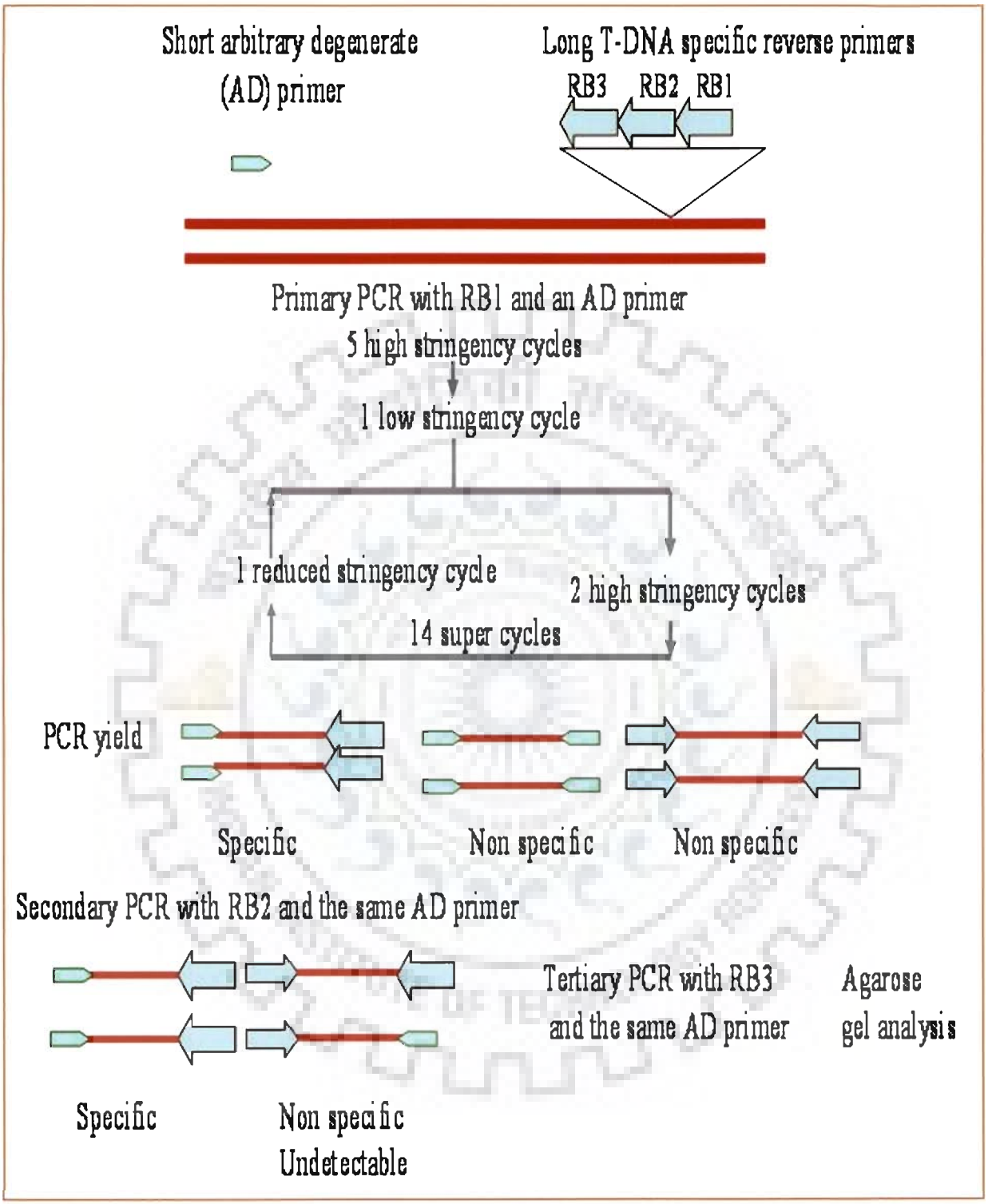

Figure 2.10 TAIL-PCR methodology used to isolate the T-DNA flanking regions of insertional mutants (Modified from Liu *et al.*, 1995).

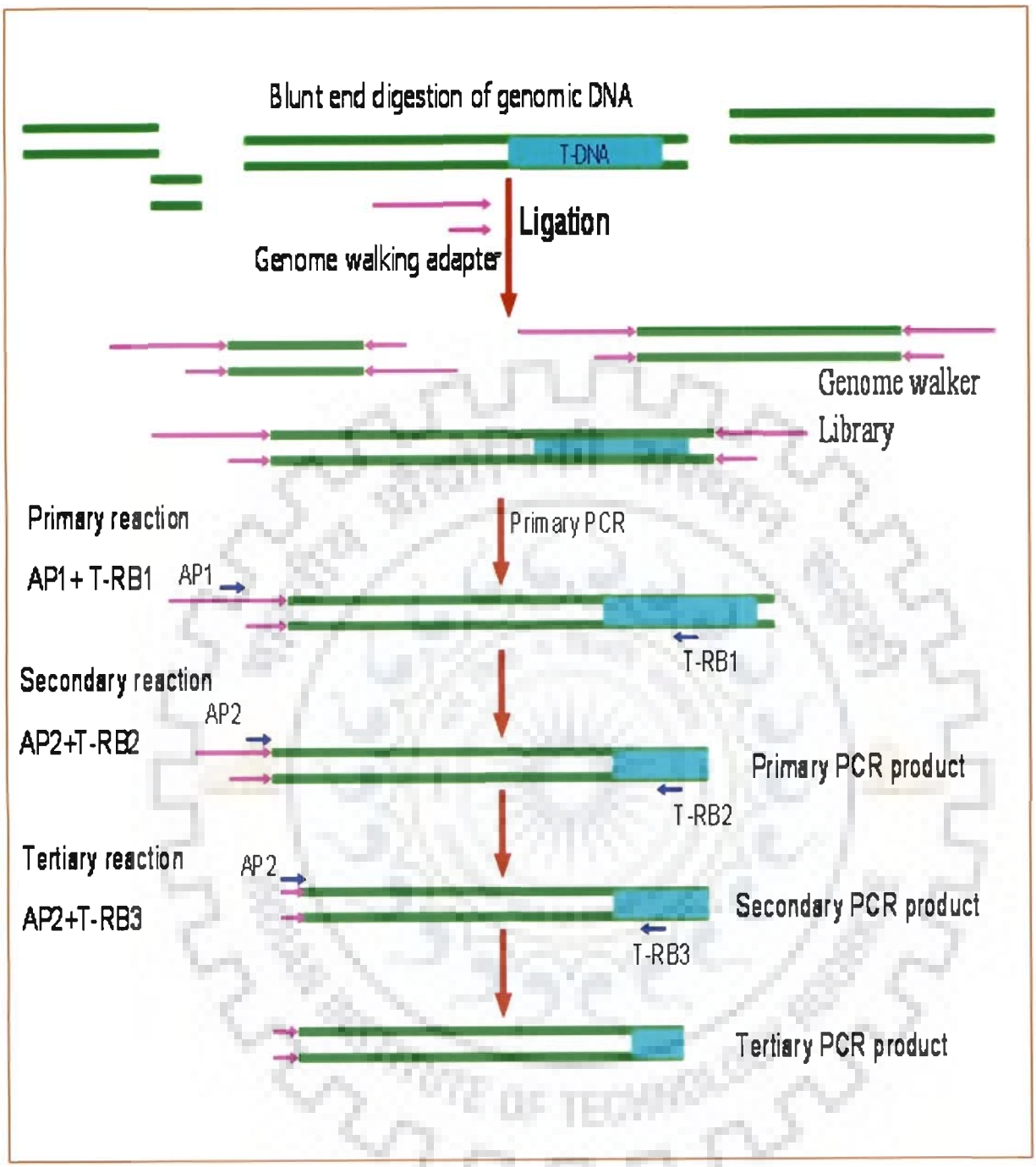

Figure 2.11 Schematic diagram for PCR genome walking which was used for isolating the T-DNA flanking regions.

Universal Genome Walking Kit from CLONTECH is fast, reliable and relatively easy to use. In the genome walking, gene and adaptor-specific nested primers are used for amplifying flanking regions. The whole genome is digested by different restriction enzymes (tetra-cutter, blunt end) and library is made by ligating the digested genomic fragments with the adapter supplied with the manufacturer's kit. The technique is accurate due to the use of two-round PCR, where the nested gene-specific primer is used in the secondary PCR, thereby increasing the probability of amplification of the desired flanking DNA sequence (Fig. 2.11). However, the genome walking strategy has a particular limitation in amplifying either smaller fragment or having no amplification of the flanking DNA. The condition arises when the restriction site is close to the upstream location of the gene specific primer in the genome. To overcome this problem, use of different restriction sites which are distant from the gene specific primer would increase the chance of obtaining templates of the flanking DNA (Rishi *et al.*, 2004).

2.11. Validation of gene function in rice:

2.11.1. Flanking Sequence Tags (FSTs):

In order to prove that a particular insertional mutation causes a certain phenotype, it must be essential to isolate additional mutant alleles for the locus or complement the mutation by introducing a wild-type copy of the respective gene into the mutant plant by using transgenic technology. The availability of additional mutant alleles would provide the quickest route to confirm the role played by the insertionally mutated gene in the observed phenotype. If the same phenotype is found to be linked to the same T-DNA insertion in several independently transformed plants, a strong argument can be made that the mutation is causing the phenotype. Generating a large collection of T-DNA-transformed lines (also called as FSTs) has the benefit of finding more than one T-DNA

insert in a given gene. Access to a large population of T-DNA-transformed lines could thus supplement the labor-intensive process of complementation analysis with transgenic approach.

So far, a number of FSTs are available in database at Rice Functional Express Database (RiceGE) developed by the Salk institute (Jung *et al.*, 2008) which has been generated by the efforts of researchers in 11 institutes in 7 countries around the world. The seeds of the mutant lines can be procured through individual suppliers like POSTECH, South Korea (Krysan *et al.*, 1999) for functional validation of the gene.

2.11.2. RNAi silencing as a tool of functional genomics in rice:

Besides insertional mutagenesis, gene silencing is an efficient tool for the determination of gene function (Anand *et al.*, 2003; Singh *et al.*, 2006b). RNA interference (RNAi) is based on sequence-specific mRNA degradation which follows the formation of double-stranded RNA (dsRNA) homologous in sequence to the targeted gene (Baulcombe, 2004). In contrast to insertional mutagenesis, RNAi allows silencing one, several, or all members of a multigene family or homoeologous gene copies in polyploids by targeting sequences that are unique or shared by several genes (Miki *et al.*, 2005). dsRNA is detected by the host plant genome as aberrant and is cleaved by the action of Dicer-like enzymes into long and short siRNAs (Tang *et al.*, 2003). These two classes of small RNAs have distinct RNA silencing functions. The short siRNA (approximately 21-mers) directs post transcriptional signaling via mRNA degradation and the long siRNA (approximately 24-mers) trigger systemic silencing and the methylation of homologous DNA (Hamilton *et al.*, 2002).

RNAi is proved to be very efficient in interfering with gene expression in various plant systems such as *Petunia hybrida*, *Arabidopsis thaliana*, *Coffea arabica*, and *Oryza sativa* (Stam *et al.*, 1997; Chuang and Meyerowitz, 2000; Lee *et al.*, 2004; Ogita *et al.*, 2004; Miki *et al.*, 2005; Isshiki *et al.*, 2001). The wide use of this powerful technique reflects its ease of application and the possibilities for genome-wide reverse genetics. When directed against viruses or endogenous genes and transgenes the gene constructs encoding intron-spliced RNA with a self-complementary hairpin (hp) structure have been shown to induce post transcriptional gene silencing with almost 100% efficiency (Smith *et al.*, 2000).

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CHAPTER 3

MATERIALS AND METHODS

3. Materials and Methods

3.1. Materials

3.1.1. Insertional mutants:

The dwarf mutant *OsGAI/Sd* used in the present study was isolated from superfine quality *indica* rice cultivar Basmati 370, transformed with T-DNA binary vector through *Agrobacterium tumefaciens* strain EHA101 containing Hm^R*Ds* cassette. The cassette Hm^R*Ds* contained *Ds* transposable element with *hpt* gene as the selectable marker. This material was obtained from the Punjab Agricultural University, Ludhiana (Dhaliwal *et al.*, 2001). Seventy two T_1 and seventeen T_2 progenies were obtained from independent transformation events in Basmati 370 using the binary vector. Out of these independent events, two were phenotypically dwarf. The first dwarf mutant was recovered from T_1 plants of B-1-6-3 transformant while the second dwarf mutant was recovered from T_1 as well as T_2 plants of B-3-1-2. The first dwarf mutant i.e., B-1-6-3 was named *OsGAI/Sd* for molecular characterization in the present investigation. Along with the dwarf mutant, two other insertional mutants viz; B-3-1 and B-2-2-3 (named *OsGAS/Sd*) were also included in the present study. These two insertional mutants were phenotypically similar to its wild type Basmati 370. The details of Hm^R*Ds* construct used for *Agrobacterium* mediated transformation of Basmati 370 is given in Fig. 3.1.

The Hm^R*Ds* construct was made by replacing the internal *HindIII* (position 1783)-*XhoI* (position 3557) fragment of 4.5 Kb *Ac* element with a 1.6 Kb 35S-hygomycin phosphotransferase (HPTII)-*ocs3'* fusion. The *Ds* construct was cloned into the binary vector pCLO111 (Dean *et al.*, 1992) which carries the left border (LB) and right border (RB) of the *Agrobacterium* T-DNA, a 1' NPTII (Neomycin phosphotransferase) selectable

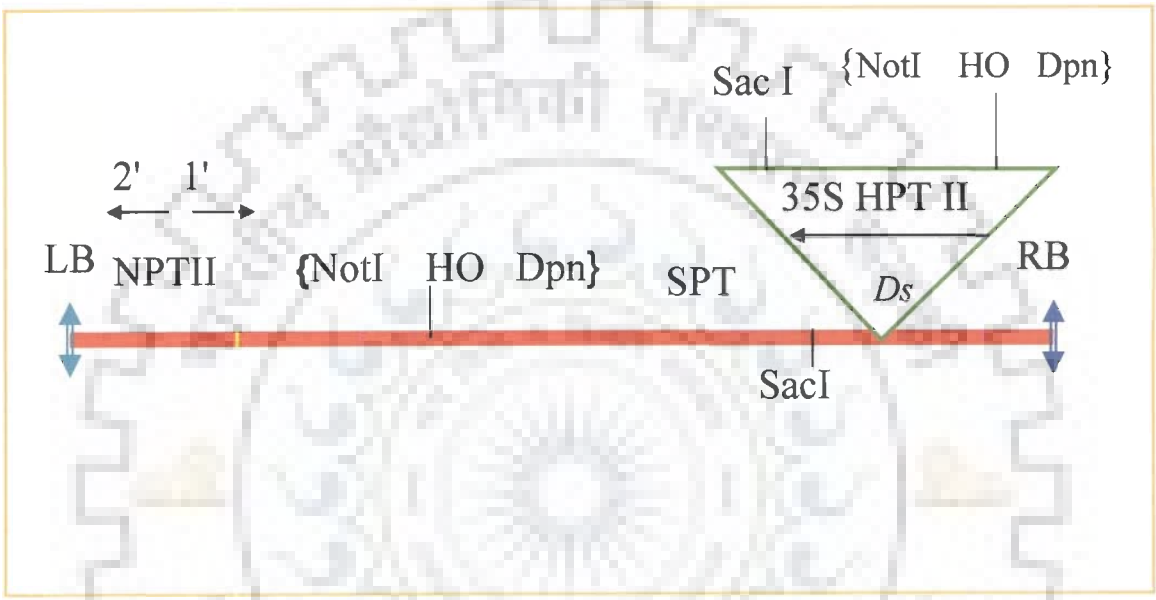

Figure 3.1 Schematic representation of the Hm^R*Ds* construct that was used for Basmati 370 transformation via *Agrobacterium* T-DNA vector. LB: Left border, RB: Right border, SPT: streptomycin phosphotransferase, NPT: Neomycin phosphotransferase, HPT: Hygromycin phosphotransferase. *Not*I, *HO* nuclease 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.

marker and 35S fusion. The elements were cloned into *XhoI*, *SacI* sites at the junction of the 35S promoter and the 5' untranslated leader of the 35S-SPT fusion. The Hm^R*Ds* also carried three rare cutter sites, *NotI*, *HO* nuclease and *DpnI/ClaI* methylase within the T-DNA and within the *Ds* element.

3.1.2. Mapping Population:

In order to tag the dwarfing gene, a mapping population was developed by crossing the dwarf mutant *OsGAI/Sd* with a distantly related non-basmati semi-dwarf cultivar PR106. The generation of mapping population was advanced to F_4 following the Single Seed Descent (SSD) method as suggested by Goulden (1939). For evaluating the inheritance of the dwarfing gene, the dwarf mutant *OsGAI/Sd* was also crossed with wild parent Basmati 370 and the generation was advanced to F_2. T-DNA insertional *OsGAI/Sd*, *OsGAS/Sd* and B-3-1, wild type Basmati 370, two semi-dwarf rice varieties viz., PR106, and Pusa Basmati 1 and the mapping population were grown in *Kharif* seasons of 2004, 2005, 2006 and 2007 at Indian Institute of Technology, Roorkee with the recommended package of practices under containment conditions. Flow chart of the development of recombinant-lines population is given in Fig. 3.2.

3.1.3. Molecular Biology Kits

QIAEXII gel elution kit	Qiagen, Valencia, CA, USA
QIAquick PCR purification kit	Qiagen, Valencia, CA, USA
iRIS® kit	IHBT, Palampur, India
Superscript™ II RT kit	Invitrogen- life technologies, CA
RNeasy MinElute cleanup kit	Qiagen, Valencia, CA, USA.

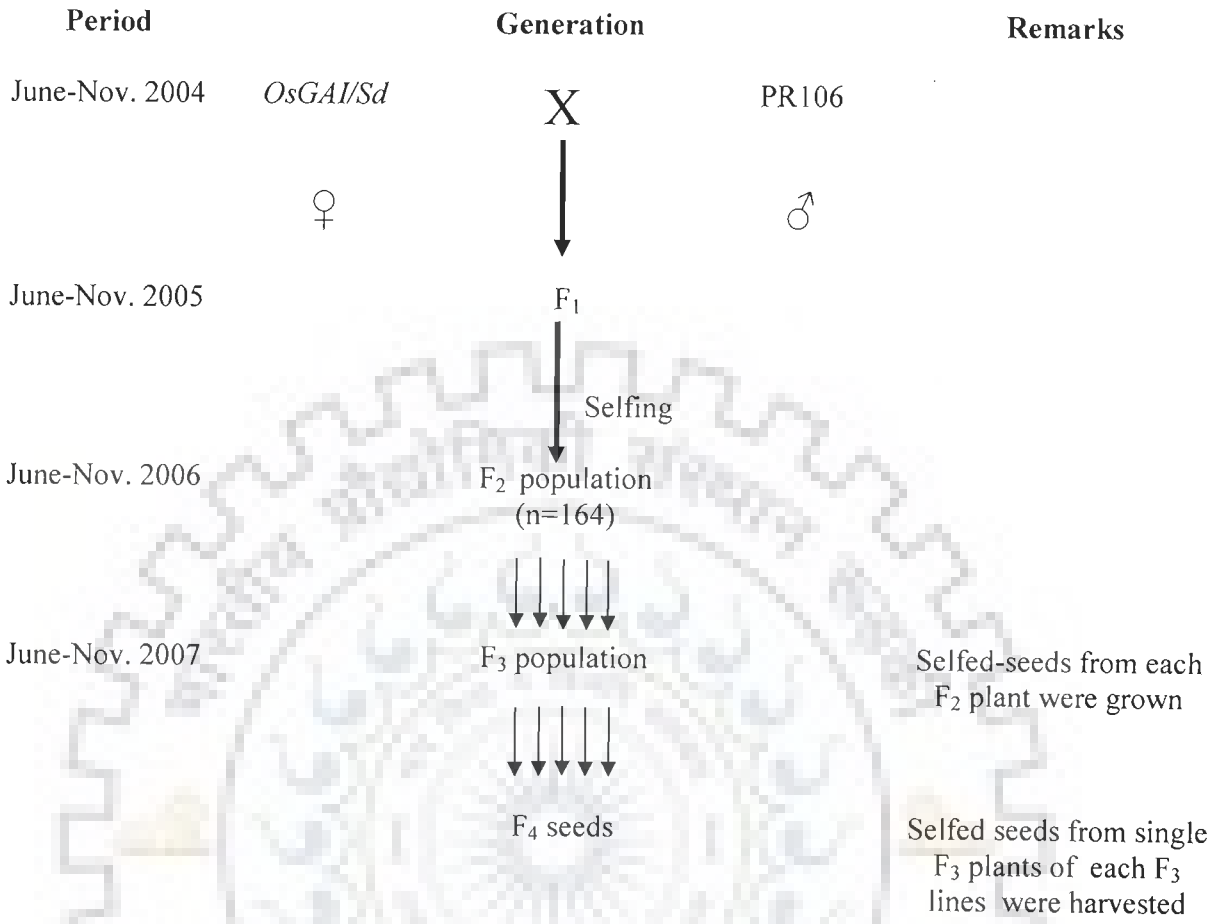

Figure 3.2 Schematic presentation of the development of mapping population.

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
DNase I	Promega Madison, WI, USA
T4 DNA ligase	Bangalore Genei, Bangalore

Ribonuclease A (Stock solution - 10 mg/ml Working solution - 10-15 mg/ml)	Sigma- Aldrich, St Louis, MO, USA

3.1.5. Antibiotics:

Hygromycin	Sigma- Aldrich, St Louis, MO, USA

3.1.6. Molecular weight markers:

100bp DNA ladder	Bangalore Genei

3.1.7. Membrane:

Hybond-N	GE Biosciences, Piscataway, NJ, USA

3.1.8. Radioisotopes:

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 40 ml of 0.5M EDTA (pH 8.0)
1X TE(Tris-EDTA buffer)	10mM Tris HCl, 1mM EDTA, pH8.0
20X SSC (pH 7.0)	1L 175.3 g NaCl 88.2 g Sodium citrate pH was adjusted to 7.0 and autoclaved
Denaturation buffer (For Southern)	1.5M NaCl 0.5N NaOH
Neutralization buffer (For Southern)	1.5M NaCl 1.0M Tris (pH 7.4)

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% β-mercaptoethanol was added before use
5X RNA gel loading buffer	0.2 M 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. DNA isolation, purification and quantification:

The genomic DNA was extracted by CTAB (cetyl-trimethyl ammonium bromide) protocol described by Murray and Thompson (1980). DNA isolation from fresh young leaves of parents, individual F_2 plants and corresponding F_3 and F_4 lines were (in bulk) were carried out. About 5-7 gm. of young, healthy and disease free leaves were grounded into fine powder in liquid nitrogen using autoclaved and chilled pestle and mortar. Before thawing, the powder was transferred into a 50 ml Oakridge tube containing prewarmed (65^0C) extraction buffer (2% w/v CTAB, 1.4 M NaCl, 20mM EDTA, 100 mM Tris HCl pH 8.0) to which 0.2% β-mecaptoethanol was added just before use. The homogenate was incubated at 65^0C for 50 to 60 minutes with occasional

mixing. To the homogenate, an equal volume of chloroform-isoamyl alcohol (24:1) was added and mixed thoroughly to ensure emulsification of the phases. The samples were centrifuged for 20 min (10000 X g, 25^0C) and supernatant were transferred to another marked centrifuge tube with the help of micropipette. To the tubes, 0.6 volume of pre-chilled isopropanol was added and mixed gently. Heat treated RNase (10 mg/mL; SIGMA) was added to a final concentration of 100µg/mL. Subsequently the samples were incubated in 37^0C water bath for 30 min. The RNase free samples were re extracted with fresh chloroform: isoamyl alcohol (24:1). The upper aqueous phase was transferred to falcon tubes using pipette. For DNA precipitation 0.6 volume of ice-chilled isopropanol was added in the sample mixture and mixed gently. In the absence of DNA precipitation, samples were incubated at 4^0C for 30 minutes. Precipitated DNA was either spooled out using a micropipette with cut-end sterilized tips or pelleted by centrifugation (5000 X g, 4^0C). Residual CTAB was removed by resuspending DNA pellet in washing solution (70% ehanol) for five minutes followed by recentrifugation (5000X g, 4^0C). Pellet was collected, air dried (37^0C) for few hours and dissolved in appropriate volume of 1X TE (Tris-EDTA buffer-10mM Tris HCl, 1mM EDTA, pH8.0). Electrophoresis (Sambrook *et at.*, 1989) was carried out for the qualitative and quantitative analysis in 0.8% agarose gel with 0.5µg/ml ethidium bromide (10mg/ml) in 1X TAE (40 mM Tris-acetate, 1mM EDTA; pH 8.0) buffer. The gel was visualized under ultraviolet light and depending upon the band intensity, the DNA concentration was adjusted to 50ng/µl with double distilled milliQ water. Spectrophotometric reading of the DNA samples was carried at 260 and 280nm wavelength and optical density (OD)

and OD_{280}/OD_{260} ratio was taken as measure of DNA purity. At 260 nm wavelength, the concentrations of DNA (OD_{260} x 50 x dilution factor) in each sample were also recorded.

3.2.2. *hpt* amplification:

The Hm^R*Ds* construct used in the T-DNA contained a Hygromycin phosphotransferase (*hpt*) gene as a selectable marker to screen out the transgenic plants in parents and the segregating generations. The coding region of the hygromycin phosphotransferase gene (*hpt*) was amplified by polymerase chain reaction using specific forward (5'-GTCTGTCGAGAAGTTTCTGATCG-3') and reverse primers (5'-GCGAGTACT TCTACACAGCCAT-3'). The reaction mix (20 µL) for PCR consisted of 50 ng of rice genomic DNA, 0.2 mM each of dNTPs, 0.2 mM of each PCR primer, 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) and 0.8 units of *Taq* DNA polymerase. Thermal cycling was done at 94°C for 30 seconds, 58°C for 2 minutes and 72°C for 1 minute. After the final cycle, the reactions were maintained at 72^0C for 5 min before completion. Reactions were conducted using PTC-100-programmable thermal cyclers (MJ Research, USA). The 950bp long amplified fragment of *hpt* gene was visualized by staining the 0.8% agarose gel with ethidium bromide from a stock of 10mg/ml.

3.2.3. Scoring of phenotypic data:

The following pre harvest observations were recorded in the F_2 population:

3.2.3.1. Plant height:

It was measured from the ground level to the tip of the terminal spikelet. It was measured at maturity stage with the help of measuring scale.

3.2.3.2. Gibberellic acid response:

Freshly prepared Gibberellic acid (GA_3) at 120ppm concentration was sprayed on the leaves of each F_2 plants as well as on 60 days old plants of dwarf mutant *OsGAI/Sd*, *OsGAS/Sd*, its wild plant Basmati 370 and two other cultivars namely, PR106 and Pusa Basmati 1. Final observation on culm elongation after GA_3 spray was taken after 10 days of application. The response of GA spray was calculated by the following formula:

$$GA_3 \text{ response } (\%) = \frac{\text{Final height after } GA_3 \text{ spray-Initial height before } GA_3 \text{ spray}}{\text{Initial height before } GA_3 \text{ spray}} \times 100$$

3.2.3.3. χ^2 test :

χ^2 test was carried out at $p<0.05$ probability at one degree of freedom. Where 'p' is the probability that the deviation of the observed from that expected is due to chance alone. χ^2 is the sum of the squared difference between observed and the expected data, divided by the expected data in all possible categories.

3.2.4. Chlorophyll estimation:

The total chlorophyll content of *OsGAI/Sd* and Basmati 370 was estimated by the method given by Arnon (1949) and Koski (1950). One gram of fresh leaf tissues were cut into small pieces and the pigment was extracted by grinding the cut tissues for 5 minutes in 100mL of 85% acetone with a mortar and pestle. The homogenate was filtered with Whatman No.1 filter paper. The filtrate was made up to 100mL volume with 85% acetone. The optical density (OD) of the extract was measured in spectrophotometer at

663nm and 644nm wavelength. The concentration of Chlorophyll a and b was calculated by the following formula:

mg chlorophyll a per gram of tissue = 1.07 (OD_{663})-0.094(OD_{644})

mg chlorophyll b per gram of tissue= 1.77(OD_{644})-0.280(OD_{663})

3.2.5. Bulk Segregant Analyses (BSA):

3.2.5.1. Detection of polymorphism by SSR (Simple Sequence Repeats):

Two hundred thirteen rice SSR or microsatellite primer pairs representing all the 12 chromosomes of rice covering both chromosomal arms for initial parental polymorphism evaluation between Basmati 370 and PR106 were selected based on IRGSP (International Rice Genome Sequencing Project), 2005; Temnykh *et al.*, 2001 and McCouch *et al.*, 2002. PCR was carried out according to Temynkh *et al.*, (2000). 20µl of reaction mixture contained 50ng of DNA, 0.1mM of each of the dNTP's, 0.25µM of forward and reverse primer, 0.6U of *Taq* DNA polymerase and 1X PCR buffer (50mM KCl, 10mM Tris-HCl, 1.5mM $MgCl_2$). The PCR conditions were 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30sec and extension at 72°C for 2 min with final extension of 72°C for 7 min.

3.2.5.2. Resolution of amplified SSR product:

2µl of 10X loading dye (0.4% W/V bromophenol blue, 0.4% W/V Xylene Cyanol FF, 50% Glycerol) was added to the 20µl PCR product. The PCR product was loaded on 2.5% LMP high resolution superfine agarose (promega) prepared with 0.5X TBE buffer (45mM Trisbase, 45mM boric acid and 1mM EDTA). Gels were stained with 1µg/ml ethidium bromide and then visualized under UV light and photographed using UVP Gel

documentation system (Model GDS7600) with GRAB-IT software programme (Annotating Grabber 32-Bits).

3.2.5.3. Identification of linked microsatellite marker and linkage analysis

Two bulks were made from the selected lines of segregating F_3 mapping population developed from *OsGAI/Sd* X PR106 cross. Positive bulks was prepared from homozygous *hpt* +ve dwarf and gibberellic acid insensitive progenies (bulk 1), while negative bulk was prepared from *hpt* -ve tall and gibberellic acid sensitive (bulk 2) F_3 lines. An equal concentration of DNA from these progenies was pooled. These two bulks along with the parents were used to identify putative SSR markers linked to the dwarfing gene. The microsatellite markers distinguishing parents and bulks were used to analyze individual plants constituting each bulk and further tested on the F_2 segregating population. Linkage analysis was done using recombination frequency between marker and mutant phonotype at each locus.

3.2.6. Thermal asymmetric interlaced PCR (TAIL PCR):

TAIL-PCR was carried out to isolate the T-DNA flanking regions of insertional mutants including *OsGAI/Sd*, B-3-1 and *OsGAS/Sd*.

3.2.6.1. Primers:

The sequence of T-DNA specific primers used for TAIL-PCR was designed in such a manner that they can amplify the flanking region of T-DNA. The T-DNA specific primers used for this purpose were same as used by Muskett et al., 2003. Sequence of a stretch of right border sequence of the plasmid vector pCLOIII and the positions of RB primers in it has been shown below (Fig. 3.3).

Figure 3.3 Right Border sequence of the plasmid vector pCLOIII

RB1: 5'-GGGGCATCGCACCGGTGAGTAAT-3'(T_m=63.7^{0}C)

RB2: 5'-AGCGAATTTGGCCTGTAGACCTCA-3'(T_m=60.4^{0}C)

RB3: 5'-TATTCGGGCCTAACTTTTGGTGTG-3' (T_m=57.4^{0}C)

In addition, three AD primers (15- or 16-mers) were designed and synthesized (Liu *et al.*, 1995):

AD1: 5'-NTCGASTWTSGWGTT-3'(64-fold degeneracy, average T_m=45.3^{0}C)

AD2: 5'-NGTCGASWGANAWGAA-3'(128-fold degeneracy; average T_m=46.6^{0}C)

AD3: 5'-WGTGNAGWANCANAGA-3'(256-fold degeneracy; average T_m=45.3^{0}C)

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

The specific primers were designed to have T_ms (60-65^{0}C) higher than those (approx.-45^{0}C) of AD primers.

3.2.6.2. PCR procedure:

20µL of primary TAIL-PCR reaction contained 0.75X PCR buffer (10 mM Tris-HCl, pH 8.3,1.5 mM $MgCl_2$), 200µM each of dNTPs, about 200ng of genomic DNA,0.8 unit of *Taq* polymerase, 0.15µM 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 RB2 and the same AD primer used in the primary reaction (1.5 μM 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 RB3 and the same AD primer used in the secondary reaction (1.5 μM 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 reaction. Primary, secondary and tertiary PCR reactions were executed as summarized in Table 3.1 using Biorad thermal cyclers.

3.2.7. Gel elution and purification of PCR product

The DNA fragment of desired length obtained in tertiary PCR reaction was excised from the agarose gel with a clean and sharp scalpel. Three volumes of QX1 (provided in the QIAquick gel extraction kit) was added to 1 volume of gel. The QXII (provided in the QIAquick gel extraction kit) resuspended by vortexing and added to the gel. The gel was incubated at 50°C for 10 min (or until the gel slice has completely dissolved). The sample was centrifuged at 10,000Xg for 30 seconds and the supernatant was removed. The pellet obtained was washed with 500μL of QX1 buffer, resuspended by vortexing and centrifuged again at 10000Xg for 30 seconds. The pellet obtained was washed with PG buffer (provided in the QIAquick gel extraction kit) by the manner mentioned above. The pellet was air dried until it became white. The entire DNA sample was again resuspended in 30μL of milliQ water with the help of tip and centrifuged for 30 seconds at 4000 rpm. The supernatant was finally collected into a clean tube using pipette.

Table 3.1 Cycle settings used for TAIL-PCR

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

3.2.8. Sequencing of PCR products:

Gel-purified TAIL-PCR products were sequenced using the ABI Big Dye terminator reaction kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. It was based on the dideoxynucleotide chain termination method (Sanger *et al.*, 1977), but using automatic sequencing machine. PCR fragments amplified with T-DNA specific primer RB3 was used as sequencing template. The fragments for sequencing were amplified under the condition: 94^0C for 20 sec, 44^0C for 1 min., and 72^0C for 2 min. The sequencing reactions were carried out using the Big Dye Terminator Ready Reaction Mix (PE Applied Biosystems) with cycle sequencing program.

3.2.9. Database search and primer designing:

With the sequenced T-DNA flanking fragments of 200-484 bp of length, NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) database was searched for nucleotide similarity by the BLASTN algorithm.

For bulk segregant analysis (BSA), the sequence of forward and reverse SSR markers spanning throughout the 12 rice chromosomes was searched from the Gramene database (http://www.gramene.org/).

Primer designing was done by using Primer3 programme (http://frodo.wi.mit.edu/) (v. 0.4.0) developed from Whitehead Institute for Biomedical Research. During primer designing certain points were considered to avoid non-specific amplification. Primer designing is always a critical and most important part of any PCR technique. For this, primer length was kept between 17 to 25 nucleotides. Its GC content was fixed around 50%. Care was taken to minimize the long run sequences with any of single nucleotide and

primer was designed in such a way that they would not form any secondary structure and would not be complementary.

Multi-alignment of protein and nucleotide sequence was carried out by Clustal W (http://www.ebi.ac.uk/clustalw/). Pair-wise alignment of two nucleotide and protein sequence was carried out by Bioedit (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) software. Search for flanking sequence tags (FSTs) was carried out using two databases namely RiceGE (http://signal.salk.edu/cgi-bin/RiceGE) and OryGeneDB http://orygenesdb.cines.fr/ OryGeneDB).

3.2.10. Confirmation of T DNA insertion at chromosome 3:

After getting the genomic sequence of wild type gene through NCBI database, the T-DNA insertion in dwarf mutant *OsGAI/Sd* was confirmed using T-DNA specific and genome specific primers. The gene specific primers were designed using PRIMER3 software in such a way that they flank the T-DNA insertion position in the gene sequence of wild type. For this, three T-DNA specific primers (RB1, RB2 and RB3) were used and two sets of nested genome-specific primers were used with the gene specific primers in all possible combinations. Nucleotide sequence of the gene specific primers was as follows:

DTF1: 5'-AGGGACGACTAAGGTGAAGAACGTC-3'

DTF2: 5'-ACGTTCGTGCACATTAATTCGTCTT-3'

DTR1: 5'-AACATGTTCGACAAAATGGCAGACT-3'

DTR2: 5'-GGAACATTAACAGGGGCAGTGAAAC-3'

Conditions used for the PCR reaction were 94°C for 3 min; 35 cycles of (Denaturation at 94°C for 30 sec; annealing at 63°C for 45sec; extension at 72^0C for 2.5 min) and an additional cycle of 72°C for 7 min using PTC-100-programmable thermal

controller (MJ Research, USA). The reaction mix (20 µL) for PCR consists of 50 ng of genomic DNA of both *OsGAI/Sd* and Basmati 370, 0.2 mM each of dNTPs, 0.2 mM of each PCR primer, 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) and 0.8 units of *Taq* DNA polymerase.

3.2.11. Genome Walking:

3.2.11.1. Restriction digestion:

Highly purified and intact genomic DNA of dwarf mutant was used for restriction digestion. Four 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* and *HpaI* separately at 37°C overnight. The digested DNA was ethanol precipitated after Phenol: Chloroform (1:1) extraction. The DNA pellet was re-suspended in the water and 500ng of completely digested DNA was used to ligate with the "Genome Walker Adaptor".

3.2.11.2. Genome walker adapter:

The sequence of the adapter used for genome was

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

3.2.11.3. Ligation:

Completely digested (500ng) DNA was ligated by T_4-DNA ligase to the genome walker adaptor. The ligated products were made to a final volume of 80uL, out of which 1uL of template DNA was used for the primary Genome walking PCR.

3.2.11.4. Genome walking PCR Conditions:

The primary PCR reactions were carried in a total volume of 20µL containing the following compounds in a final concentration: 1X *Taq* DNA polymerase buffer, 1.5mM

$MgCl_2$, 0.2mM of each (dATP, dTTP, dCTP and dGTP), 0.2µM of each primer (adaptor-specific and T-DNA specific) and 1U of DNA Polymerase. The primary PCR conditions were 94°C for 3 min; 10 cycles of step down conditions [denaturation at 94°C for 30s; annealing at 70°C to 65°C (-0.5°C /cycle) for 45s; extension at 72°C for 2.5 min]; 30 cycles of (denaturation at 94°C for 30s; annealing at 65°C for 45s; extension at 72^0C for 2.5 min) and an additional cycle of 72°C for 7 min using Applied Biosciences 2720 Thermal Cycler, USA. T-DNA specific primer (RB1 5'-GGGGCATCGCACCGGTGAGTAAT-3') and an adaptor specific primer (AP1: 5'-GTAATACGACTCACTATAGGGC-3') were used for the primary PCR reaction. The primary PCR product was checked on agarose gel and diluted to fifty fold with sterile distilled water. 1µL of the diluted primary 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 final concentration of different components: 1X *Taq* DNA Polymerase buffer, 1.5mM $MgCl_2$, 0.2µM of each primer, 1µL of diluted first PCR reaction as template and 0.8U of *Taq* DNA polymerase. The secondary PCR conditions were same as of primary PCR conditions. T-DNA specific primer (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 and 1X TBE buffer. Tertiary PCR was performed using T-DNA specific primer (RB3: 5'-TATTCGGGCCTAACTTTTGGTGTG-3') and an adaptor-specific primer (AP2: 5'-ACTATAGGGCACGCGTGGT-3'). The secondary PCR product was diluted to fifty fold

with sterile distilled water and 1µl of diluted secondary PCR product was used as template to perform tertiary PCR with similar set of secondary PCR conditions.

3.2.12. RT (Reverse Transcription) PCR:

RT-PCR involves isolation of total RNA followed by RT in order to get a representative cDNA sample. In the next step, transcripts were amplified with gene specific primers and the products were separated on a gel. The different steps followed for performing RT-PCR are as follows:

3.2.12.1. Total RNA isolation:

Root and shoot samples for 10 days old seedlings of the dwarf mutant and wild type Basmati 370 were freeze dried in liquid nitrogen. The freeze dried samples were then kept at -80^{0}C for storage. The total RNA from seedlings was isolated by the *iRIS*® kit obtained from IHBT, Palampur, India. The protocol for isolation of RNA using *iRIS*® system was as per the manufacturer's instruction. The RNA pellet obtained in the final step was washed with 70% ethanol (prepared with DEPC-treated water). The air dried pellets was then dissolved in 20µL of RNase-free water.

3.2.12.2. DNase treatment and cleaning:

DNA was removed by DNase treatment, 10µl reaction consisting of RNA in DEPC treated water 8µl, RQ1 RNase free DNase 1 U/µg, 1µl 10X reaction buffer, incubated at 37°C for 30 min. Resulting RNA was extracted with phenol: chloroform: isoamyl alcohol (25:24:1) and precipitated with sodium acetate and ethanol. RNA was pelleted, washed with 70 % ethanol and resuspended in DEPC-treated water. RNA was further cleaned up using RNeasy MinElute cleanup kit. The eluted RNA was quantified by using spectrophotometer at 260 nm wavelength. To check the RNA quality the optical density

(OD) reading of RNA samples were also taken at 280nm and 260nm wavelength and the quality of RNA was judged on the basis of OD_{260}/OD_{280} ratio. The presence of protein contaminants or light scattering by any precipitates decreases the ratios of OD_{260}/OD_{280} (Teare *et al.* 1997).

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 and run at 50V. Gel was visualized and photographed.

3.2.12.4. cDNA synthesis:

mRNA was reverse transcribed using the superscript™ II RT Kit (invitrozen). 20µl reaction was set using 1µg of total RNA, oligo $(dT)_{12-18}$ (500 µg/mL), 1µL dNTP mix 10mM each. The mixture was heated to 65°C for 5 min and quick chilled on ice and after brief centrifuge. To the reaction mixture 4µL of 5 X first strand buffer; 2µL of 0.1M DDT and RNase OUT (40U/µL) were added. The contents of the tube was mixed thoroughly and incubated at 42°C for 2 min. 1µL (200 U) of Super Script II RT was added and mixed by pipetting gently up and down. The mixture was set for final incubation at 42°C for 90 min. The reaction was inactivated by heating the reaction mixture at 70°C for 15 min. 2µL of this cDNA was used for the RT-PCR.

3.2.12.5. PCR conditions:

PCR reaction was carried out in 20µL contains 20ng of DNA, 0.2mM of each of the dNTP's, 10 pmole of forward and reverse primer (primer sequence), 0.8 U of *Taq* DNA polymerase and 1X PCR buffer (50mM KCl, 10mM Tris-Cl, 1.5mM $MgCl_2$). The PCR amplification was performed in PTC-200 thermalcycer (M J Research, USA) with the

following thermal profile initial denaturation step of 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 20 sec, annealing at 62°C for 20 sec and extension at 72°C for 30 sec with last cycle of only extension of 72°C for 5 min.

3.2.12.6. Primers:

Normalization of the signals obtained for the gene of interest is necessary to compare the expression of gene in different tissues. In this study, Actin gene (Os03g50890) was used as a reference gene for assessing the efficiency of RNA isolation and PCR condition.

One set of gene specific primers based on the coding sequence of the candidate gene was used for RT-PCR.The sequences of the primer were as follows:

Forward-DRTF: 5'- AATTCGGTTTCCCTCCAGCAG-3' (Tm=62^{0}C)

Reverse-DRTR: 5'- CACTGATGCCATTCTTTCAACTCC-3' (Tm=59.5^{0}C)

The sequences Actin gene forward and reverse primers were as follows:

Actin-F: 5'- ATCCTTGTATGCTAGCGGTCGA-3'

Actin-R: 5'- ATCCAACCGGAGGATAGCATG-3'

3.2.13. Southern Blotting:

Genomic DNA was isolated from dwarf mutant *OsGAI/Sd* and wild type Basmati 370, by CTAB method and the concentration was quantified by NanoDrop spectrophotometer (NanoDrop Tech). 1.5 µg of genomic DNA was digested with 10 U of four restriction enzymes (*HindIII, DraI, EcoRV* and *EcoRI*) individually at 37°C for overnight. The resultant fragments were separated by electrophoresis through 0.9% agarose gel, using the 1X TAE buffer (Sambrook *et al.*, 1989) at 20 V for 24 hrs until the bromophenol blue dye had migrated to the appropriate position. Gel was electrophoresed at

low voltage for longer period for better separation of fragments. Gel was stained with ethidium bromide, rinsed in distilled water, and photographed. The gel was destained in distilled water and DNA was purinated in 0.25 N HCl for ten minutes and then neutralized with 0.4N NaOH. The DNA from gel was transferred to a Biodyne/Hybond N^+ membrane (Amersham Inc.) by a disposable gel transfer system using 0.4N NaOH solvent. Three sheets of Whatman 3MM filterpapers were placed in such a way that the edges were dipped into the tray containing 0.4 M 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 (15 cm thick) overnight. This arrangement allowed the DNA to transfer to the membrane. *hpt* specific PCR product 950bp length was amplified by using *hpt* specific primers (*hpt*F: 5'-GTCTGTCGAGAAGTTTCTGATCG-3' and *hpt*R: 5'-GCGAGTACTTCTACACAGCCAT-3'). The PCR product was eluted form gel and purified using the QIAGEN QIAquick PCR purification kit (USA). PCR product (25 ng) was made upto 8 µl with sterilized double distilled water for 10 min to denature and added 2 µl of hexanucleated buffer, 3 µl of dNTP (A,G,T), 2 µl of Klenow fragment and 5 µl of radiolabelled α-P^{32} dCTP were added. The mixture was then incubated for 1 hour and purified through spun columns containing Sephadex G50. The blot was prehybridized with prehybridization buffer, (7% Sodium Dodecyl Sulphate, 1% Bovine serum albumin and 0.5 M phosphate buffer at pH 7.2) at 65°C for 2 hours. To the radiolabel the probe, 30 µl of buffer (1% SDS) was added and probe was denatured by adding equal volume of 0.4 N NaOH. Denatured probe was then added to the blot and incubated for 20 hr at 65 ^{0}C. After hybridization membrane was washed at 65^{0}C for 30 min. each in 2X SSC and 1X

SSC followed by washing for 1 hour in 0.5X SSC (1XSSC: 0.15 M NaCl plus 0.015 M sodium citrate). All washing solutions contained 0.1% (w/v) SDS. The hybridized blot was exposed to X ray-film to reveal the specific hybridization signals.

3.2.14. Histological observations:

For histological examination, leaves and stems were fixed in FAA fixative. 100ml of FAA solution contained ethyl alcohol: 50 ml, glacial acetic acid: 5ml, formaldehyde (40%): 10 ml and water: 35 ml. FAA fixed excised leaves and stems were dehydrated in a graded ethanol series (30%, 50%, 70%, 90% and 100%) and xylene. To support the tissues in sectioning, the tissues were embedded in paraffin wax (Sdfine) at 60^0C, and sectioned to 10 µM thickness on a rotary microtome. Ribbons obtained from paraffin sections were mounted on slides, hydrated and dehydrated in a graded ethanol series. The tissues were stained with toluidine blue O (0.05%, w/v) dye as per the method suggested by Johansen (1940). Toluidine blue O (TBO) is a metachromatic stain, which produces different colors depending on the polymer to which it adheres. With TBO, the primary walls (ground tissue, phloem subtype of vascular tissue) were stained purple and lignified secondary walls of xylem tracheids and vessels (a subtype of vascular tissue) were stained blue, while some other cells may take on a greenish color. Longitudinal and transverse sections of stem and leaf of dwarf mutant and Basmati 370 were observed under a light microscope (Axiostar plus 1169-151, Carl Zeiss Co., Oberkochen, Germany) at different magnifications.

3.2.15. Scanning Electron microscopy (SEM):

Surface imaging of leaves and stems of *OsGAI/Sd* and Basmati 370 was done using scanning electron microscope. Various steps that followed for sample preparation for SEM were as follows:

1. Primary fixation: The sample was immersed in 2.5% glutaraldehyde for two hours at room temperature.
2. Dehydration: 50% ethanol for 5 min., 70% ethanol for 30 min. (two changes), 90% ethanol for 30 min. (two changes) , 100% ethanol for 30 min. (two changes), absolute alcohol: amyl acetate (3:1) for 30 min., absolute alcohol: amyl acetate (2:2) for 30 min., absolute alcohol: amyl acetate (1:3) for 30 min., and amyl acetate for 30 min.
3. Critical Point Drying: For 40 minutes.
4. Mounting: Sample was mounted onto metal stub with double sided carbon tape.
5. Sputter Coating: A thin layer of gold metals over the sample was applied using an automated sputter coater. These processes take about 10 minutes. Spur coated samples were then placed under electron microscope and the surface images of the plant samples were taken at 200X magnification.

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CHAPTER 4

RESULTS

4. Results

4.1. Plant morphology:

The gross morphology of Basmati 370, its mutant *OsGAI/Sd* and *OsGAS/Sd* have been shown in Fig. 4.1. The height of wild type Basmati 370 was almost double to that of its dwarf mutant *OsGAI/Sd.* The dwarf mutant had high tillering capacity, small panicle size and slightly smaller grains. The mutant plants were photoinsensitive and healthy with broad and dark green leaves. In wild-type Basmati 370, concomitant with the change from vegetative to reproductive growth, the upper two to four internodes start to elongate in succession from base to top. Fig. 4.1.1b shows the first, second and third internodes of Basmati 370 and dwarf mutant *OsGAI/Sd*, respectively. The length of second internode of the dwarf plants was approximately half to that of Basmati 370. Basmati 370 with a height of approximately 160 cm has narrow leaves, less tiller number and longer grains as compared to that of the dwarf mutant. Panicle size and number of spikelets were less in dwarf mutant as compared to Basmati 370. The filled mature grains in Basmati 370 were longer than that in *OsGAI/Sd* (Fig. 4.1.2c). 40-45% seed sterility was also observed in the *OsGAI/Sd* mutant. In dwarf mutant, the fertile grains took more time to mature as compared to Basmati 370. The dwarf phenotype was observed even at 10 days old seedling stage (Fig. 4.1.2a). The roots of Basmati 370 were found to be more fibrous as compared to dwarf mutant (Fig. 4.1.2b). The grain L/B ratio of *OsGAI/Sd* was 4.8 while that of Basmati 370 was 6.00 (Fig. 4.1.2c and 4.1.2d).

Morphology of B-3-1 was very much similar to Basmati 370 with respect to plant height, number of tillers (average 12) and days to flowering (~125 days). However, *OsGAS/Sd* had lower grain L/B ratio (3.75) than Basmati 370 (6.00) (Fig. 4.1.3a and

4.1.3b). The plant height of *OsGAS/Sd* was approximately 110 cm at the time of maturity. Average number of tillers in *OsGAS/Sd* was 13 while days to 50% flowering were similar to Basmati 370.

4.2. Anatomy of the *OsGAI/Sd* dwarf mutant:

In order to clarify how the *OsGAI/Sd* gene functions in the internode elongation and leaf development, light microscopy of second internode, leaf sheath and fourth leaf from 75 days old plants of dwarf mutant and Basmati 370 was carried out. The tissues were fixed in FAA fixative for the comparative histological study. Before sectioning, the dehydrated tissues were embedded in paraffin wax which served as the supporting medium for the tissues. It is evident from the images taken at different magnifications (Fig. 4.2.1 and 4.2.2), that the cells in mutant plant were packed densely and their cell size was smaller than its wild counterpart. The cell length was smaller but the width was more in the dwarf mutant.

4.2.1. Anatomy of the second internode and leaf sheath:

In the longitudinal section (LS) of the second internode (Fig. 4.2.1a and 4.2.1b), the length of cells in certain layers has been reduced to half of the wild type plants. In the transverse section (TS) of second internode (Fig. 4.2.1c and d), there was a difference between the number of layers of cell in dwarf mutant (~17) and Basmati 370 (~12). Cell density (number of cells per unit area) in the internode was more in dwarf mutant compared with Basmati 370. The cell diameter was smaller in the dwarf mutant and it remained almost same for all the parenchymatous cells as compared to progressive increase in cell diameter from peripheri to the center, in case of Basmati 370 stem parenchymatous cells.

Figure 4.1.1 Comparative morphology of Basmati 370 and *OsGAI/Sd*. (a) Left: Basmati 370 (height: 160 cm), Right: *OsGAI/Sd* (height: 80 cm). (b) First, second and third internode of Basmati 370 (left) and *OsGAI/Sd* (right). (c) Second internode of Basmati 370 (left) and *OsGAI/Sd* (right). (d) Fourth leaf of Basmati 370 (left) and *OsGAI/Sd* (right).

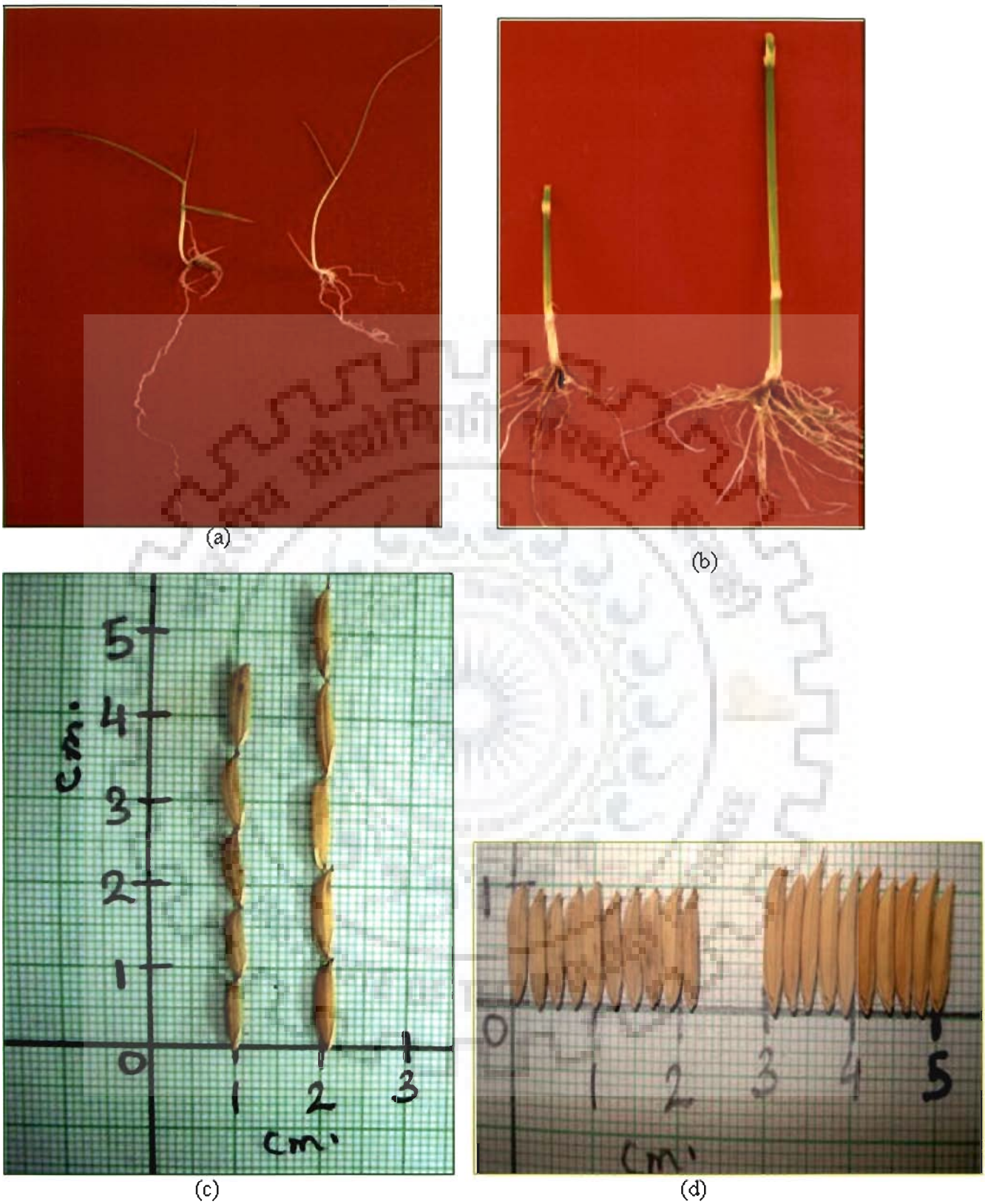
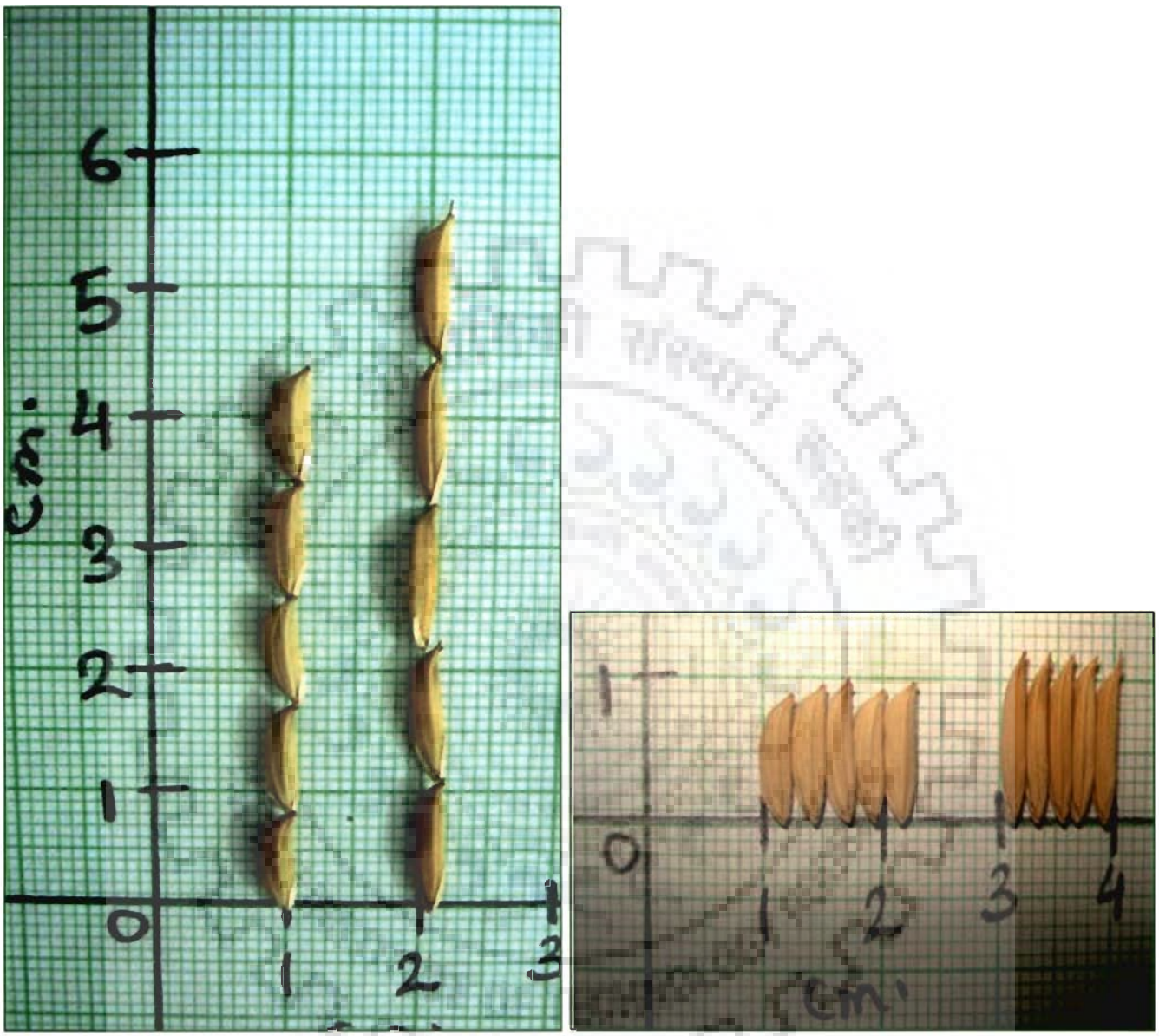


Figure 4.1.2 Comparative morphology of Basmati 370 and *OsGAI/Sd*. (a) 10 days old seedling of *OsGAI/Sd* (left) and Basmati 370 (right). (b) A culm of *OsGAI/Sd* (left) Basmati 370 (right). (c) Grain length of *OsGAI/Sd* (left) and Basmati 370 (right). (d) Grain breadth of *OsGAI/Sd* (left) and Basmati 370 (right).



(a) (b)

Figure 4.1.3 Comparative seed morphology of *OsGAS/Sd* and Basmati 370. (a) Seed length in *OsGAS/Sd* (left) and Basmati 370 (right). (b) Seed breadth of *OsGAS/Sd* (left) and Basmati 370 (Right).

The size of cells of vascular bundle of stem was found to be less in Basmati 370 and the starch grain deposition was more in the cells of *OsGAI/Sd* than Basmati 370. The TS of leaf sheath at the same magnification showed a reduction in cell length and breadth in dwarf mutant as compared to Basmati 370 (Fig. 4.2.1e and f).

4.2.2. Anatomy of the fourth leaf:

Number of cells in the leaf xylem was found to be more in dwarf mutant as compared to Basmati 370. In the dwarf mutant leaves, the cells were densely packed with chloroplasts, which gave them a dark green appearance when stained with Toluidine blue (Fig. 4.2.2a and b). The size of cells in vascular bundles of fourth leaves was longer in Basmati 370. The diameter of the bulliform cells were smaller in dwarf compared to Basmati 370 (Fig. 4.2.2c and d) in the LS of leaf blade across bulliform cells. In TS of mid-vein, the number of air-spaces in Basmati 370 was less than in the dwarf mutant (Fig. 4.2.2e and f).

4.3. Scanning Electron Microscopy (SEM):

SEM of longitudinal sections of second internodes was carried out to see the difference of surface structure of 75 days old plants of Basmati 370 and dwarf mutant *OsGAI/Sd*. Images in Fig. 4.3a and 4.3b show longitudinal sections through the vascular cells and cortex cells. Starch granules were visible inside vascular cells (shown by arrows). Deposition of starch granules was found to be more in dwarf mutant as compared to Basmati 370, which may be due to slower vegetative growth of the dwarf mutant. The cells in Basmati 370 were longer than that in *OsGAI/Sd*. Fig. 4.3c and 4.3d

show the SEM of the leaf (adaxial) epidermis of Basmati 370 and *OsGAI/Sd.* No significant differences were observed between the leaves except longer trichomes in Basmati 370 as compared to broad and short trichomes in dwarf mutant plant (shown by arrows.

4.4. Chlorophyll content:

Chlorophyll (Chl) is a photosynthetic pigment which is an essential component in plant photo system. Chl content is positively correlated with photosynthetic rate. All Chl b and most of the Chl a molecules have been shown to be light harvesting pigments. Cells with a higher Chl content can collect and transfer more light energy; consequently their photosynthetic efficiency is high. The Chl a and Chl b contents in dwarf mutant *OsGAI/Sd* were found to be 4.38 and 1.0 mg/g of leaf tissues, respectively, while in Basmati 370 the Chl a and Chl b contents were 3.86 and 0.895 mg/g of leaf tissues, respectively. These differences in chlorophyll contents are evident from Fig. 4.4 where the leaves of dwarf mutant appear to be darker green as compared to Basmati 370 under field conditions.

4.5. Response of the dwarf mutant to exogenous Gibberellic Acid (GA_3)

Observations on the percentage increase in plant height upon the foliar spray of gibberellic acid (GA_3) at 120 ppm concentration on 60 days old plants (Table 4.1) showed that the two semi-dwarf varieties PR106 and Pusa Basmati 1, having the same dwarfing gene *sd1* from Dee-Geo-Woo-Gen (DGWG) source, responded to the exogenous supply of gibberellic acid. The varieties PR106 and Pusa Basmati 1 showed 57 and 44 percent increase in plant height over the untreated plants after 10 days of application. Even the traditional tall variety Basmati 370 and one of its insertional

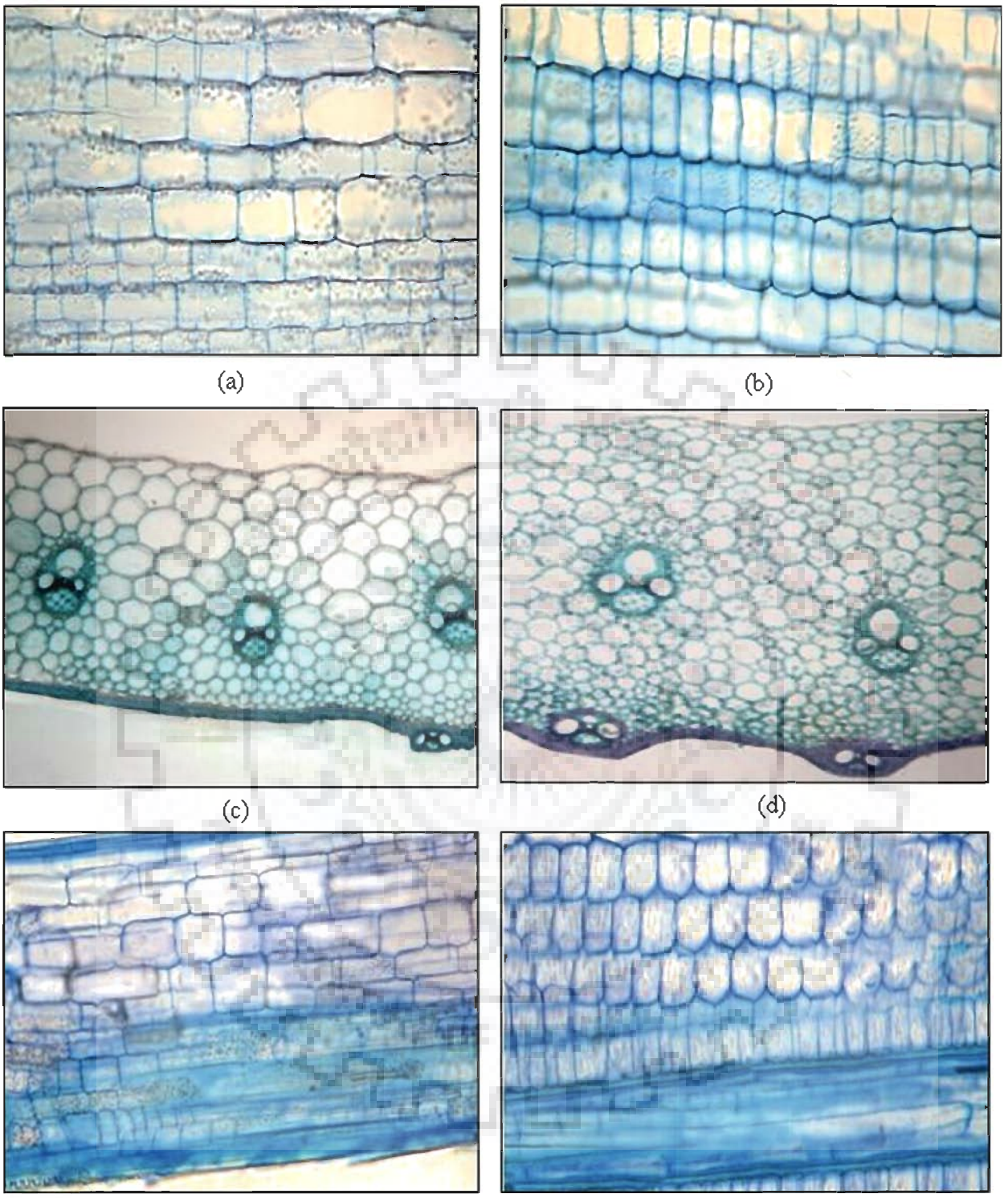


Figure 4.2.1 Comparative anatomy of Basmati 370 and *OsGAI/Sd*. (a) LS of second internode in Basmati 370 and (b) *OsGAI/Sd*, at 10X magnification. (c) TS of second internode in Basmati 370 and (d) *OsGAI/Sd*, at 10X magnification. (e) LS of leaf-sheath in Basmati 370 and (f) *OsGAI/Sd*, respectively at 40X magnification.

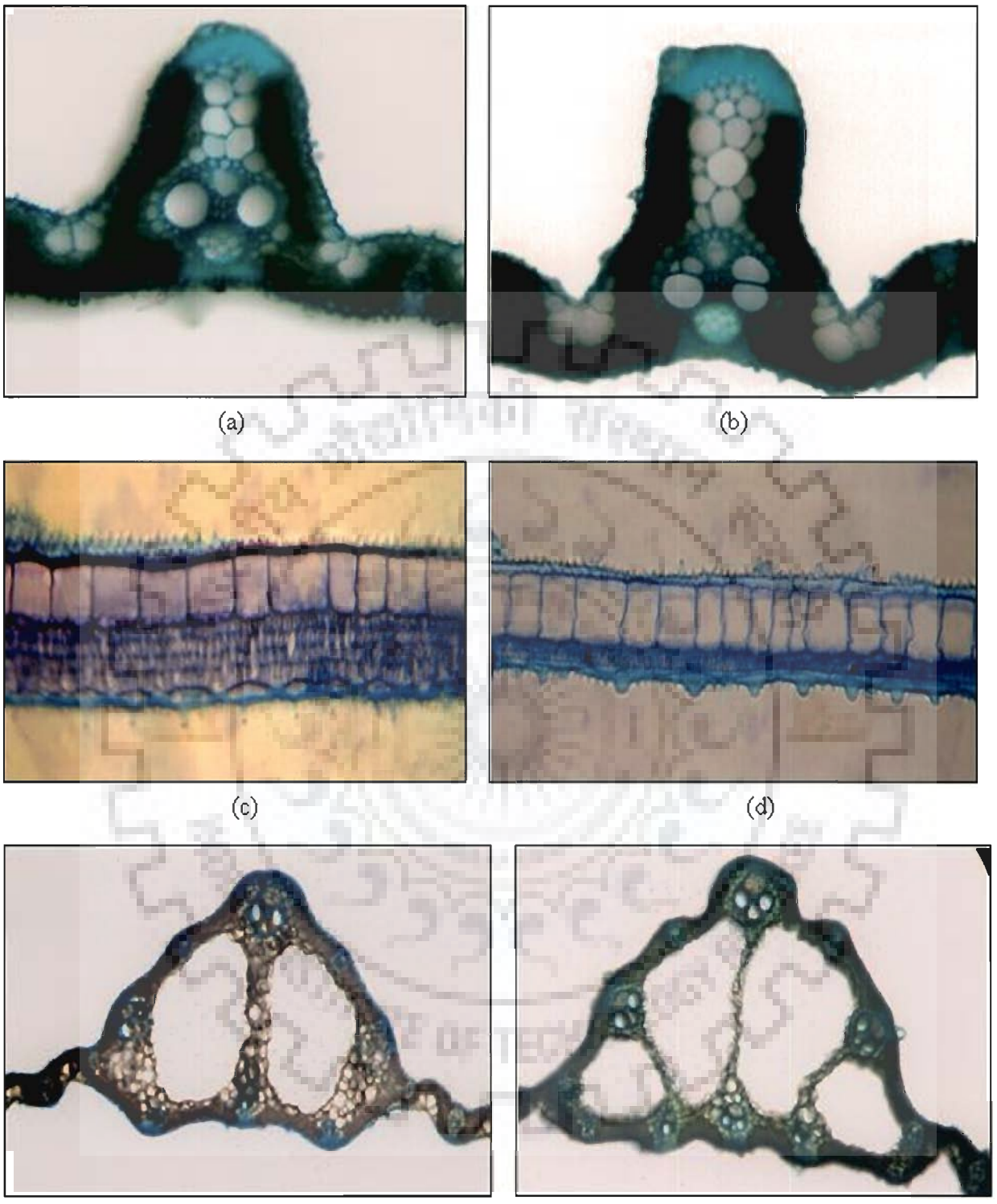


Figure 4.2.2 Comparative anatomy of Basmati 370 and *OsGAI/Sd*. (a) TS of leaf–blade of fourth leaf in Basmati 370 and (b) *OsGAI/Sd*, at 10X magnification. (c) LS of leaf blade across the bulliform cells in Basmati 370 and (d) *OsGAI/Sd* at 40X magnification. (e) TS of mid-vein in Basmati 370 and (f) *OsGAI/Sd* at 20X magnification.

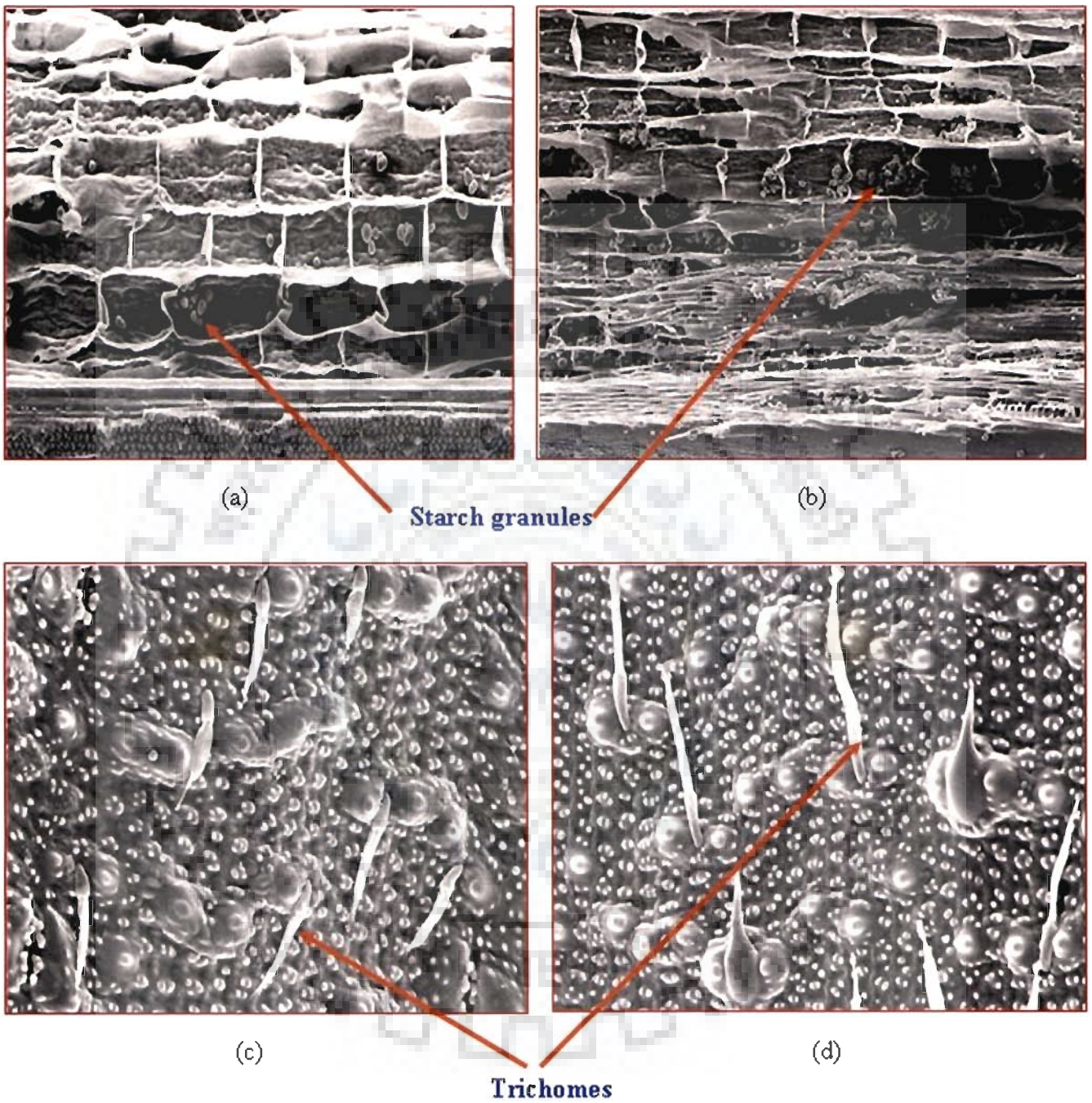


Figure 4.3 SEM of Basmati 370 and dwarf mutant *OsGAI/Sd*. (a): Surface image of longitudinal section of second internode in Basmati 370 and (b) *OsGAI/Sd*. (c): adaxial surface of the fourth leaf in Basmati 370 and (d) *OsGAI/Sd*. All the images were taken at 200X magnification.

Figure 4.4 Contrast of darker green leaves of dwarf mutant (*OsGAI/Sd*) (with high chlorophyll content) and light green leaves of Basmati 370.

Table 4.1 Plant height of different rice varieties and dwarf mutants before and after GA3 application at 120 ppm

Variety/Mutant	Average plant height (cm)		% Increase in height over control
	Before application	10 days after GA3 application	
PR106	90.75	142.5	57.01
Pusa Basmati 1	92.6	134.2	44.00
Basmati 370	110.2	144.8	31.00
OsGAS/Sd	92.4	120.1	30.00
OsGAI/Sd	65.9	72.34	9.78

mutants, *OsGAS/Sd* indicated 30 percent increase in plant height after GA_3 spray. On the other hand, plant height of *OsGAI/Sd* mutant remained almost same with only 10% increase after exogenous gibberellic acid application. This lack of response to GA clearly shows that the dwarfing mutant *OsGAI/Sd* is gibberellic acid insensitive while all the cultivars with and without *sd1* were GA_3 sensitive.

4.6. Phenotypic evaluation of the F_2 population

In order to tag the dwarfing gene, a mapping population was developed by crossing the GA-insensitive dwarf mutant *OsGAI/Sd* with a non-basmati GA-sensitive semi-dwarf cultivar PR106. Inheritance of dwarfing gene was also studied by crossing *OsGAI/Sd* with its wild type Basmati 370. The F_1 plants obtained from both the crosses were dwarf and insensitive to GA_3 spray which indicated the dominance of GA-insensitive dwarfing gene over GA-sensitive dwarf and tall phenotype. 164 F_2 plants from *OsGAI/Sd* X PR106 cross and 107 F_2 plants from *OsGAI/Sd* X Basmati 370 cross were scored for plant height and sensitivity to exogenous GA_3 spray. The percentage increase in height after GA_3 treatment among the 164 F_2 plants segregated into 115 GA-insensitive, and 49 GA-sensitive plants with a good fit with expected 3:1 ratio (Table 4.2; Fig. 4.5a). The dwarf: tall and GA-insensitive *vs.* GA-sensitive plants in F_2 plants of *OsGAI/Sd* X Basmati 370 cross were segregated in 3:1 ratio for both height and GA-sensitivity (Fig. 4.5b and 4.5c; Table 4.2), indicating the presence of dominant and monogenic mode of inheritance for the traits. All the dwarf plants were also GA_3 insensitive indicating the pleiotropic effect of the mutant controlling both the traits. Cut-off point for grouping the segregating F_2 plants for plant height and GA-responsiveness in both of the crosses was in the range of mid-parental value, i.e., for plant height it was

110 cm and for response to gibberellic acid was 30.5%. The average plant height of dwarf mutant, PR106 and Basmati 370 at flowering stage were 80cm, 110 cm, and 160 cm, respectively.

Tall (GA sensitive) and dwarf (GA insensitive) plants were found among the progenies obtained by selfing the dwarf mutant parent *OsGAI/Sd*. These tall plants were positive for *hpt* gene in PCR amplification. The GA sensitivity and plant height of these tall plants were similar to the Basmati 370.

Table 4.2 F_2 segregation for response to GA treatment and plant height in crosses of *OsGAI/Sd* with PR106 and Basmati 370

Trait	Cross (*OsGAI/Sd* X PR106)				
	Number of plants				
% increase in height after GA spray*	GA insensitive	GA sensitive	Ratio	χ^2 value at 1 *df*	
				Calculated	p<0.05
	115	49	3:1	2.08	3.84
	Cross (*OsGAI/Sd* X Basmati 370)				
	Number of plants				
Plant Height** (cm)	Dwarf	Tall	Ratio	χ^2 value at 1 *df*	
				Calculated	p<0.05
	79	28	3:1	0.06	3.84
% increase in height after GA spray*	GA insensitive	GA sensitive	3:1	2.26	3.84
	87	20			

* Cut-off point for % increase in height after GA spray in classifying GA-insensitive and GA-sensitive plants was 30.5% as that for Basmati 370.

** Cut-off point for plant height in classifying dwarf and tall plants was 110 cm at the mid parental value.

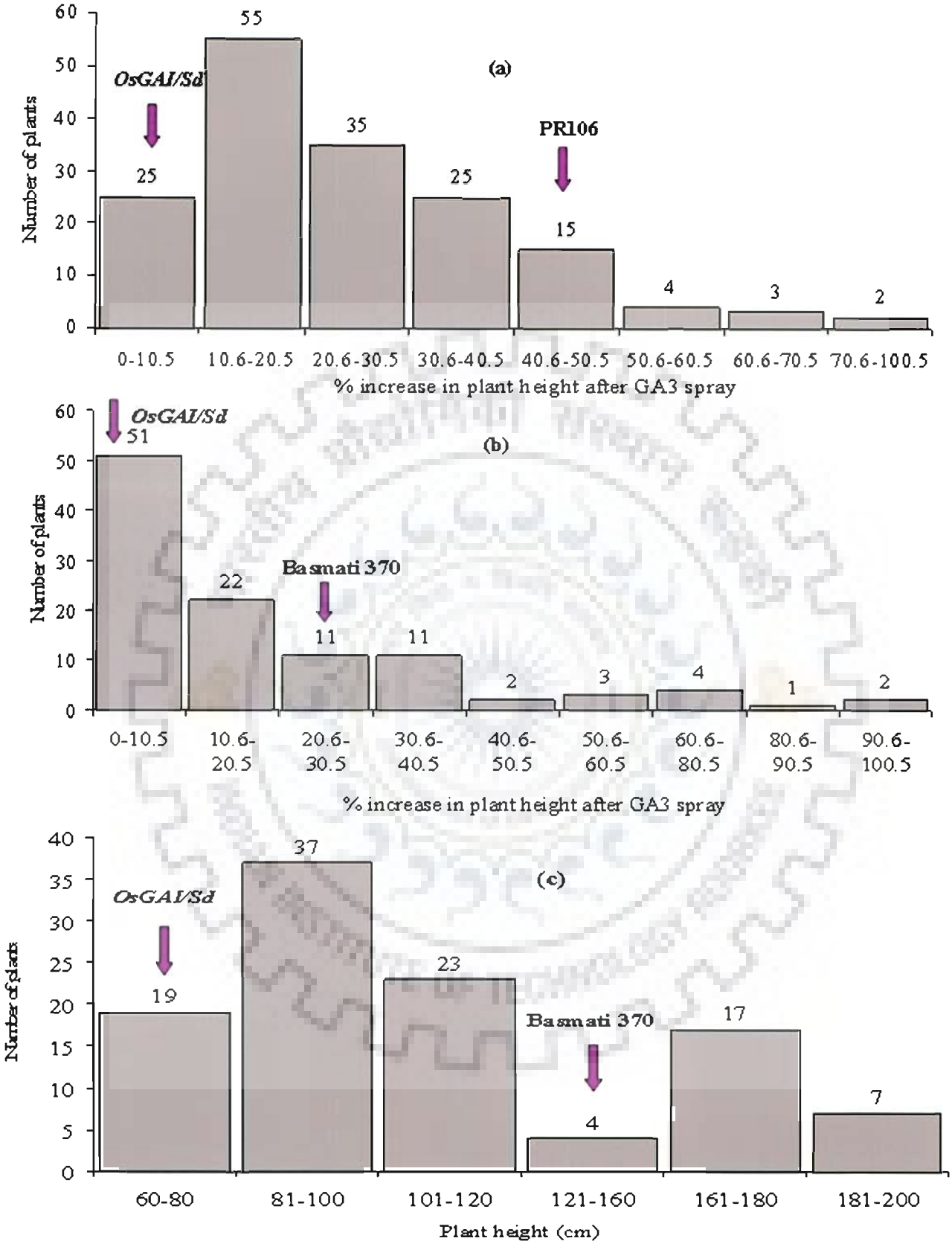


Figure 4.5 Frequency distribution of F2 plants for GA3 response in *OsGAI/Sd* X PR106 (a) and in *OsGAI/Sd* X Basmati 370 (b) and for plant height in *OsGAI/Sd* X Basmati 370 (c).

4.7. Use of Hygromycin phosphotransferase (*hpt*) gene as selectable marker:

Selfed seeds of three insertional mutants namely, B-3-1 and *OsGAS/Sd* and *OsGAI/Sd* containing *hpt* gene as the selectable marker along with wild type Basmati 370 were screened for resistance to hygromycin at different concentrations i.e., 30, 50, 60, 80 and 120 ppm. For selection of transgenic and homozygous plants, hygromycin at 80 ppm concentration was found suitable for completely inhibiting the seed germination in Basmati 370. All the seeds of insertional mutants B-3-1, *OsGAS/Sd* and *OsGAI/Sd* were resistant to hygromycin during germination (Fig. 4.6a) indicating the presence of T-DNA insertion because the *hpt* gene is inserted within the RB and LB in Hm^R*Ds* construct used for transformation. The presence of *hpt* gene in the insertional mutant *OsGAI/Sd* was also confirmed by using an oligonucleotide primer pair, based on the sequence of *hpt* gene. The primers amplified a fragment of *hpt* gene of the size of 950 bp (Fig. 4.6b) in dwarf mutant and not in Basmati 370. All the plants obtained from the hygromycin resistant seedlings were positive in PCR amplification for *hpt* gene. F_2 plants of *OsGAI/Sd* X PR106 cross were screened for *hpt* gene amplification. Out of 164 F_2 plants, 118 were found to be *hpt* positive and 46 were found negative for *hpt* gene (Fig. 4.6c; Table 4.4) showing segregation in 3:1 ratio (χ^2= 0.812, $p<0.05$) indicating single copy insertion and dominant expression of the *hpt* gene in the genome of dwarf mutant. The F_2 seeds, when grown in hygromycin at 120 ppm concentration, also segregated at 3:1 (χ^2= 0.24, $p<0.05$) for germinated (147) *vs.* ungerminated (53) seeds in petri-plates (Table 4.3).

4.8. Bulk segregant analysis (BSA):

164 F_2 plants obtained from *OsGAI/Sd* X PR106 cross were scored for plant height, sensitivity to exogenous GA_3 spray, days to 50% flowering and tiller number

(Appendix I). Normal frequency distribution for these phenotypes in F_2 population validated the population to be used in Bulk Segregant Analysis (BSA) for mapping the dwarfing gene.

Table 4.3 Germination of F_2 seeds of *OsGAI/Sd* X PR106 on 80ppm hygromycin

Germination on 80 ppm Hygromycin			χ^2 value at 1 *df*	
Germinated	Non-germinated	Ratio	Calculated	p<0.05
147	53	3:1	0.24	3.84

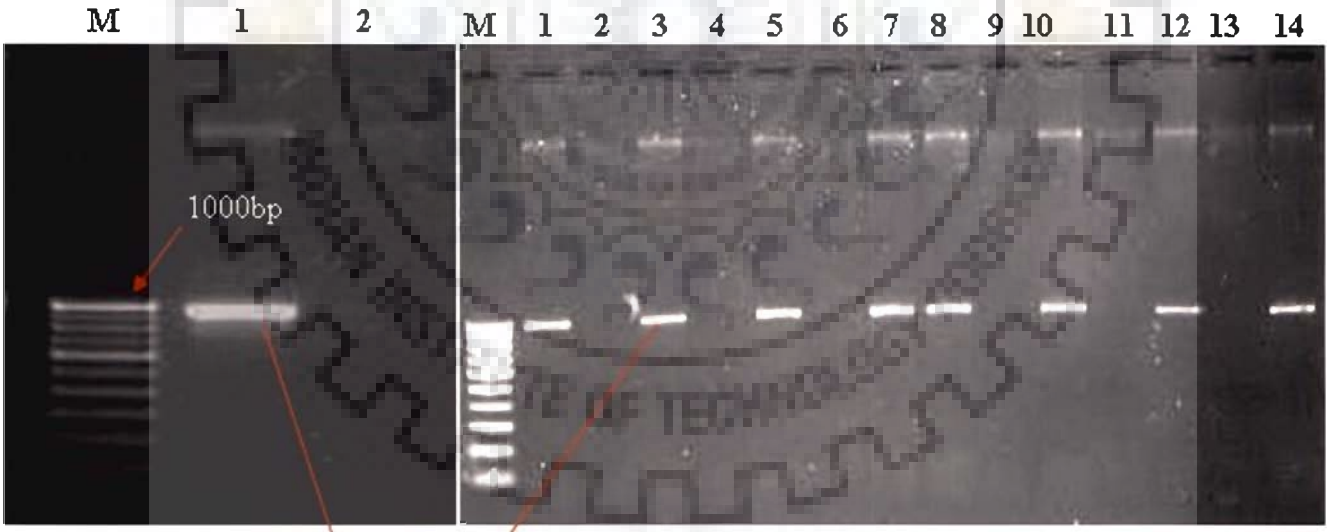
Table 4.4 PCR amplification of *hpt* in F_2 plants of *OsGAI/Sd* X PR106

hpt amplification			χ^2 value at 1 *df*	
hpt + ve	*hpt* - ve	Ratio	Calculated	p<0.05
118	46	3:1	0.812	3.84

4.8.1. Screening for parental polymorphism:

Survey for parental polymorphism was conducted for identifying the SSR markers polymorphic between the tall parent Basmati 370 and semi-dwarf parent PR106. A total of 213 SSR primers uniformly distributed across 12 rice chromosomes were selected as based on the SSR maps of rice (Temnykh *et al.*, 2001 and McCouch *et al.*, 2002). Of the 213 SSR primers tested, 95 (44.60%) exhibited polymorphism between Basmati 370 and PR106. Chromosome-wise location of polymorphic markers between the parental lines is presented in Fig. 4.7.

(a)



(b) 950 bp *hpt* (c)

Figure 4.6 Characterization of insertional mutants for hygromycin resistance.
(a): Germination of seeds in petri plates at 80ppm conc. of hygromycin.
Basmati 370 (left) and *OsGAI/Sd* (right).
(b): PCR amplification of 950 bp fragment of *hpt* gene in *OsGAI/Sd* (lane1) and Basmati 370 (lane2).
(c): A representative gel picture of the amplification of *hpt* gene in F2 plants of *OsGAI/Sd* X PR106 cross (lane 3-14).

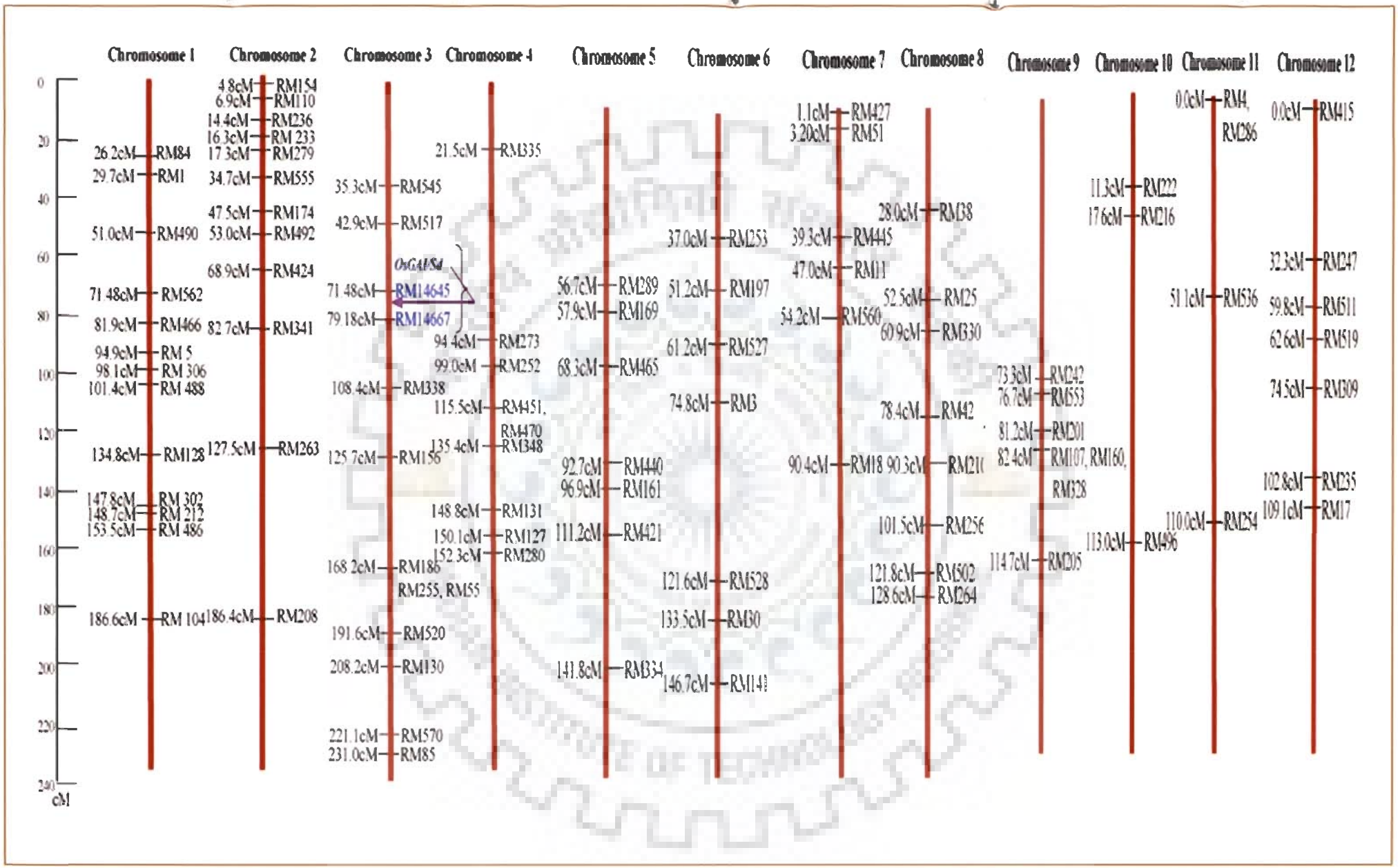


Figure 4.7 SSR markers of 12 chromosomes of rice showing polymorphism between Basmati 370 and PR106. Location of dwarfing gene on chromosome 3 is marked by blue colour (arrow).

4.8.2. Identification of SSR markers linked to dwarfing gene through Bulked Segregant Analysis (BSA):

A total of 95 SSR markers showing polymorphism between the mutant and PR106 were used for mapping the dwarfing gene through BSA. Positive bulk was composed of eight homozygous *hpt* positive, dwarf and gibberellic acid insensitive F_3 progenies while negative bulk included nine *hpt* gene negative, tall, and gibberellic acid sensitive F_3 progenies. In BSA, the SSR markers RM14645 and RM14667, located on chromosome 3 at a physical distance of 7148123bp and 7633850bp (IRGSP, 2005), respectively, showed polymorphism between bulks (Fig. 4.8 and Fig. 4.9). These markers were further used to genotype the individual F_3 progenies constituting the *hpt* positive and *hpt* negative bulks (Fig. 4.8.1 and Fig. 4.9.2). The amplification pattern obtained clearly indicated the association of these markers with *hpt* gene which has been used as a selectable marker in the T-DNA used for the insertional mutagenesis of Basmati 370 in the present study.

4.8.3. Mapping of the dwarfing gene by genotyping of F_2 population:

A total of 164 individual F_2 plants were used for genotyping with the two microsatellite markers viz., RM14645 and RM14667 located on chromosome 3. Co-segregation analysis of individual markers using the marker and mutant genotype of 164 individual F_2 plants was carried out with the help of recombination frequency between marker and *hpt* gene at each locus (Table 4.5). Out of 164 F_2 plants, the linkage analysis with the SSR marker RM14645 and RM14667 identified 2 and 11 recombinants, respectively. Based on the data on recombination frequency, the marker RM14645 and

RM14667 were mapped at distance of 1.21cM and 6.49cM, respectively from the T-DNA insertion containing *hpt* gene (Fig. 4.10) on chromosome 3.

4.9. Southern hybridization:

The genomic DNA was digested with *EcoRV, EcoRI, DraI and HindIII,* Southern blotted and probed with α-P^{32} dCTP radio labeled 950 bp fragment of T-DNA specific *hpt* gene. The analysis detected *hpt* gene fragments which were of different sizes for each restriction enzymes used and thus allowed the detection of T-DNA copy number. The hybridization patterns have been shown in Fig. 4.11 which indicated single T-DNA insertion in the dwarf mutant *OsGAI/Sd.* The segregation of positive verses negative plants in PCR amplification for the *hpt* gene in F_2 plants of *OsGAI/Sd* X PR106 cross in 3:1 ratio also confirmed the single copy insertion of T-DNA in *OsGAI/Sd.*

4.10. Thermal asymmetric interlaced PCR (TAIL-PCR):

TAIL-PCR involves a series of reactions that are intended to map T-DNA insertion within a genome. The main components of the three reactions are the AD (Arbitrary Degenerate) primers, nested T-DNA border primers, and DNA from the T-DNA insertional line that is to be mapped for flanking sequences. AD primers are degenerate primers that anneal throughout the genome. The border primers are specific for the left and right borders of the T-DNA. From the primary reaction to the tertiary, the nested border primers get closer to the edge of the T-DNA. That is why a 'shift' is visible when running a gel with the secondary and tertiary reactions next to each other. In the primary reaction, one low-stringency PCR cycle is run to create one or more annealing sites for the AD primers near the target sequence. A specific product is then preferentially amplified over nonspecific ones by interspersion of two high-stringency PCR cycles with

Figure 4.8 Identification of putative linked SSR marker RM14645 for dwarfing gene *OsGAI/Sd* through Bulked Segregant Analysis (BSA): Lane M: 100bp ladder, 1: Basmati 370, 2: PR106, 3: *hpt* positive bulk and 4: *hpt* negative bulk.

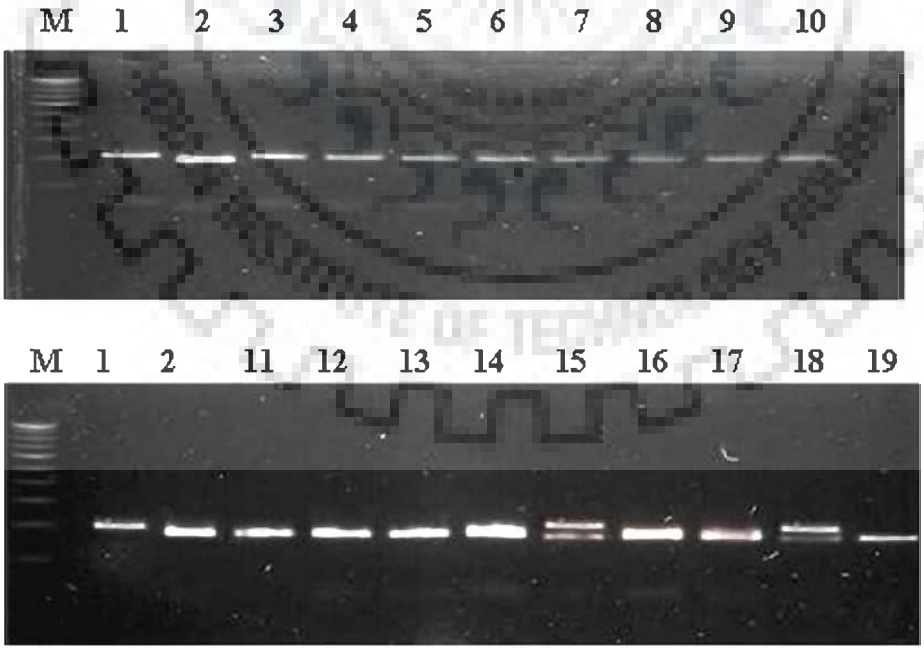


Figure 4.8.1 Screening of *hpt* positive and negative F3 progenies constituting the bulks using RM14645, Lane M: 100bp ladder, 1: Basmati 370, 2: PR106, lanes 3-10: *hpt* positive lines, lanes 11-19: *hpt* negative F3 progenies.

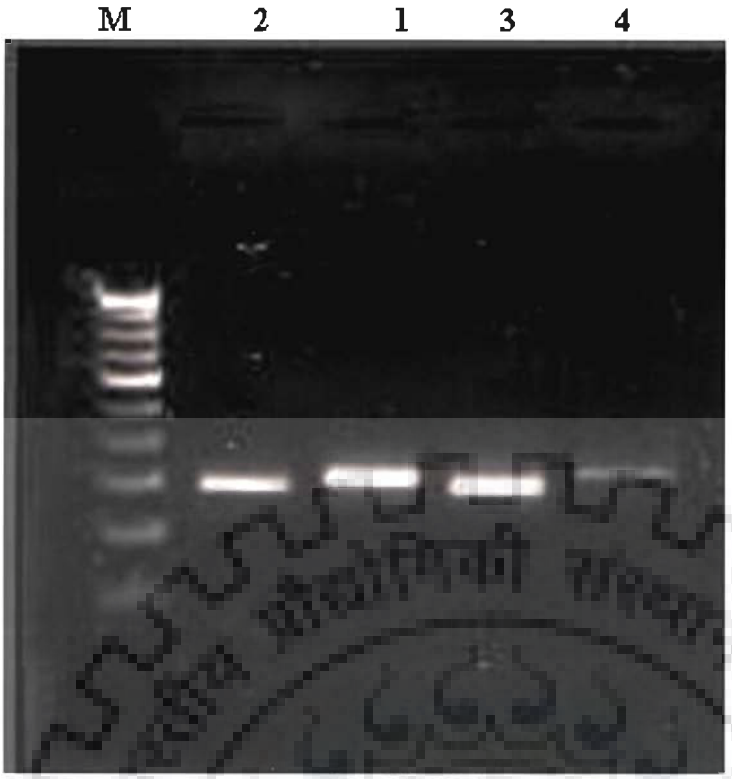


Figure 4.9 Identification of putative linked SSR markers for dwarfing gene *OsGAI/Sd* through Bulked Segregant Analysis (BSA): Lane M: 100bp ladder, 1: Basmati 370, 2: PR106, 3: *hpt* positive bulk and 4: *hpt* negative bulk.

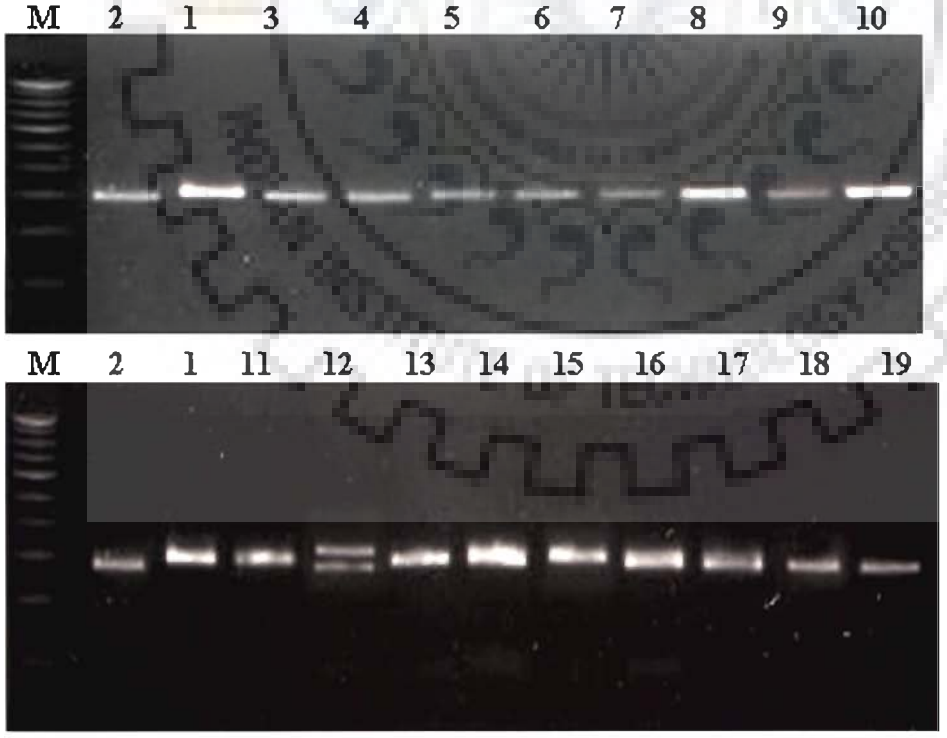


Figure 4.9.1 Screening of *hpt* positive and negative F3 progenies constituting the bulks using RM14667. Lane M: 100bp ladder, 1: Basmati 370, 2: PR106, lanes 3-10: *hpt* positive lines, lanes 11-19: *hpt* negative F3 progenies.

Table 4.5 Recombination frequency in 164 F_2 plants with two linked markers

Marker	Basmati 370 allele but *hpt* -ve	Homozygous for PR106 allele but *hpt* +ve	Total number of recombinants	Recombination frequency (%)
RM14645	0	2	2	1.21
RM14667	2	9	11	6.49

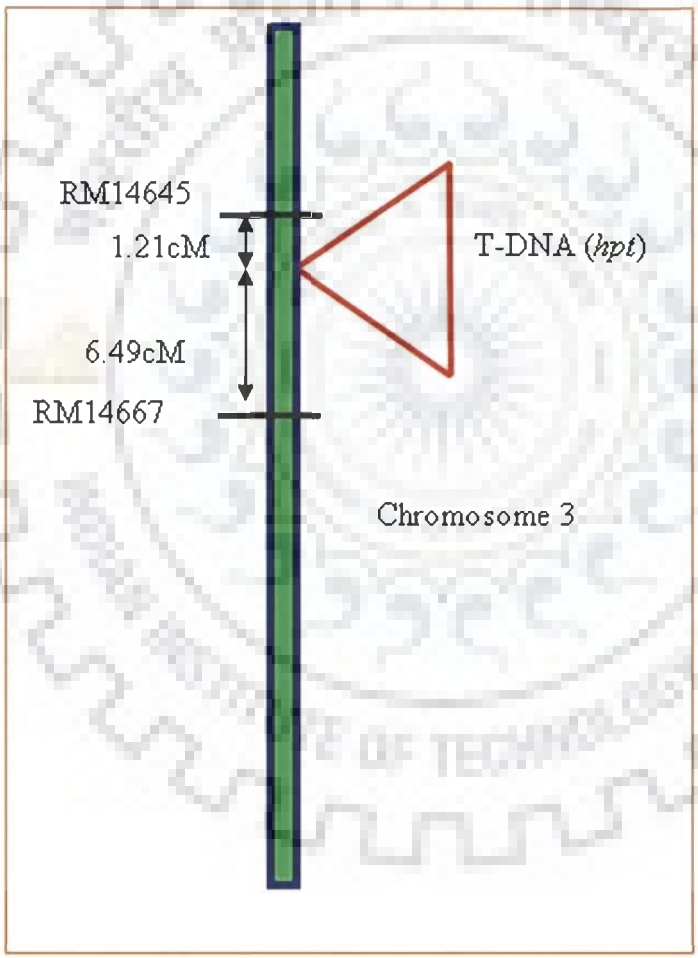


Figure 4.10 Linkage map of chromosome 3 showing the genetic distance of linked markers RM14645 and RM14667 in relation to T-DNA insertional site of *OsGAI/Sd* mutant.

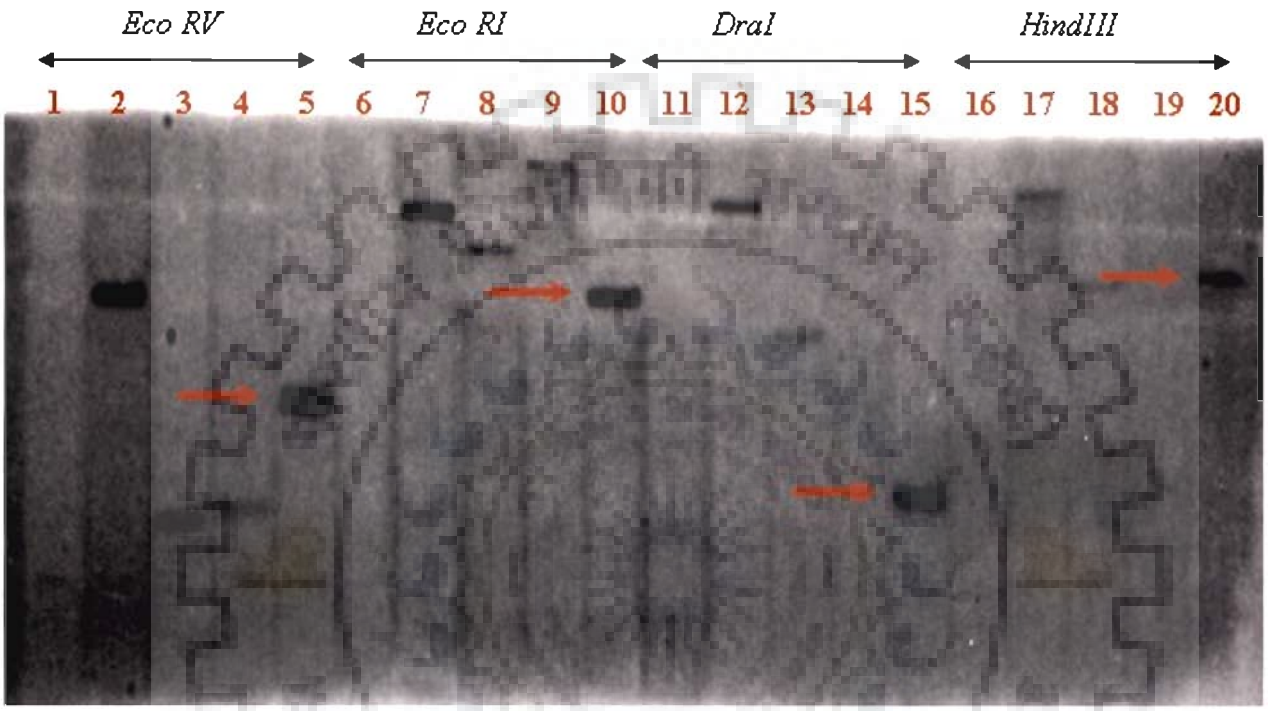


Figure 4.11 Southern hybridization of Basmati 370 insertional mutants, including dwarf mutant (*OsGAI/Sd*) using four different restriction enzymes and *hpt* as labeled probe. *EcoRV*: Lane 1: Basmati 370, Lane 5: *OsGAI/Sd* (Arrow). *EcoRI*: Lane 6: Basmati 370, Lane 10: *OsGAI/Sd* (Arrow). *DraI*: Lane 11: Basmati 370, Lane15: *OsGAI/Sd*. *HindIII*: Lane 16: Basmati 370, Lane 20: *OsGAI/Sd* (Arrow).

one reduced-stringency PCR cycle. The nested PCR amplifications help to achieve higher specificity.

Three T-DNA specific right border primers (RB1, RB2 and RB3) were used (Muskett *et al.*, 2003) for TAIL-PCR to identify flanking genomic sequences. Sequences flanking the T-DNA were amplified from total genomic DNA by TAIL-PCR as per the protocol given by Liu *et al.* (1995) (Fig. 2.10, Table 3.1), using three arbitrary degenerate (AD) primers previously designed for *Arabidopsis* (Liu *et al.*, 1995; Liu and Whitter, 1995). The fidelity of TAIL-PCR amplification was judged by the appearance on 1.2% agarose gels of secondary and tertiary PCR by the difference of 30-50 nucleotides as expected from the location of nested T-DNA specific primers. The size of the tertiary TAIL-PCR products ranged from 350bp to 500bp (~500 bp with AD1, ~450bp with AD2 and ~350bp with AD3) (Fig. 4.12).

Amplified flanking sequences from tertiary TAIL-PCR from RB3 and AD1, AD2 and AD3 were run on 1.2% agarose gel, eluted and purified using the Qiagen QIAEXII gel elution kit (USA). The PCR products were eluted in 30μL of water. About 80 ng of the purified PCR-amplified DNA was used for cycle sequencing with the T-DNA specific RB3 primer (5'-TATTCGGGCCTAACTTTTGGTGTG-3'; T_m=57.4^{0}C) under the conditions specified by the manufacturer (Applied Biosystems, Foster City, CA.). The sequencing results with AD1, AD2 and AD3 showed the 484bp, 456bp and 342bp sequences, respectively. The sequences then used to query the NCBI Gene Bank of *Oryza sativa* japonica cv. Nipponbare nucleotide sequence database by using BLASTN and BLASTX programs (Altschul *et al.*, 1990, 1997). The efficiency of the sequencing reaction was checked by aligning these three sequences by multiple sequence alignment

tool. The similarity percentage between the aligned sequences ranged from 75% to only upto 92% which may be due to difference in the length of the fragments sequenced and different sequencing errors.

4.10.1. Genomic location of insertion sites

Assigning location and function to the T-DNA sequence is an essential step. The similarity search of the flanking sequences (AD1-RB3, AD2-RB3 and AD3-RB3) using BLAST was done. All showed significant alignment with the *Oryza sativa* japonica cv. Nipponbare with only a single hit at chromosome 3 (Fig. 4.13). Similarly, 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 the cultivars.

No results were obtained using primers based on left border (LB) of the T-DNA. This may be because of the loss or modification of T-DNA left border. After doing BLASTN with whole sequence of rice Nipponbare genome in NCBI database, the T-DNA insert was found to present on chromosome 3 (Fig. 4.15) in the gene region, namely, Anaphase Promoting Complex/Cyclosome (APC/C) subunit having gene bank accession number Os03g0236900 spanning 7178921 to 7187583 bp. The main features of corresponding mRNA and protein sequences searched have been given in section 4.13.

4.11. Genome walking:

In order to know the flanking sequence of the T-DNA, PCR genome walking was also performed on the dwarf mutant and Basmati 370, prior to the results obtained through TAIL-PCR. But, in spite of digesting the genomic DNA of the dwarf mutant with four different enzymes i.e. *EcoRV*, *DraI*, *RsaI* and *HpaI*, no specific DNA sequences were amplified even up to tertiary reactions. Choice of restriction enzyme is an important

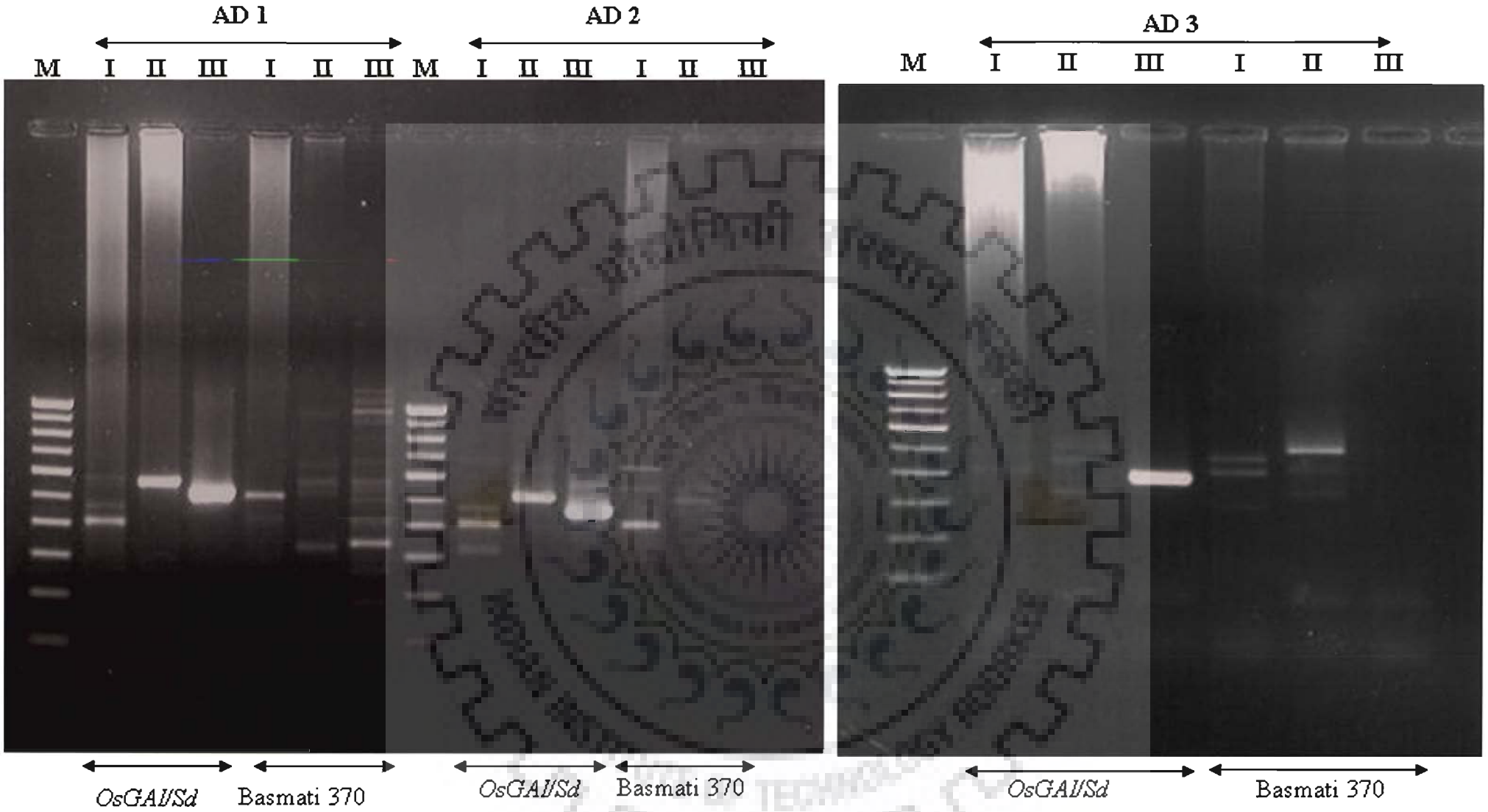


Figure 4.12 TAIL-PCR products amplified from T-DNA insertional dwarf mutant. Lane: M=100bp DNA ladder; I, II, and III: products of primary, secondary and tertiary reactions, respectively.

BLAST result (AD1-RB3)

Subject:*Oryza sativa* (japonica cultivar-group) genomic DNA, chromosome 3, Score = 737 bits (399), Expect = 0.0, Identities = 447/468 (95%), Gaps = 12/468 (2%), Strand=Plus/Minus

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Sbjct  7180174  TCTCTCCTCCGCTTGGGATTTTTCTGCGCTGCGGCGGCGGTGGTGGGCGGCGCTGCTGGA  7180115

Query  73       GGGAAACCGAATTTTGTATCCCACATTCGCCACTAGGGTTCGCGGCGGAGAGTGGTGGTT  132
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Sbjct  7180114  GGGAAACCGAATTTTGTATCCCACATTCGCCACTAGGGTTCGCGGCGGAGAGTGGTGGTT  7180055

Query  133      TTTGGAAATTTCTCACGGGGAAAAAAAAATGGAGTCCCGACCCGCCGCGGATCGCACCAG  192
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Sbjct  7180054  TTTGGAAATTTCTCACGGGGAAAAAAAAATGGAGTCCCGACGCGCCGCGGATCGCAGCAA  7179995

Query  193      GTGATCGATGGATGGAACGTGCAAGCAGTATGGCTTATGGGGCCACGGGCCCACGGCCCA  252
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Query  253      CAACATATGCAAACGAACAAGAAAGCTTGTATAGGAGAGGACACACGGGCCAGGCCCATC  312
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Query  313      ATCTGGCCGGATTCGGATTCTACCTCTGTGAAATTGCGATCAAAACCTACCGGAAATGTA  372
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Query  373      TAAGATTCCGTGGGTAT-CAAATGGTTGATTTTGGCCAGCTTACATTGACGTCTCTT-AC  430
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Query  431      CCTTAGCTCGTCCCGTGTTTGAATTTACTTTCCTATTATTATAAACAC  478
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Sbjct  7179761  C-TTAG-TCGTCCC-TGTTTGAATTTACTTTTCTATTATTATTAACAC  7179717
```

BLAST result (AD2-RB3)

Subject: *Oryza sativa* (japonica cultivar-group) genomic DNA, chromosome 3.Score = 632 bits (342), Expect = 6e-180. Identities = 391/414 (94%), Gaps = 5/414 (1%). Strand=Plus/Minus

```
Query  30       TCTCTCCTCCGCTTGGGATTTTTCTGCGCTGCGGCGGCGGTGGTGGGCGGCGCTGCTGGA  89
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Sbjct  7180174  TCTCTCCTCCGCTTGGGATTTTTCTGCGCTGCGGCGGCGGTGGTGGGCGGCGCTGCTGGA  7180115

Query  90       GGGAAACCGAATTTTGTATCCCACATTCGCCACTAGGGTTCGCGGCGGAGAGTGGTGGTT  149
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Sbjct  7180114  GGGAAACCGAATTTTGTATCCCACATTCGCCACTAGGGTTCGCGGCGGAGAGTGGTGGTT  7180055

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Sbjct  7180054  TTTGGAAATTTCTCACGGGGAAAAAAAAATGGAGT-CCCGACGCGCCGCGGATCGCAGCA  7179996

Query  209      GGTGATCGATGGATGGAACGTGCAAGCAGTATGGCTTATGGGCCACGGGCCCACGGCCCA  268
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Sbjct  7179995  AGTGATCGATGGATGGAACGTGCAAGCAGTATGGCTTATGGGCCACGGGCCCACGGCCCA  7179936

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Sbjct  7179935  CAACATATGCAAACGAACCAGAAAGCCTGTATAGGAGAGGACACACGGGCCAGGCCCATC  7179876

Query  329      ATCTGGCCGGATTCGAAATCTACCTCTGTGAAATTGCAATCAAAACCTACGGAAAGGTAT  388
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Sbjct  7179875  ATCTGGCCGGATTCGGAATCTACCTCTGTGAAATTGCGATCAAAACCTACGGAAATGTAT  7179816

Query  389      GGAGTGCG-GCGCACCCACACTGTTGATTTGGCCAGTTTACATTGACTTTCTTC  441
                 || | || | | |  || | |||||||| ||||| |||||||||| ||||||
Sbjct  7179815  AGATTTCGTG-GTATACAAA-TGTTGATTTTGCCAGCTTACATTGACGTTCTTC  7179764
```

BLAST result (AD3-RB3)

Subject: *Oryza sativa* (japonica cultivar-group) genomic DNA, chromosome 3. Score = 475 bits (257), Expect = 7e-133. Identities = 302/324 (93%), Gaps = 2/324 (0%). Strand=Plus/Minus

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Query  14       TCTCTCCTCCGCTTGGGATTTTTCTGCGCTGCGGCGGCGGTGGTGGGCGGCGCTGCTGGA  73
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Query  74       GGGAAACCGAATTTTGTATCCCACATTCGCCACTAGGGTTCGCGGCGGAGAGTGGTGGTT  133
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Sbjct  7180114  GGGAAACCGAATTTTGTATCCCACATTCGCCACTAGGGTTCGCGGCGGAGAGTGGTGGTT  7180055

Query  134      TTTGGAAATTTCTCACGGGG----------GGGATTCCCAACCCCCCGCGAATCCCACCAG  193
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Sbjct  7180054  TTTGGAAATTTCTCACGGGGAAAAAAAAAATGGAGTCCCGACGCGCCGCGGATCGCAGCAA  7179995

Query  194      GGGATCAATGGATGGAACGTGCAACCATTATGGTTTATGGGCCACGGGCCCACGGCCCAC  253
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Sbjct  7179994  GTGATCGATGGATGGAACGTGCAAGCAGTATGGCTTATGGGCCACGGGCCCACGGCCCAC  7179935

Query  254      AACATATGCAACCAAACCAAAAAGCCTGTATAGGAAG-GGACACACGGGCCAGCCCCATC  312
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Query  313      ATCTGGCCGGATCCGAAATCTACC  336
                |||||||||||| || ||||||||
Sbjct  7179875  ATCTGGCCGGATTCGGAATCTACC  7179852
```

Figure 4.13 Alignment of the AD1-RB3, AD2-RB3 and AD3-RB3 T-DNA flanking sequencing with the of *Oryza sativa* japonica cv. Nipponbare using BLASTN.

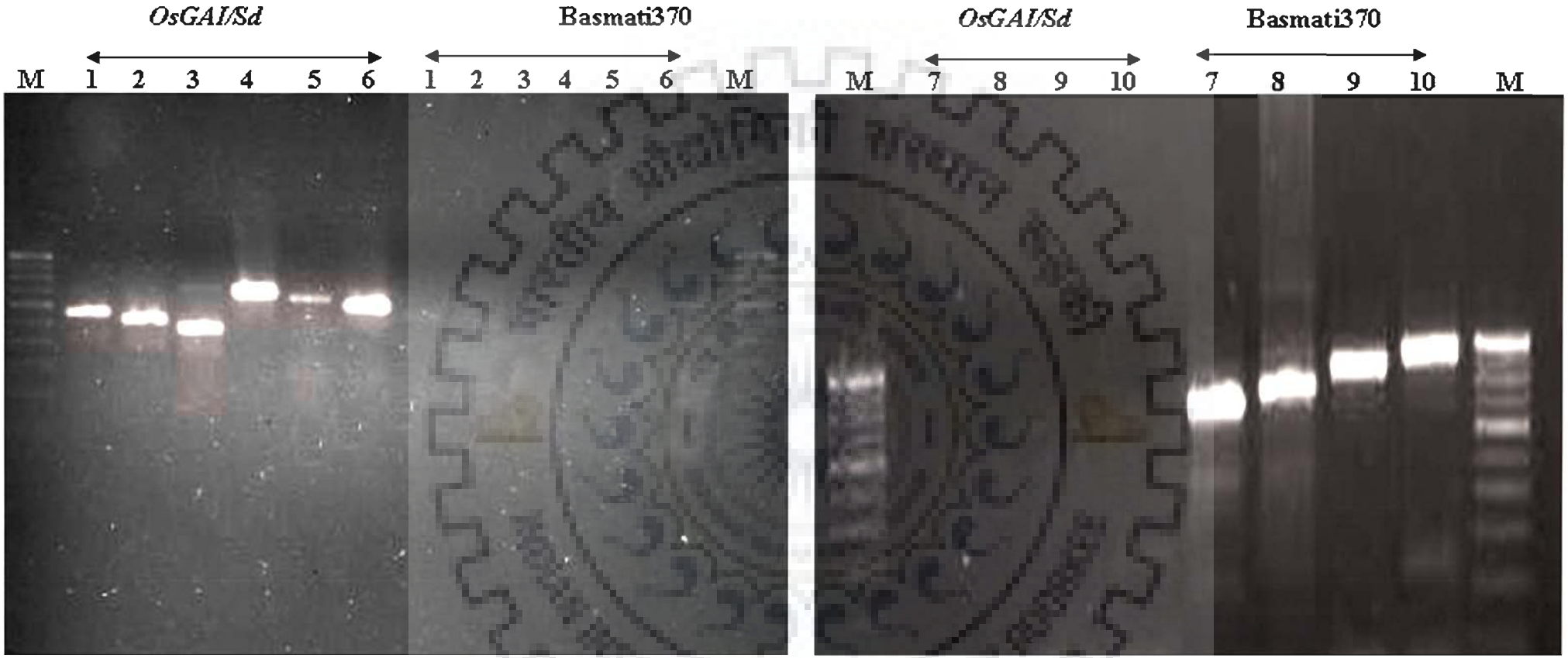


Figure 4.14 Amplification with gene specific primer and T-DNA specific primers using different primer combinations in Basmati 370 and *OsGAI/Sd*. M= 100bp ladder, Lanes: 1-10: T-DNA specific primer and gene specific primer combinations. See Table 4.6 (Sl.No. 1-10) for primer lanes.

feature that has an effect on the size of flanking sequence to be identified. This is based on the location of the restriction sites flanking to the T-DNA specific primer sequence. The failure of the genome walking experiment was mainly due the choice of restriction enzymes used for digesting the genomic DNA in the *OsGAI/Sd*. In this study, the hexacutter and tetra cutter restriction enzymes were used which generated blunt ends of the genomic DNA. The restricted fragments were ligated with the genome walker adaptor which was provided with manufacturer's (CLONTECH) kit. After getting the sequence of flanking region of the T-DNA by TAIL-PCR, presence of the four restriction sites *EcoRV*, *DraI*, *RsaI* and *HpaI* were searched by NEB cutter software. It was found that the restriction sites were either very far away (for *RsaI* site) upstream (upto 2 Kb) of the T-DNA specific primer or there were no restriction sites available for the *EcoRV*, *DraI* and *HpaI* to digest the same genomic region upstream of the insertion site in dwarf mutant.

4.12. Validation of the insertion at chromosome 3:

In order to validate the genomic location of the T-DNA insertion at 7180174th bp position on chromosome 3, two sets of nested gene specific forward and reverse primers flanking the insertion site were designed using Prime3 software based on the genomic sequence of the candidate gene from NCBI database (Table 4.6). The primers were designed in such a way that they should amplify about 980 bp sequences in the wild type Basmati 370. The location of forward (DTF1 and DTF2) and reverse (DTR1 and DTR2) gene specific primers in the genomic sequence has been shown in Fig. 4.15 and Fig. 4.19. The nested T-DNA specific right border primers (RB1, RB2 and RB3) which have been used in TAIL-PCR were used with gene specific primers in all possible combinations. Gel picture of the amplified product using these primer combinations in dwarf mutant

OsGAI/Sd and Basmati 370 have been given in Fig. 4.14. The Table 4.6 shows the expected product size in Basmati 370 and dwarf mutant in PCR by using combinations of gene specific primer with T-DNA specific primers in Basmati 370 and dwarf mutant.

Desired lengths of fragments were amplified using the combination of nested forward primers with the T-DNA specific primers in case of *OsGAI/Sd* but absent in Basmati 370. Moreover, desired fragments were amplified using gene specific nested forward and reverse primers in Basmati 370 but absent in dwarf mutant. No amplification in *OsGAI/Sd* was due to the insertion of about 10 Kb T-DNA fragment at 7180174th position on chromosome 3 which can not be amplified in normal PCR. While deciding the length of the desired fragment to be amplified, the base pair lengths to be amplified with T-DNA primers were also added.

Table 4.6 Primer combination and PCR product expected size in Basmati 370 and *OsGAI/Sd*

Sl. No.	Primer combination	Expected size in Basmati370 (bp)	Expected size in *OsGAI/Sd* (bp)
1	DTF1+RB1	-	600
2	DTF1+RB2	-	562
3	DTF1+RB3	-	517
4	DTF2+RB1	-	657
5	DTF2+RB2	-	617
6	DTF2+RB3	-	572
7	DTF1+DTR1	712	>10kb
8	DTF1+DTR2	768	>10kb
9	DTF2+DTR1	870	>10kb
10	DTF2+DTR2	926	>10kb

One combination of gene specific primer and T-DNA specific primer (DTF1+RB2) was also screened in the 164 F_2 plants (*OsGAI/Sd* X PR106) for analyzing the segregation of T-DNA insertion in the population. It was found that the ratio of number of plants positive (117) *vs.* negative (47) in PCR amplification with DTF1+RB2

primer combination was 3:1 (χ^2:1.17, $p<0.05$). Fig. 4.16 is one of the gel photographs of the PCR amplification with DTF1+RB2 primer combination in the F_2 population.

4.13. Transcription analysis:

To examine the expression of the GA-insensitive dwarfing gene present in different tissues of *OsGAI/Sd* mutant, reverse transcription PCR (RT-PCR) analysis was performed with total RNA isolated from roots and shoots. Seeds of Basmati 370 and *OsGAI/Sd* were germinated for 10 days on moistened filter paper in sterile petri-plates. Roots and shoots were collected into liquid nitrogen and stored at -80^{0}C prior to use. The *iRIS*® kit supplied from IHBT, Palampur, India was used for RNA isolation.

The transcript of *OsGAI/Sd* gene was detected in shoot as well as in roots (Fig. 4.17). To investigate the physiological changes in gene expression, the relative expression of a target gene versus a reference gene is necessary. For the purpose of uniform loading of RNA samples and equal efficiency of enzymes involved in RT-PCR reactions, use of the non-regulated housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), albumin, actins, tubulins, cyclophilin, 18S rRNA and 28S rRNA is needed as a reference gene (Kim *et al.*, 2003). Actin gene (Os03g50890) in the present study was selected as reference gene for RT-PCR based on orthology search in rice and other plant species using BLASTN programme. One set of primer (DRTF and DRTR) pair for RT-PCR was designed using PRIMER3 software on exonic regions (shown in the cDNA sequence of the candidate gene in Fig. 4.20). The primer designed was based on the mRNA sequence of the candidate gene obtained from the NCBI database.

The cDNA was synthesized and RT-PCR was set up using the actin as the reference gene. A fragment of 350bp was amplified using the mRNA specific primers in the wild type Basmati 370, whereas amplification was obtained using cDNA of dwarf mutant. 200bp fragment of actin gene was amplified in every RT-PCR indicated the uniformity in loading of RNA samples and equal efficiency of enzyme reactions in the RT-PCR. Lack of any amplification indicated the loss of expressions of wild type gene in the dwarf mutant *OsGAI/Sd* but expression of this gene was present in both root and shoots of Basmati 370 (Fig. 4.18).

4.14. Salient characteristics of the candidate gene in *Oryza sativa* cv. Nipponbare:

The salient features of the candidate gene were determined from all the information present in NCBI database. The candidate gene encoding APC/C subunit contains 16 exons and 15 introns. The T- DNA insertion was found to be at the second exon at 7180174^{h} bp position on chromosome 3 (Fig. 4.15 and Fig. 4.19). In the KEGG link (NCBI) the candidate gene (Os03g0236900) showed its orthology to sixth subunit of APC/C complex. The 8.6 Kb length of the candidate gene encodes by 728 amino acid protein containing a conserved tetratricopeptide repeat (TPR) domain. The molecular weight of the encoded protein from Unipro database search was found to be 80.638 kDa. The TPR domain typically contains 34 amino acids as the consensus sequence which is found in a variety of organisms including bacteria, cyanobacteria, yeast, fungi, plants, and humans in various sub cellular locations. Multiple sequence alignment of different TPR domain containing amino acid sequences PhSPY (locus id-Y17720) in petunia, LeSPY (AJ312093) in tomato, AtSPY (ATU62135) in *Arabidopsis*, HvSPY (AF035820) in barley and APC6 (NM_001056026) in rice was performed using ClustalW multiple

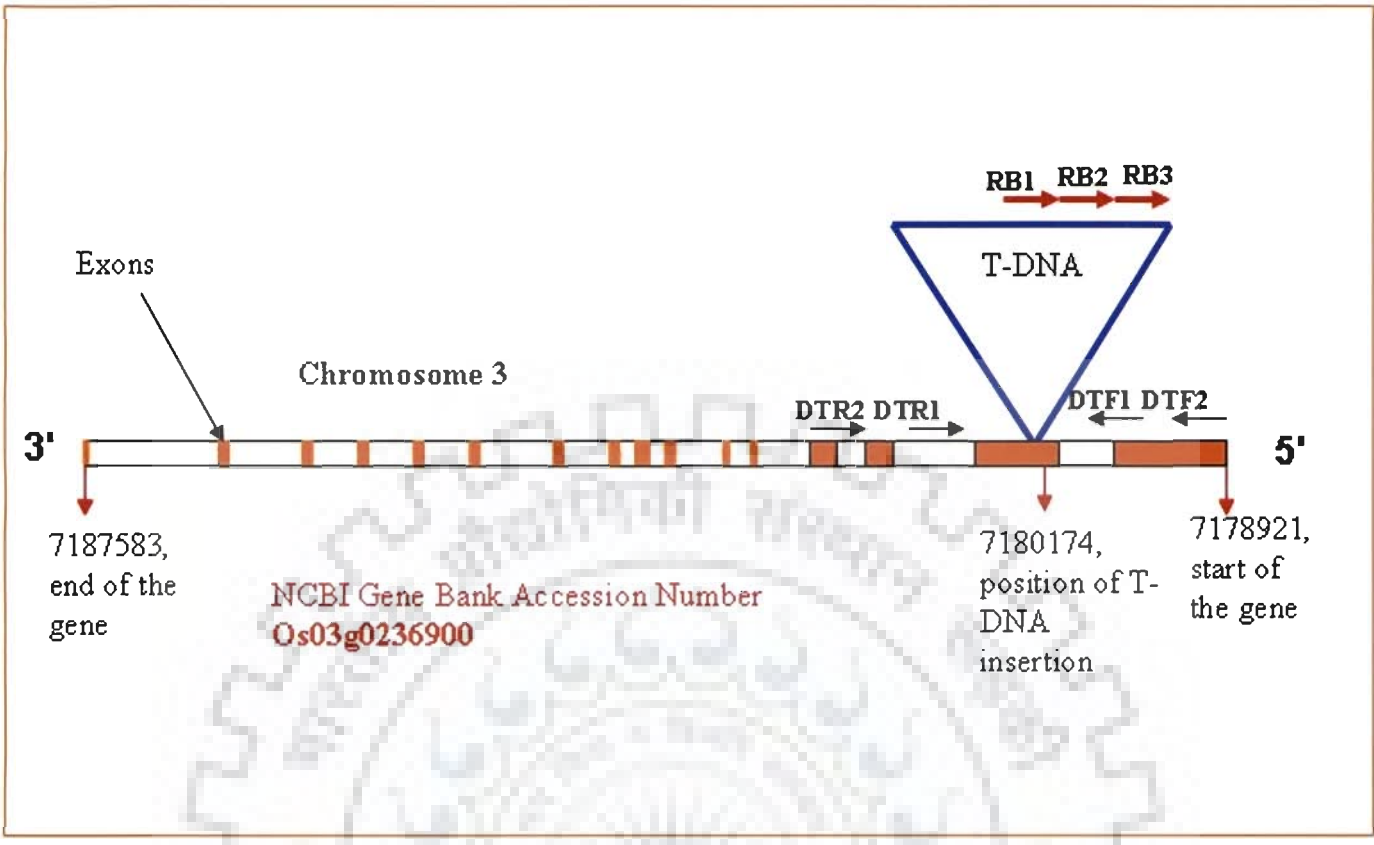


Figure 4.15 Diagrammatic presentation of T-DNA insertion on chromosome 3 in dwarf mutant of Basmati 370.

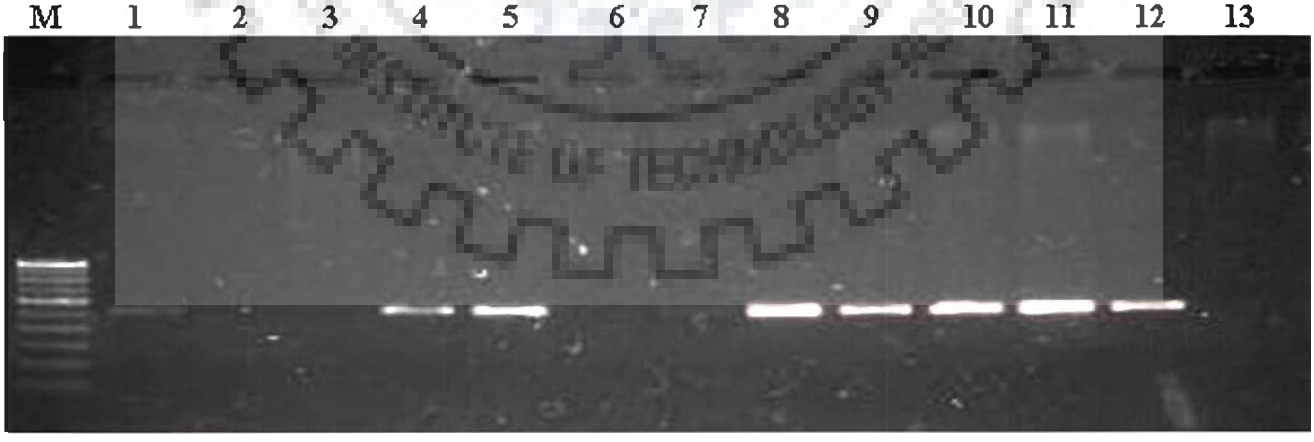


Figure 4.16 PCR amplification with DTF1+RB2 primer combination in 164 F2 plants of *OsGAI/Sd* X PR106 cross. Lanes: M: 100bp ladder. 1: *OsGAI/Sd*, 2=Basmati 370, 3-13: individual F2 plants.

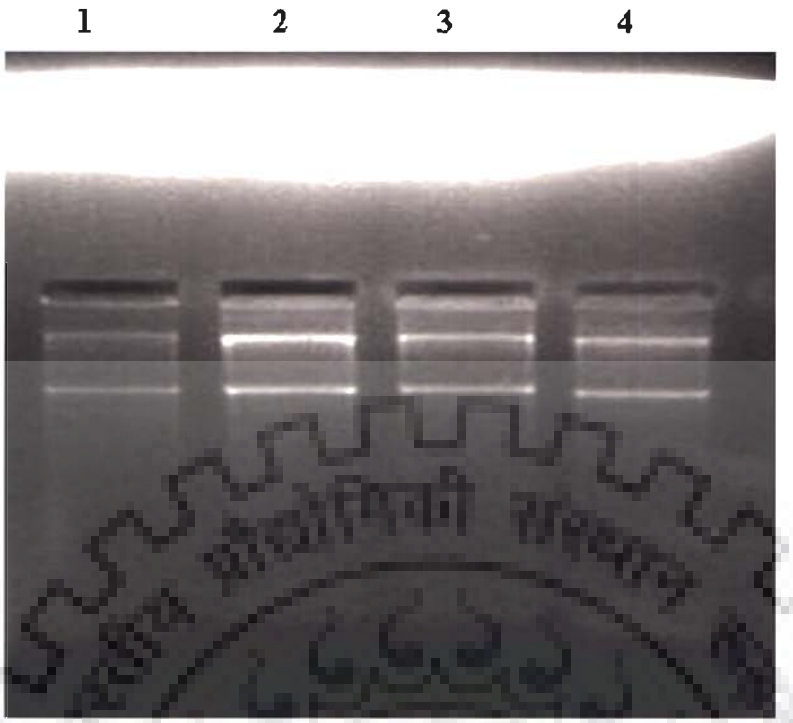


Figure 4.17 Gel electrophoresis of total RNA isolated from different tissues of 10 days old tissues of Basmati 370 and dwarf mutant. Lane1: Basmati 370 roots, Lane2: *OsGAI/Sd* roots, Lane3: Basmati 370 shoots, Lane4: *OsGAI/Sd* shoots.

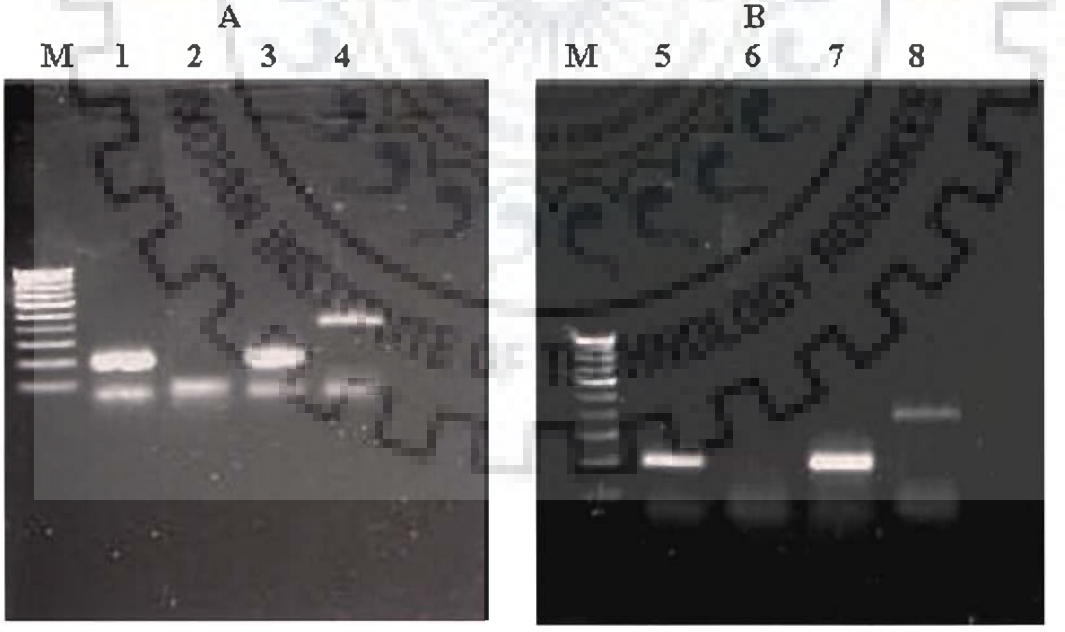
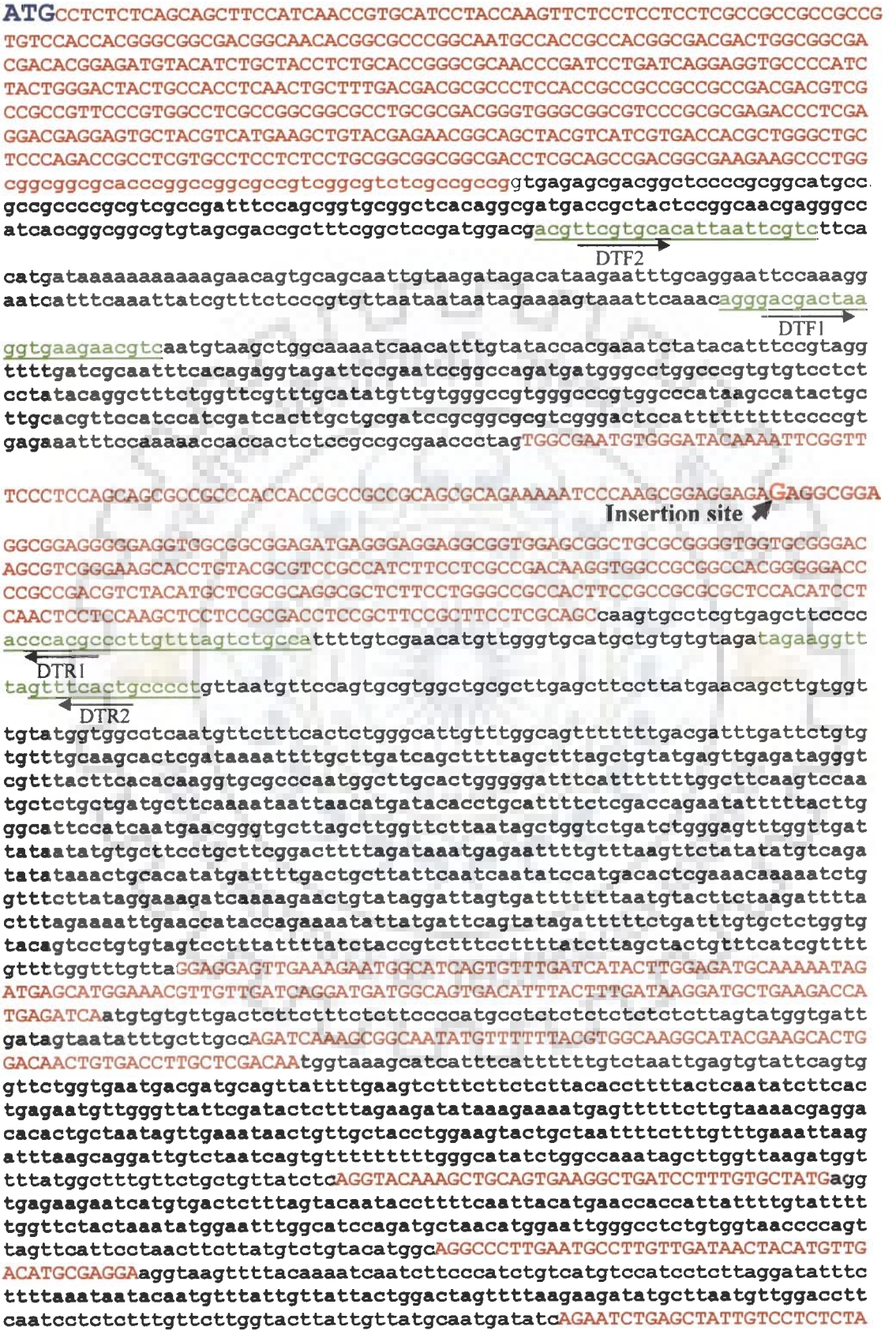


Figure 4.18 RT-PCR analyses in the roots (A) and shoots (B) of Basmati 370 and dwarf mutant *OsGAI/Sd* using actin as the reference gene. M =100 bp ladder. Lane: 1, 3, 5 and 7: actin, Lane: 2 and 6: *OsGAI/Sd*, Lane: 4 and 8: Basmati 370.


DTF2
DTF1
Insertion site
DTR1
DTR2

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AACTTGCAGAGCaggtaataacttgagaaatcctaccgagactaattttcttatgaggcttccactctga
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aaggaggtttaatgtggtaatagtagttggtcagatgctgaaaatacatcctgtatttgtaatattggat
gggttatagaaaatgagatattatcatgcacaacttccttttacttagtgaatactgtttgttgtagctc
acaatgagagcctatcatggagagtatgttatttgcaaagttttatttggcaaggcataatagttttgtc
atcatttttttaaaccccctttttctttctgctgtgatgcAGTTCTTCACGCAAGCAAAATCTATCTGCCC
ATCTGATCCGCTTATATATAACGAGATGGGGGTTGTAGCTTATAATATGAAAgagtaagagtttctctgt
tctaaaggtttcttttgtctgcttatcctattattattggaaccagtgatgctccactttcccttgtaag
atgctttgctctcatgaaagggaaagcattcctataagttctatatctgatattcctataggaaaaatgt
tatgctaacatggatgttttgaaagatgtccatgatatacaaggttgatttggtggttctgaaattttga
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ttcatgttaaggttagcatgtagaaactggctacaccggaggaactttgggataaaagaagaataaaaga
aggggaatggctccaggataaataagaaaggataggctattatgatgaactttgggggagaaaaaatgttt
catctttcttgattgatttgtaacggatgctttgctgttcccttgtatcgacaagatcttcacatagta
ttcagtgaagtaaacccaagtaagagagcaactaatttttttcccccttagtttccaactaatgctgcacc
agctgggccctgcattaacgcaatcacgataacaagtgtgttttatgcatttatagtatgatcattagag
tttgaaacgtgtgattgatgtccatgatccatatacaagtttggtttttatttgcttttcaaattctctt
caacttataaaatttcatcccattagatttggttctctgctgaaaaaaatgtttgacacatccagttcta
gtggtttcagtaaaaagaactgtttgtgctgctattaaagtagttgagaatttgatattctgtttttttt
aaaaaaaatgtttAGGTATCAAAAAGCAGTTCAGTGGTTTGAGCTAACACTGGAGCATACTTCATCCTCC
TTGAATGAAATGTGGGAACCAACATTGGTGAATCTTGGGCATGCATTGCGGAAACTCaagtaagggaacc
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tagatttgtcaagttccaatcccatttatatgctcaatttatgatttttgttatacatgagagaatggga
aaggaagtgttgatttagttcagtcatctcagttgtgcagctgtggttgctttgtttgaagacctttttat
tacagactagaacaaaataaatcagcaatatgccagtgttattatttgggccagtgtgtgtgagggtaag
ttcgaacttgtggatttctttgtatccagagccagtcaagcctaaattgcatgaaactttaataagaaaa
tatgccttagattttgtgcctagcatatcctgacctgaatagcatttctttgcgatgaacagcagctttg
ctgttagttttgactgatggaataataattaaccgaattatactggttctctttctttttggttgttgtc
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TATCATATTATGAAAAGGCACTCACCTTTCAAACCAAAAGTTTGAGCGCGTTTGCTGGTCTTGCTTATAC
TTACCACCTTAtggtactcttctgttcctttcttatccactgctttgaatggtactatctctgttatgct
gcactggcatgctgtaaaggcgatgtatcatgtgcatagggcagaagttctgcaaattctgacaaaagaa
cctggaagatgatacgtgttagatcatggtctttccacatagacttatatttaattatctattggatatc
tatatgatcagaagttcaaaactaagttttatgtaacctgctacttcaattacaacatcaattcatatat
gtgtgctgagcgtttaccgttgaaacatcgggatacttggtatttgttagttcccataggcataagtgag
aaacaactagacaaggcatagagatgccattggaagaatcgagaacagagtaatgagagggtgatttaag
ttctagtagtagtgttttcaggaaaaaaaatttcggtcagagattccttgctaatttacatgaaaatcac
gaaggacaattgacctttaagcttaagttgcatttgtagaagcaatatttttagttttaagaatgagac
ctgatatgcttgattcattgtttcAGTTTGACACTTCCACAGTTACTTTCTGA

Figure 4.19 Genomic sequence of the candidate gene (Os03g0236900) from *Oryza sativa* cv. Nipponbare. Position 7178921-7187583bp of *Oryza sativa* genomic DNA, of chromosome 3 (Black colored base pairs in small letters are intronic and red colored capital letters base pairs are exonic regions in the sequence).

ATGCCTCTCTCAGCAGCTTCCATCAACCGTGCATCCTACCAAGTTCTCCTCCTCCTCGCCGCCGCCGCCG
TGTCCACCACGGGCGGCGACGGCAACACGGCGCCCGGCAATGCCACCGCCACGGCGACGACTGGCGGCGA
CGACACGGAGATGTACATCTGCTACCTCTGCACCGGGCGCAACCCGATCCTGATCAGGAGGTGCCCCATC
TACTGGGACTACTGCCACCTCAACTGCTTTGACGACGCGCCCTCCACCGCCGCCGCCGCCGACGACGTCG
CCGCCGTTCCCGTGGCCTCGCCGGCGGCGCCTGCGCGACGGGTGGGCGGCGTCCCGCGCGAGACCCTCGA
GGACGAGGAGTGCTACGTCATGAAGCTGTACGAGAACGGCAGCTACGTCATCGTGACCACGCTGGGCTGC
TCCCAGACCGCCTCGTGCCTCCTCTCCTGCGGCGGCGGCGACCTCGCAGCCGACGGCGAAGAAGCCCTGG
CGGCGGCGCACCCGGCCGGCGCCGTCGGCGTCTCGCCGCCGTGGCGAATGTGGGATACAAAATTCGGTTT

DRTF

CCCTCCAGCAGCGCCGCCCACCACCGCCGCCGCAGCGCAGAAAAATCCCAAGCGGAGGAGAGAGGCGGAG
GCGGAGGGGGAGGTGGCGGCGGAGATGAGGGAGGAGGCGGTGGAGCGGCTGCGCGGGGTGGTGCGGGACA
GCGTCGGGAAGCACCTGTACGCGTCCGCCATCTTCCTCGCCGACAAGGTGGCCGCGGCCACGGGGGACCC
CGCCGACGTCTACATGCTCGCGCAGGCGCTCTTCCTGGGCCGCCACTTCCGCCGCGCGCTCCACATCCTC
AACTCCTCCAAGCTCCTCCGCGACCTCCGCTTCCGCTTCCTCGCAGCCAAGTGCCTCGAGGAGTTGAAAG

DRTR

AATGGCATCAGTGTTTGATCATACTTGGAGATGCAAAAATAGATGAGCATGGAAACGTTGTTGATCAGGA
TGATGGCAGTGACATTTACTTTGATAAGGATGCTGAAGACCATGAGATCAATATCAAAGCGGCAATATGT
TTTTTACGTGGCAAGGCATACGAAGCACTGGACAACTGTGACCTTGCTCGACAATGGTACAAAGCTGCAG
TGAAGGCTGATCCTTTGTGCTATGAGGCCCTTGAATGCCTTGTTGATAACTACATGTTGACATGCGAGGA
AGAATCTGAGCTATTGTCCTCTCTAAAATTTGGAAAAGAAGATGGGTGGCTCTCAGCATTCTACTCTTGT
TTGATAAGGAAGCATGAAAAAGAATATATAGTGGAAGCAAAGTTCAAGGAATTTGAACGAGAATCTTGTA
GTATTTCATCTTTGAGTTCAGGACTGACACTGAAAAATAATATTGACGTGTTGGCTTGCAAAGCTGAATA
CTATCATCAGAGTGGAGAGTACCAAAAATGTTTCGAACTCACATCTGCGTTACTTGAAAGGGACCCTTTT
CATTTGAAATGCACGTTAGTTCATTTGGCAGCTGCAATGGAGCTTGGCCATTCAAATGACCTTTATATTT
TGGCCTGCAATCTAGTGAAGGACTATCCTCAGAACTGTGGTTCCATCTGTAGAGCTCTTTCCTGGTTTGC
TGTTGGGTGTTATTACTACTGTATTAAGAAGTATGATCAAGCACGCAGATACTTTGGCAAAGCTACAGGG
TTAGATGGGACATTTCCTCCTGCTTGGATTGGTACTGGTATTGCTTATGCTGCACAGGAGGAGGGTGATC
AAGCAATGGCTGCATTTCGGACAGCAGCTCGGCTATTTCCTGGATGTCATCTGCCAACTTTATACATGGG
CATGCAATATTTGAATGCACAATTTCAAACTTGCAGAGCAGTTCTTCACGCAAGCAAAATCTATCTGCCC
ATCTGATCCGCTTATATATAACGAGATGGGGGTTGTAGCTTATAATATGAAAGAGTATCAAAAAGCAGTT
CAGTGGTTTGAGCTAACACTGGAGCATACTTCATCCTCCTTGAATGAAATGTGGGAACCAACATTGGTGA
ATCTTGGGCATGCATTGCGGAAACTCAAGAAATATCAAAAGGCAATATCATATTATGAAAAGGCACTCAC
CTTTCAAACCAAAAGTTTGAGCGCGTTTGCTGGTCTTGCTTATACTTACCACCTTATGTTTGACACTTCC
ACAGTTACTTTCTGA

Figure 4.20 *Oryza sativa* cv. Nipponbare cDNA sequence of candidate gene Os03g0236900. The positions of forward and reverse primers used in RT-PCR have been indicated by arrows.

MPLSAASINRASYQVLLLLAAAAVSTTGGDGNTAPGNATATATTGGDDTEMYICYLCTGRNPILIRRCPI

YWDYCHLNCFDDAPSTAAAADDVAAVPVASPAAPARRVGGVPRETLEDEECYVMKLYENGSYVIVTTLGC

SQTASCLLSCGGGDLAADGEEALAAAHPAGAVGVSPPWRMWDTKFGFPPAAPPTTAAAAQKNPKRRREAE

AEGEVAAEMREEAVERLRGVVRDSVGKHLYASAIFLADKVAAATGDPADVYMLAQALFLGRHFRRALHIL

NSSKLLRDLRFRFLAAKCLEELKEWHQCLIILGDAKIDEHGNVVDQDDGSDIYFDKDAEDHEINIKAAIC

FLRGKAYEALDNCDLARQWYKAAVKADPLCYEALECLVDNYMLTCEEESELLSSLKFGKEDGWLSAFYSC

LIRKHEKEYIVEAKFKEFERESCSISSLSSGLTLKNNIDVLACKAEYYHQSGEYQKCFELTSALLERDPF

HLKCTLVHLAAAMELGHSNDLYILACNLVKDYPQNCGSICRALSWFAVGCYYYCIKKYDQARRYFGKATG

LDGTFPPAWIGTGIAYAAQEEGDQAMAAFRTAARLFPGCHLPTLYMGMQYLRMHNFKLAEQFFTQAKSIC

PSDPLIYNEMGVVAYNMKEYQKAVQWFELTLEHTSSSLNEMWEPTLVNLGHALRKLKKYQKAISYYEKAL

TFQTKSLSAFAGLAYTYHLMFDTSTVTF

Figure 4.21 Amino acid sequence of APC6 encoded by the candidate gene Os03g0236900 (*Oryza sativa* cv. Nipponbare). Blue colored amino acids with underlined arrows indicate the position of TPR domain.

sequence alignment (http://www.ebi.ac.uk/). The amino acid sequences were downloaded from NCBI which contained different number of TPR domain. The alignment score was highest between PhSPY and LeSPY. APC6 showed highest alignment with LeSPY (Fig. 4.22).

4.15. The anaphase-promoting complex/cyclosome (APC/C):

The position the TPR conserved domains in amino acid sequence of APC6 have been given in Fig. 4.21. A phylogenetic tree was created using DRAWGRAM tool of Clustal W multiple-alignment by Biology Workbench (http://workbench.sdsc.edu/) (Fig. 4.23). The tree was based on multiple sequence alignment of the 728 amino acid (encoded by the candidate gene) with the 11 different protein amino acid sequences which showed homology to the sequence in ClustalW multiple sequence alignment. The amino acid sequence (NP_001049491) was found to be most closely related to ABF94847 from *Oryza sativa*. The nearest relatives of the protein sequence encoded by the candidate gene were *Schizosaccharomyces pombe* (NP_593301) and *Drosophila melanogaster* (NP_477397). Rest other amino acid sequences did not form cluster with the queried amino acid sequence (Fig. 4.23).

In KEGG link from NCBI database, the candidate gene was found to be the sixth subunit of holo-APC/C enzyme which is an Ubiquitin-protein ligase (E3) that plays role in mitosis by ubiquitinating the regulatory proteins and thereby targeting them for proteolysis by the 26S proteasome (Fig. 4.24). Main function of APC6 (or Cdc 6) is to create linkage with another APC subunit Doc1 (or APC10) to cause holo-enzyme to come partially closure to the substrate for its degradation.

4.16. Orthologous and paralogous gene search:

Homologous genes may be either paralogous or orthologous. If the two sequences are generated by duplication, then they are paralogous, in contrast, orthologous sequences are the result of speciation events. With duplicated genomic segments comprising of about 27-65% of the total rice genome, rice is likely to have more number of paralogous genes as compared to other cereal crops. Among 67 paralogous genes searched through database (KEGG), the candidate gene showed highest identities (0.400 score) with NCBI gene ID Os03g0618300, located from 23252738 -23250755 bp (1984bp in length) position of chromosome 3. The gene ID Os03g0618300 encodes an isopencillin N-synthase family protein containing members of the 2-oxoglutarate and Fe (II)-dependant oxygenase superfamily as the conserved domains but without any TPR domain. Pair-wise alignment of APC6 and Os03g0618300 from *Oryza sativa* has been shown in Fig. 4.25. Among 523 hits of orthologous genes in different species, the *Arabidopsis thaliana* NCBI gene entry AT1G78770 (Anaphase Promoting Complex component 6) showed highest identity score of 0.724. Pair-wise alignment of APC6 (*Oryza sativa*) and AT1G78770 from *Arabidopsis thaliana* has been shown in Fig. 4.26.

4.17. TAIL-PCR of two other insertional mutants:

TAIL-PCR was also used to isolate the flanking region of T-DNA in the other two insertional mutants B-3-1 and *OsGAS/Sd* which were developed by T-DNA insertion in Basmati 370 by an *A. tumefaciens* strain EHA101. For the TAIL-PCR, the degenerate primer AD1 having 64 fold degeneracy was used for B-3-1 while AD2 having 128 fold degeneracy was used for *OsGAS/Sd.*


PhSPY MAWTEKDVENGKESDSLGNNGFLKGVQSSSDSKGSPVRISPVKKSFEGKDAITYANILRS 60
LeSPY MAWTEKDVENGKESESLGNNGFLKGGQSSSGSKGSPGRISHVKKIFEDKDAITYANILRS 60
AtSPY MVGLEDDTE--RERSPVVENGFSNGSRSSSSSAG---VLSPSRKVTQGNDTLSYANILRA 55
HvSPY MESLQ-----GKESN---------GAVPVCNGGGGAAAPPAKQQLPEGTDALRYANILRS 46
APC6 ----------MPLSAASIN 9
PhSPY RNKFVDALAIYESVLQKDSGSIESLIGKGICLQMQNMGRLAFESFAEAIKLDPQNACALT 120
LeSPY RNKFVDALAIYESVLEKDSKSIESLIGKGICLQMQNTGRLAFESFSEAIKVDPQNACALT 120
AtSPY RNKFADALALYEAMLEKDSKNVEAHIGKGICLQTQNKGNLAFDCFSEAIRLDPHNACALT 115
HvSPY RNKFADALQLYTTVLDKDGANVEALIGKGICLQAQSLPRQALDCFTEAVKVDPKNACALT 106
APC6 RASYQVLLLLAAAAVSTTGGDGNTAPGNATATATTGGDDTEMYICYLCTGRNP----ILI 65
PhSPY HCGILYKDEGRLVEAAESYQKALKADPSYKPAAECLAIVLTDIGTSLKLAGNSQEGIQKY 180
LeSPY HCGILYKDEGRLVEAAESYEKALKADPSYTPAAECLAIVLTDIGTSLKLAGNTQEGIQKY 180
AtSPY HCGILHKEEGRLVEAAESYQKALMADASYKPAAECLATVLTDLGTSLKLAGNTQEGIQKY 175
HvSPY HCGMIYKDEGHLVEAAEAYQKARSADPSYKAASEFLAIVLTDLGTSLKLAGNTEDGIQKY 166
APC6 RRCPIYWDYCHLNCFDDAPSTAAAADDVAAVPVASPAAPARRVGGVPR----------- 113
PhSPY YEAIKIDSHYAPAYYNLG-VVYSEMMQYDMALNCYEKAAIERPMYAEAYCNMGVIYKNRG 239
LeSPY YEAIKIDSHYAPAYYNLG-VVYSEMMQYDMALNCYEKAALERPMYAEAYCNMGVIFKNRG 239
AtSPY YEALKIDPHYAPAYYNLG-VVYSEMMQYDNALSCYEKAALERPMYAEAYCNMGVIYKNRG 234
HvSPY CEALEVDSHYAPAYYNLG-VVYSEMMQFDVALTCYEKAALERPLYAEAYCNMGVIYKNRG 225
APC6 -ETLEDEECYVMKLYENGSYVIVTTLGCSQTASCLLSCGGGD----------LAADG 159
PhSPY DLESAIACYERCLAVSPNFEIAKNNMAIALTDLGTKVKLEGDINQGVAYYKKALYYNWHY 299
LeSPY DLESAIACYERCLAVSPNFEIAKNNMAIALTDLGTKVKLEGDINQGVAYYKKALCYNWHY 299
AtSPY DLEMAITCYERCLAVSPNFEIAKNNMAIALTDLGTKVKLEGDVTQGVAYYKKALYYNWHY 294
HvSPY ELDAAIACYDRCLTISPNFEIAKNNMAIALTDLGTKVKIEGDINQGVAYYKKALFYNWHY 285
APC6 EEALAAAHPAGAVGVSPPWRMWDTKFGFPPAAPPTTAAAAQKNPK---RRREAEAEGEVA 216
PhSPY ADAMYNLGVAYGEMLKFDMAIVFYELAFHFNPHCAEACNNLGVIYKDRDNLDKAVECYQM 359
LeSPY ADAMYNLGVAYGEMLKFDMAIVFYELAFHFNPHCAEAGNNLGVIYKDRDNLDKAVECYQL 359
AtSPY ADAMYNLGVAYGEMLKFDMAIVFYELAFHFNPHCAEACNNLGVLYKDRDNLDKAVECYQM 354
HvSPY ADAMYNLGVAYGEMLNFEMAIVFYELALHFNPRCAEACNNLGVIYKDRDNLDKAVECYQM 345
APC6 AEMREEAVERLRGVVRDSVGKHLYASAIFLADKVAAATGDPADVYMLAQALFLGRHFRRA 276
PhSPY ALTIKPNFSQSLNNLGVVYTVQGKMDAAASMIEKAIIANPTYAEAYNNLGVLYRDAGNIS 419
LeSPY ALSIKPNFSQSLNNLGVVYTVQGKMDAAASMIEKAIIANPTYAEAYNNLGVLYRDAGNIS 419
AtSPY ALSIKPNFAQSLNNLGVVYTVQGKMDAAASMIEKAILANPTYAEAFNNLGVLYRDAGNIT 414
HvSPY ALSIKPNFSQSLNNLGVVYTVQGKMDAAASMIEKAILANPTYAEAYNNLGVLYRDAGSIT 405
APC6 LHILNS--SKLLRDLRFRFLAAKCLEELKEWHQCLIILGDAKIDEHG--NVVDQDDG--- 329
PhSPY LAIEAYEQCLKIDPDSRNAGQNRLLAMNYINEGSDDKLYEAHRDWGWRFMRLYQQYNSWD 479
LeSPY LAIEAYEQCLKIDPDSRNAGQNRLLAMNYINEGTDDKLYEAHRDWGRRFMKLYPQYTSWD 479
AtSPY MAIDAYEECLKIDPDSRNAGQNRLLAMNYINEGLDDKLFEAHRDWGWRFTRLHPQYTSWD 474
HvSPY LSVQAYERCLQIDPDSRNAGQNRLLAMNYIDEGSDDKLYDAHREWGKRFMKLYAQYTSWD 465
APC6 -------SDIYFDKDAEDHEINIKAAICFLRGKAYEALDNCDLARQWYKAAVKADPLCYE 382
PhSPY NSKDPERQLVIGYVSPDYFTHSVSYFIEAPLAYHDYANYKVVIYSAVVKADAKTNRFRDK 539
LeSPY NSKVPERPLVIGYVSPDYFTHSVSYFIEAPLAHHDYTNYKVVVYSSVVKADAKTNRFRDK 539
AtSPY NLKDPERPITIGYISPDFFTHSVSYFIEAPLTHHDYTKYKVVVYSAVVKADAKTYRFRDK 534
HvSPY NPKVADRPLVIGYVSPDFFTHSVSYFVEAPLTHHDYTKCKVVVYSGVVKADAKTLRFKDK 525
APC6 ALECLVDNYMLTCEEESELLSSLKFGKED--------GWLSAFYSCLIRKHEKEY----- 429

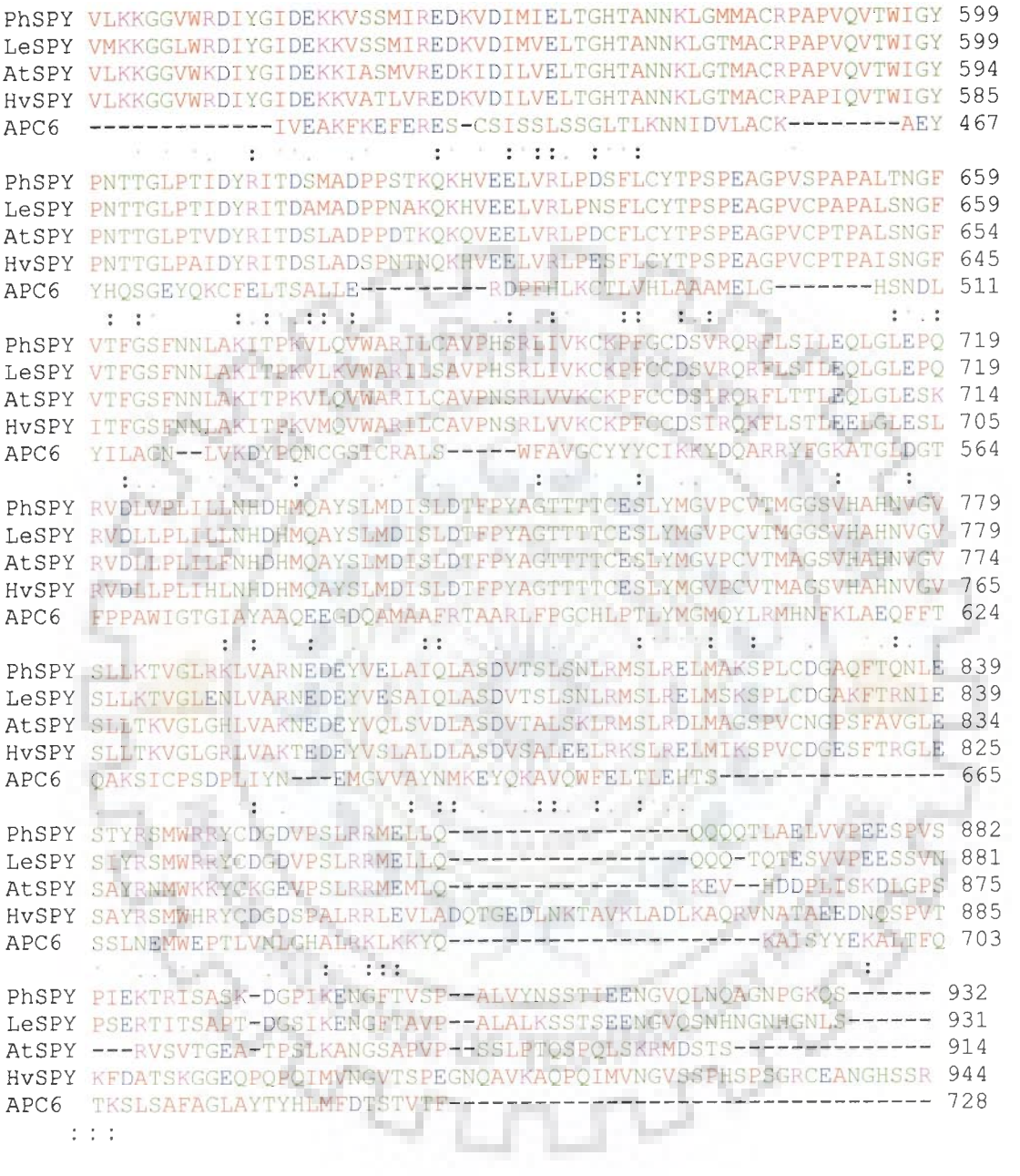

Figure 4.22 Multiple sequence alignment of TPR domain containing amino acid sequences from *Abrabidopsis thaliana* (AtSPY), *Lycopersicon esculentum* (LeSPY), *Petunia hybrida* (PhSPY), *Hodeum vulgare* (HvSPY) and APC6 from *Oryza sativa*, cv. Nipponbare. * Conserved amino acid residues in all the five sequences.

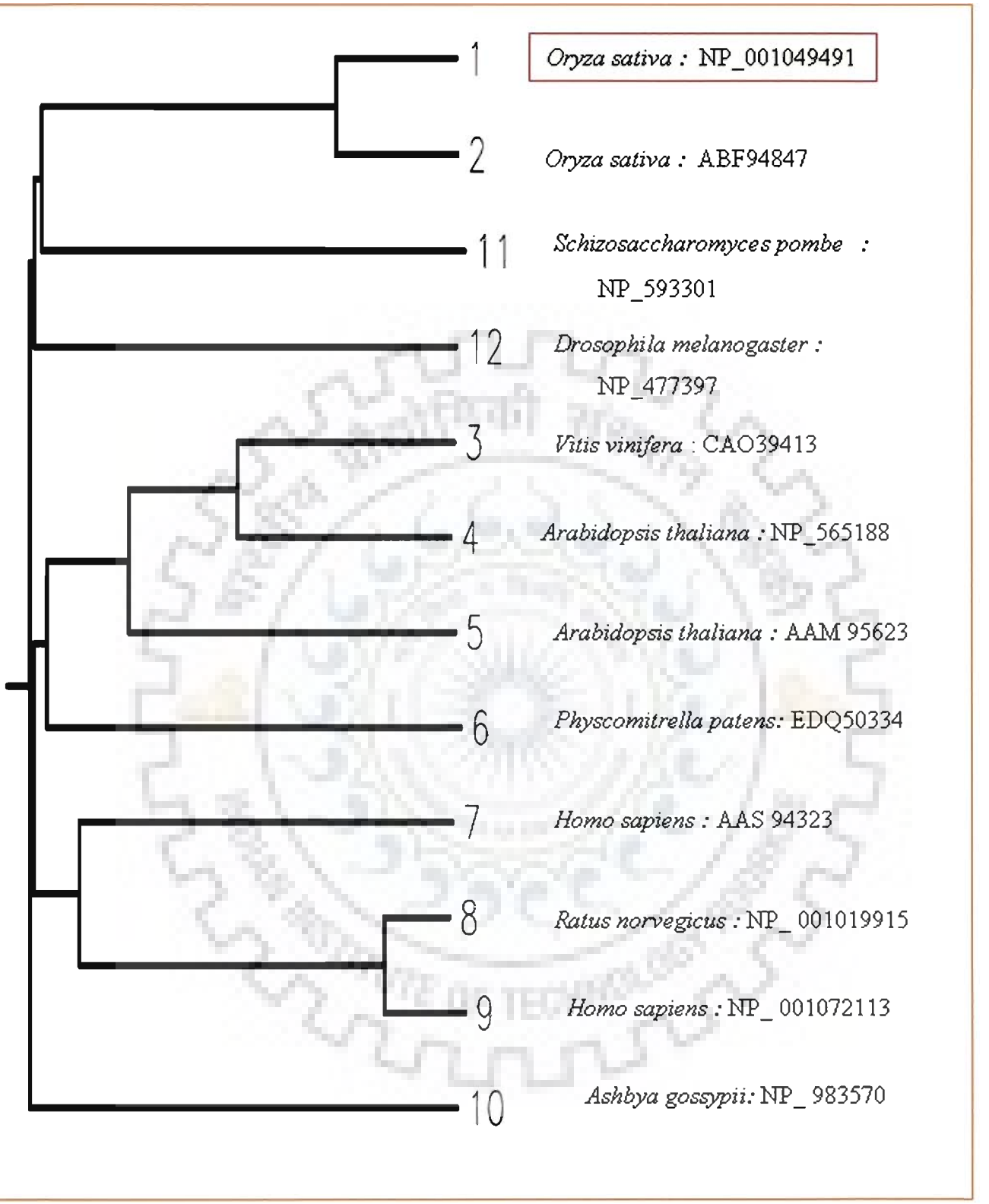

Figure 4.23 Phylogenetic rooted tree of twelve protein amino acid sequences showing homology to *Oryza sativa* gene bank accession number NP_001049491 (red box).

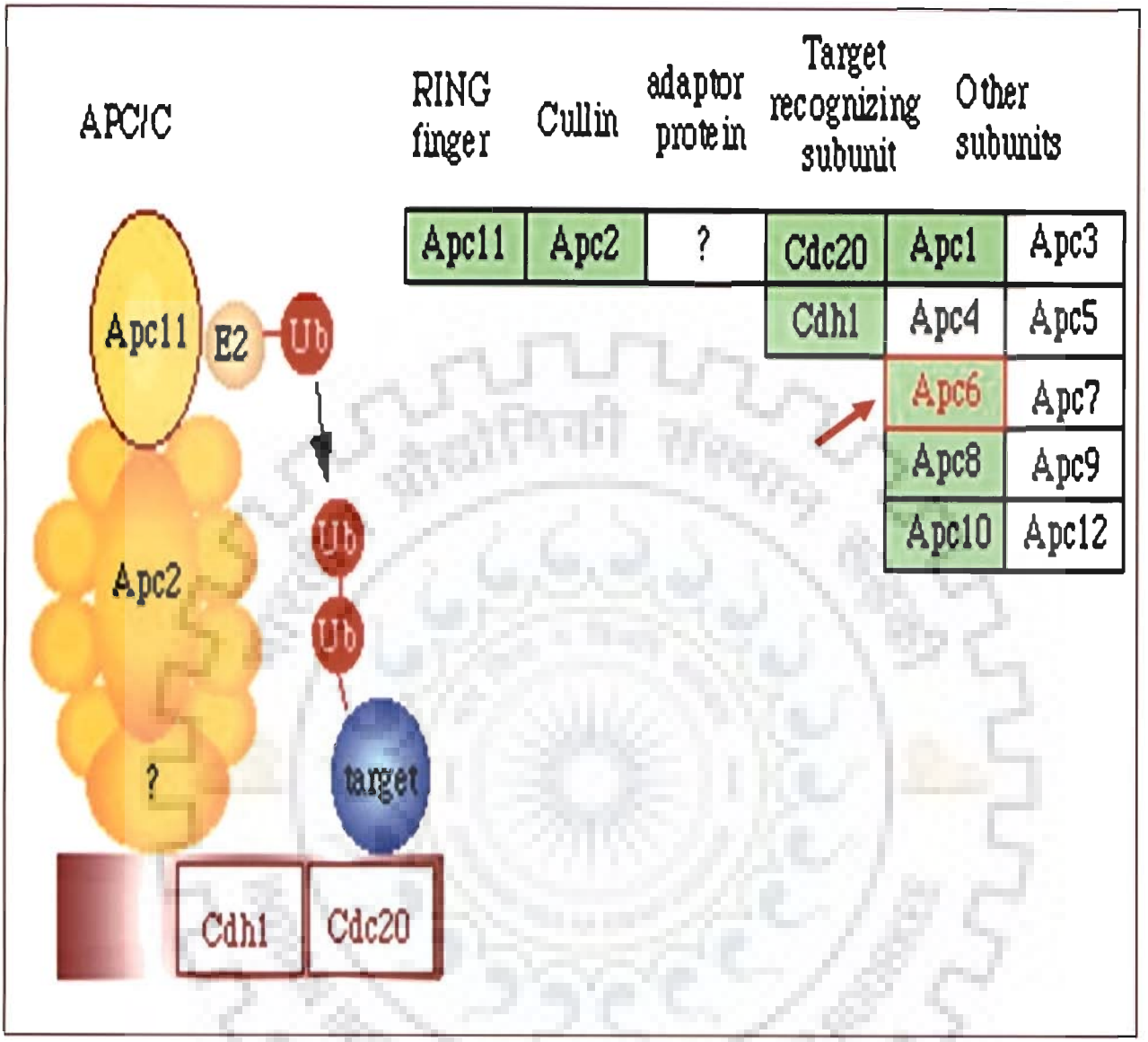

Figure 4.24 Role of Anaphase Promoting Complex/Cyclosome subunit in proteolysis of a target protein from KEGG link (NCBI). The different subunits of APC/C complex reported so far have been shown. The APC6 has been marked in red arrow because of its putative role in the dwarfness of *OsGAI/Sd* mutant in the present study.

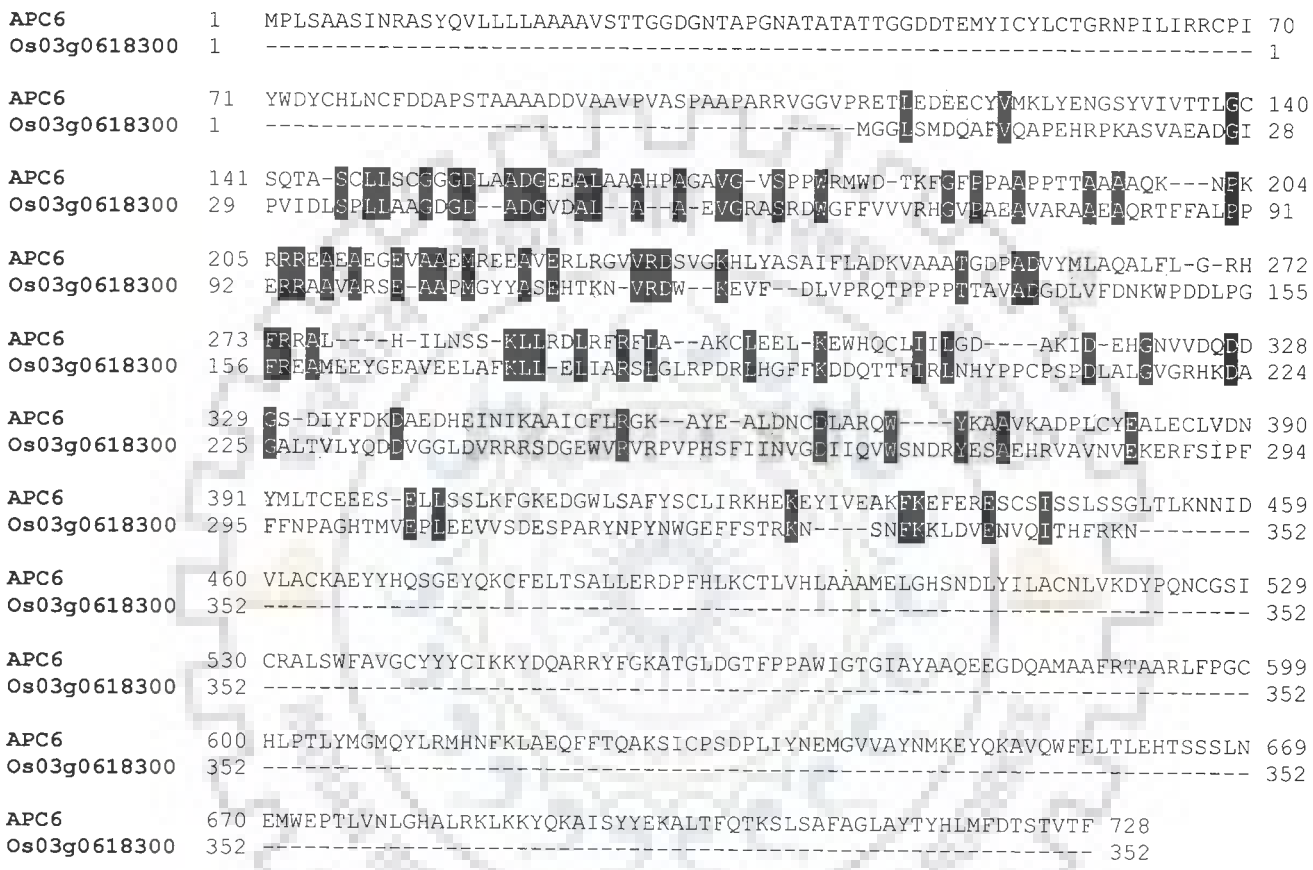

Figure 4.25 Pair-wise alignment of amino-acids encoded by APC6 and Os03g0618300 gene from Oryza *sativa* cv. Nipponbare showing highest identities among paralogous sequences. Identical matches are dark colored while similar matches are light dark colored.

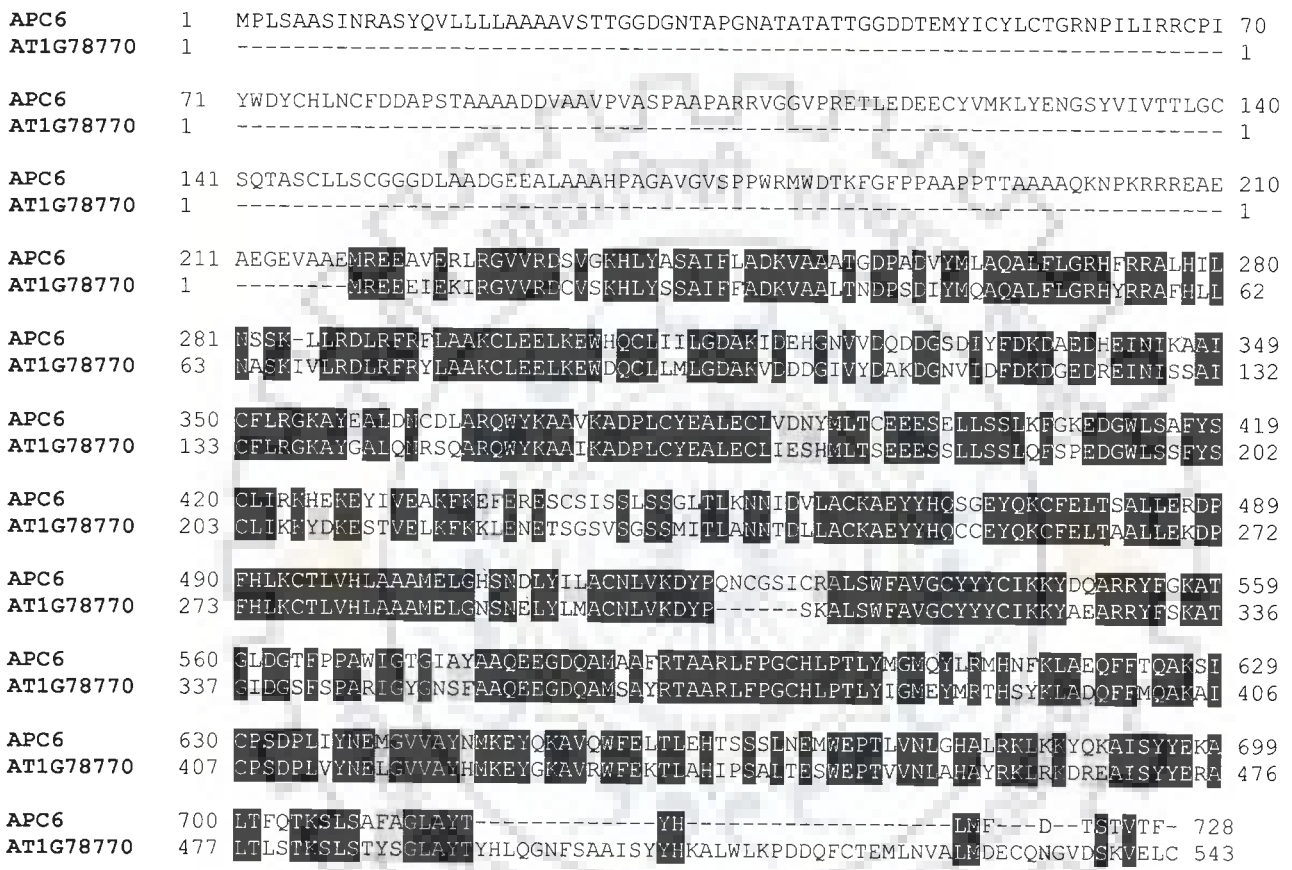

Figure 4.26 Pair-wise alignment of APC6 (*Oryza sativa*) and AT1G78770 from Arabidopsis thaliana showing highest identities among Orthologous sequences. Identical matches are dark colored while similar matches are light dark colored.

Primary, secondary and tertiary TAIL-PCR reactions were carried out using an AD primer from one side and three T-DNA specific nested primers (RB1, RB2 and RB3) on the other side in three consecutive reactions. A single specific fragment of 240bp in B-3-1 and 300bp in *OsGAS/Sd* were amplified in the tertiary reactions of TAIL-PCR under similar conditions as done in the TAIL-PCR of dwarf mutant *OsGAI/Sd* (Fig. 4.27).

Both the tertiary PCR products were purified and got sequenced by Axygen India Pvt. Ltd. The sequencing was carried out using T-DNA specific RB3 primer under the conditions specified by the manufacturer. After sequencing, 208 bp and 248 bp sequences were obtained in B-3-1 and *OsGAS/Sd* mutants, respectively. These sequences were aligned with the total genome sequence of *Oryza sativa* cv. Nipponbare in NCBI database using BLAST tool. With a single hit, the nucleotide sequence of B-3-1 aligned on chromosome 11 (Fig. 4.28) and that of *OsGAS/Sd* aligned on chromosome 3 (Fig. 4.29) of Nipponbare nucleotide sequence database. The T-DNA insertion in B-3-1 was found at 15587862 bp position in the non-coding region of chromosome 11 while the position of insertion in *OsGAS/Sd* was at 27661369 bp position in the non-coding region of chromosome 3. In both the mutants, the obtained sequences of T-DNA flanking region matched with the uncharacterized sequences on chromosome 3 and 11. The sequences encoding a hypothetical protein were present in 5' and 3' ends of the putative sequence in NCBI data base.

4.18. Validation of gene function through database search of Flanking Sequence Tags:

In order to validate the function of the candidate genes, search was made for Flanking Sequence Tags (FSTs) from two online databases namely, http://signal.salk.edu/cgi-bin/RiceGE and http://orygenesdb.cines.fr/ OryGeneDB). No FST was found in RiceGE

database (Fig. 4.30) throughout the whole stretch of the candidate gene on chromosome 3 (7178921bp-7187583bp). But in OryGeneDB, four FSTs namely DX888666, A20869, A32498, C09262 and CZ552601 showed 100% identity with gene Os03g13370.1 with a putative function in cell division cycle protein 16 (or APC6) in *Oryza sativa* japonica cultivar (Table 4.7). Among these, an FST A32498 located in exonic region and generated by T-DNA insertion would be a good candidate for functional validation of the candidate gene identified in this study. Seeds will be ordered from POSTECH and will be grown in field to find out phenotypic similarities if any between the plants from FST source and the *OsGAI/Sd* mutant. FST search for *OsGAS/Sd* and B-3-1 was made upto 1 kb flanking upstream and downstream region from insertion site, using RiceGE database. No FST in B-3-1 was found while in *OsGAS/Sd* two FSTs namely PFG-1C-5452R: B03444-2707 from Hwayoung T-DNA right border and PFG-1B-064441.L: A032892715 from Donjin T-DNA left border was found.

Table 4.7 Result of FST search for the candidate query gene NC_008396.1 in OrygeneDB database

FST	**Plant Name**	**Orientation**	**Region**	**Source**	**Mutagen**
DX888666	RdSpm1170_3.1	Forward	Promoter	UCD	*Ds*
A20869	3A-12007	Reverse	Promoter	Postech	T-DNA
A32498	3A-60930	Forward	Exon	Postech	T-DNA
C09262	0	Forward	Promoter	Postech	T-DNA
CZ552601	M0004324	Forward	Promoter	TRIM	T-DNA

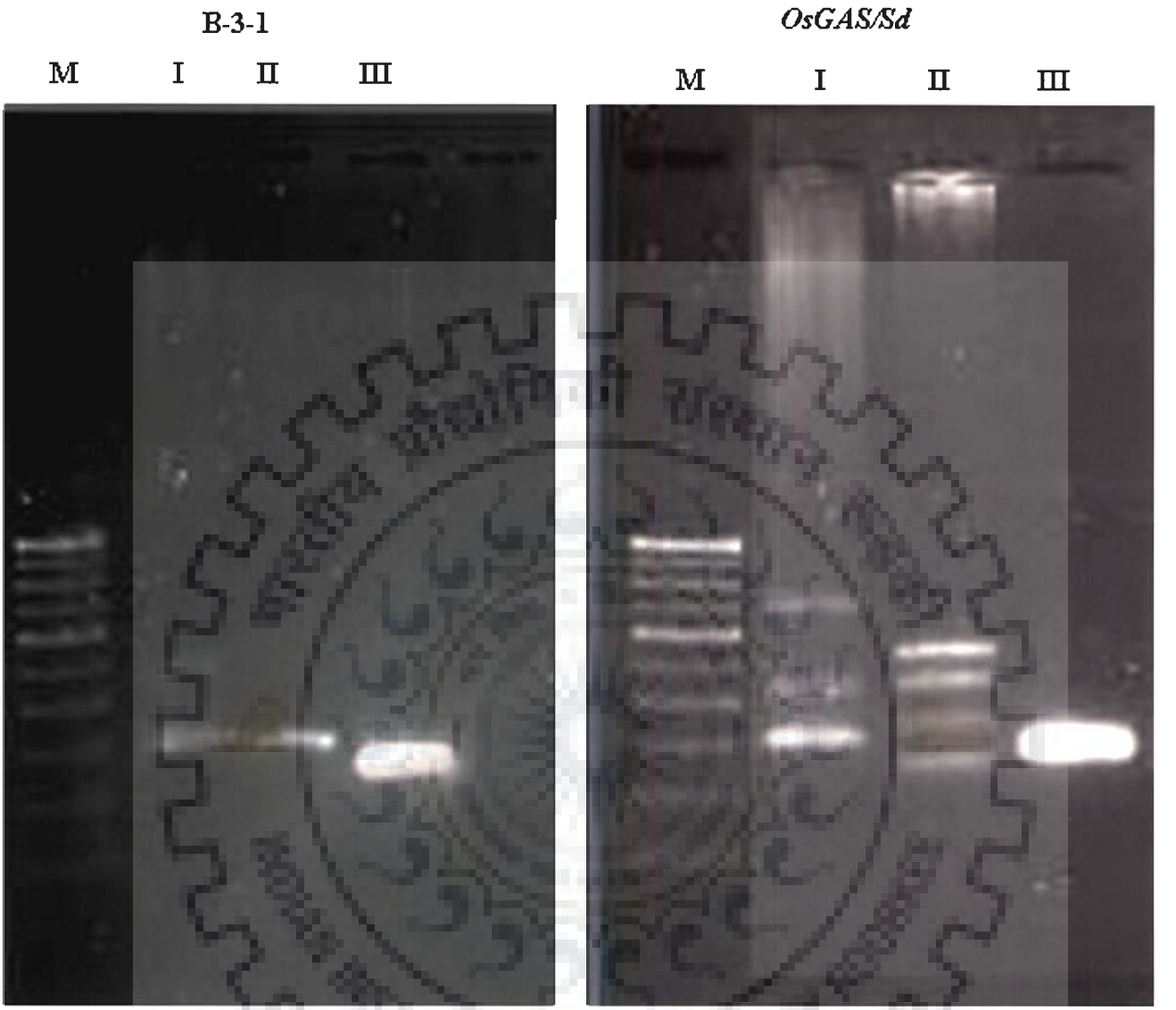

Figure 4.27 TAIL-PCR of two insertional mutants B-3-1 (left) and *OsGAS/Sd* (Right). Lanes: I, II and III indicate the primary, secondary and tertiary PCR products using T-DNA specific RB1, RB2 and RB3, respectively and an degenerate primer AD1 (for B-3-1) and AD2 (for *OsGAS/Sd*). M=100 bp ladder.

> ref|NC_008404.1| *Oryza sativa* (japonica cultivar-group) genomic DNA, chromosome 11, Length=2838694

Features flanking this part of subject sequence:
7568 bp at 5' side: hypothetical protein
29590 bp at 3' side: hypothetical protein

Score = 335 bits (181), Expect = 3e-90
Identities = 190/194 (97%), Gaps = 2/194 (1%)
Strand=Plus/Minus

```
Query  16        ATCG-AATCATGACGACGACCTTTTGTTTATTGACTGATTTAATTTCAATGGGGATGCAT  74
                 |||| || ||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  15588054  ATCGAAAACATGACGACGACCTTTTG-TTATTGACTGATTTAATTTCAATGGGGATGCAT  15587996

Query  75        ACAGCTGGGTTTTGGTGGGTTTACCATTTCTTCACCAGAAGTCAGAAGATCATCTCTGCA  134
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15587995  ACAGCTGGGTTTTGGTGGGTTTACCATTTCTTCACCAGAAGTCAGAAGATCATCTCTGCA  15587936

Query  135       TCGTGGGTTGAGTTCTTTTCAACAACTCGAAATGATAAATTTCTCTTTTTTAAGCATGTT  194
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15587935  TCGTGGGTTGAGTTCTTTTCAACAACTCGAAATGATAAATTTCTCTTTTTTAAGCATGTT  15587876

Query  195       TCTCGAACTCCAAA  208
                 |||||||||| |||
Sbjct  15587875  TCTCGAACTCTAAA  15587862
```

Figure 4.28 Sequence alignment of 208bp sequence flanking T-DNA in the insertional mutant B-3-1 with total genomic sequence of *O. sativa* present in NCBI database. The query sequence obtained through TAIL-PCR matched with chromosome 11.

>ref|NC_008396.1| Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 3, Length=36192742

Features flanking this part of subject sequence:
498 bp at 5' side: hypothetical protein
554 bp at 3' side: hypothetical protein

Score = 372 bits (201), Expect = 1e-101
Identities = 226/237 (95%), Gaps = 6/237 (2%)
Strand=Plus/Plus

```
Query  15        GTTCTTGTTCTTGG-TA-TATACCGGGCCTACTCCAACGCGTTGAGATAGTGGATGTTGG  72
                 ||| ||||| |||| | || ||||||||||||||||||||||||| ||||||||||||
Sbjct  27661369  GTTTTTGTT-TTGGAAAGTAAACCGGGCCTACTCCAACGCGTTGAGAAAGTGGATGTTGG  27661427

Query  73        ACGTCGGATCGAAAACGGAGATGTGCATGTGCTGGTTTCACTAACCAAATCGAGTATGAA  132
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27661428  ACGTCGGATCGAAAACGGAGATGTGCATGTGCTGGTTTCACTAACCAAATCGAGTATGAA  27661487

Query  133       AGAACTTAAACACAGGCACAATTTGATCGTATAATCTCTCAAAATAATTTGGTGGACAAA  192
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  27661488  AGAACTTAAACACAGGCACAATTTGATCGTATAATCTCTCAAAATAATTTGGTGGACAAA  27661547

Query  193       ATTTCTAGACGACCAAACATCGTGTCGGCAACAGGGTGTTC-TCTTCACATCGACAA  248
                 |||||||||||||||||||||||||||||||||||||||| | ||||  |||||||
Sbjct  27661548  ATTTCTAGACGACCAAACATCGTGTCGGCAACAGGGTGTTCAT-TTCAG-TCGACAA  27661602
```

Figure 4.29 Sequence alignment of 248bp sequence flanking T-DNA in the insertional mutant *OsGAS/Sd* with total genomic sequence of *O. sativa* present in NCBI database. The query sequence obtained through TAIL-PCR matched with chromosome 3.

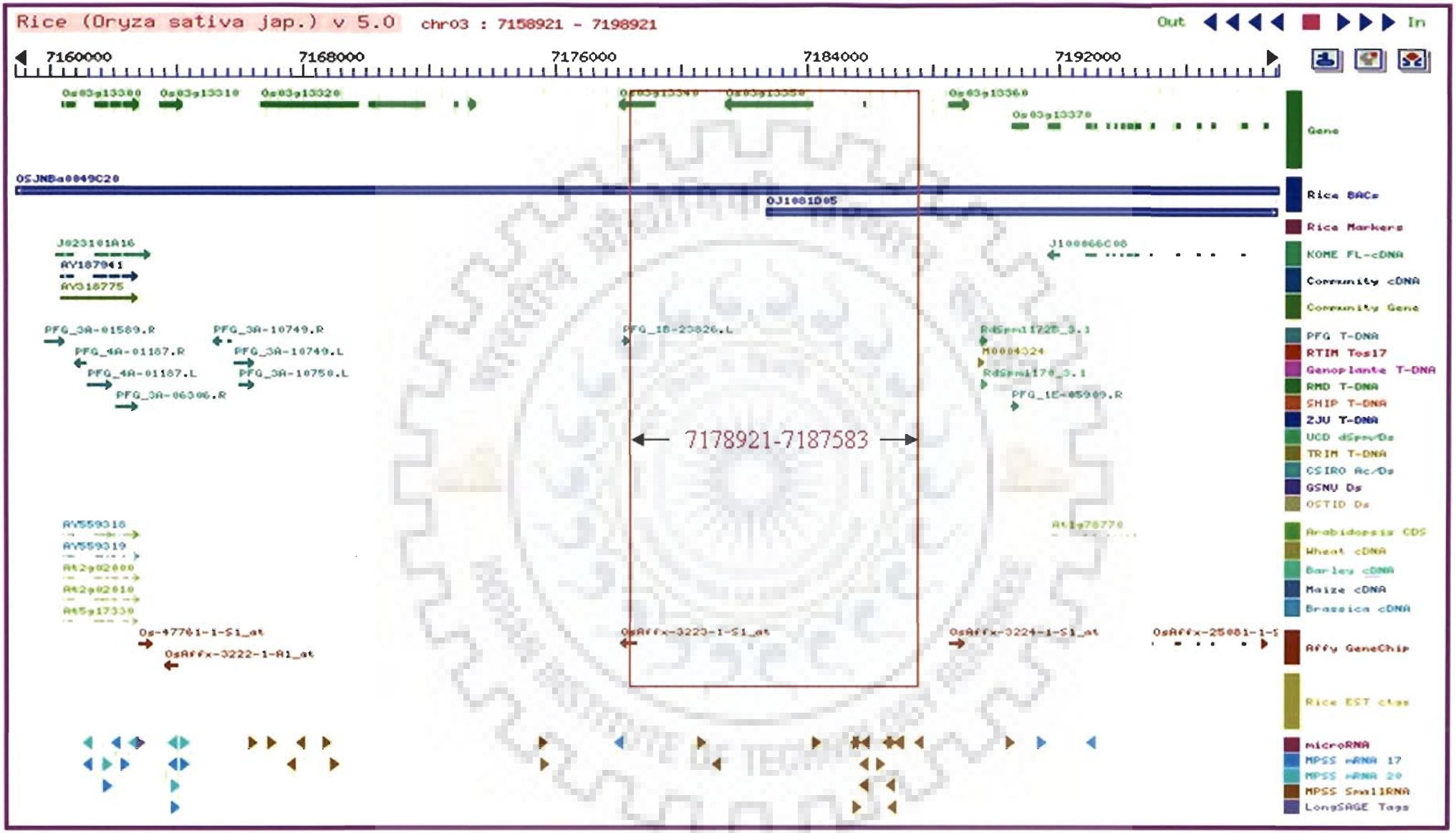

Figure 4.30 Result of FST search for the candidate gene on chromosome 3 for *OsGAI/Sd* in RiceGE database

CHAPTER 5

DISCUSSION

Discussion

Basmati, a premium quality superfine and fragrant rice cultivar, has a major share in the total rice export from India, which increased from 5.8 lakh metric tonnes in 1997-98 to 10 lakh metric tonnes (23% of the total rice export) during 2006-07. Basmati 370, a wonder Basmati rice cultivar, with high export value, continues to reign for its unique quality characteristics till today. But due to its tall stature, it lodges at the time of harvesting. Hence development of dwarfness while keeping all its exquisite quality characteristics intact would help in increasing the yield and income from this variety. The rice genome has been recently sequenced, and a large collection of full-length cDNAs and expressed sequence tags (ESTs) is available (Feng *et al.*, 2002; Sasaki *et al.*, 2002; Yu *et al.*, 2005). Now there is a focus to systematically identify and assign the functions to the newly identified rice genes (Shimamoto and Kyozuka, 2002). For functional genomics in rice, T-DNA insertional mutagenesis using *Agrobacterium tumefaciens* mediated transformation provides a more rapid way to clone a gene due to its low copy number and random insertions throughout the genome (Delseny *et al.*, 2001). Moreover, the *Agrobacterium* mediated transformation procedure in rice has been well established and the generation of T-DNA insertion lines is no longer a problem.

In the present study, three independent T-DNA insertional transformants of Basmati 370 (Dhaliwal *et al.*, 2001) namely, B-1-6-3 (named *OsGAI/Sd*), B-3-1 and B-2-2-3 (named *OsGAS/Sd*), were used for their phenotypic and molecular characterization. The Hm^{R}*Ds* construct used to generate these mutants contains hygromycin phosphotransferase (*hpt*) gene as the selectable marker. The presence of T-DNA insertion in these mutants was confirmed by using *hpt* specific primers for PCR amplification of

950bp *hpt* fragment. The mutants were germinated at 80ppm hygromycin whereas the wild type Basmati 370 seeds failed to germinate at the same hygromycin concentration. The *hpt* gene was also used as a probe to determine the single copy of T-DNA insertion in the genome of *OsGAI/Sd* mutant in Southern hybridization using four different restriction enzymes namely: *HindIII, DraI, EcoRV* and *EcoRI.* There are several reports of using *hpt* gene as a selectable marker to determine the presence of T-DNA insertions and its copy number in the genome of rice (Nakagawa *et al.*, 2000; Greco *et al.*, 2003; Forkan *et al.*, 2004; Fu *et al.*, 2001) and *Arabidopsis* (Tani *et al.*, 2004).

The height of *OsGAI/Sd* (80 cm) was approximately half to that of its wild type parent Basmati 370 (approx. 160 cm) while the height of semi-dwarf B-2-2-3 (*OsGAS/Sd*) was approximately 110 cm at the time of maturity. *OsGAI/Sd* mutant showed only upto 10% increase in plant height after GA_3 treatment in contrast to 30-55% increase in the height after the GA_3 treatment in *OsGAS/Sd*, Basmati 370, PR106 and Pusa Basmati 1. The phenotype of B-3-1 was almost similar to its wild type parent Basmati 370. The most significant physiological effects of exogenous gibberellic acid (GA_3) on plants is to break dwarfism and stimulate the elongation of genetically dwarf genotypes, as these dwarf lines generally have a low level of endogenous GA_3 in their tissues (Singh *et al.* 1978; Suge, 1990). GA responsive and non-responsive dwarf plants can be identified easily in varieties having gibberellic acid insensitive gene and the *sd1* gene by the foliar application of GA_3 onto the plants. Such an approach to rapidly identify different dwarfing genes was also extended to other dwarfing sources in rice (Kumar and Singh, 1967). This indicated that the *OsGAI/Sd* is a GA-insensitive mutant like the previously reported *GID1* (Sasaki *et al.*, 2001), *GID2 (*Sasaki *et al.*, 2003*), SLR1*

(Itoh *et al.*, 2002) and *D1* (Ashikari *et al.*, 1999) mutants in rice, which are related to mutations in GA-signal transduction pathway. The *OsGAS/Sd* was found to be sensitive to gibberellic acid. Several GA-sensitive mutants like *sd1* have been identified in rice with defect in different GA-biosynthetic genes (Sakamoto *et al.*, 2004).

Several differences were found in the histological sections of the internode, leaf and leaf sheath in the *OsGAI/Sd* and Basmati 370. Cell elongation is associated with mitotic activity in cells of the meristematic tissues. From various observations, it can be hypothesized that the cellular differences between *OsGAI/Sd* and Basmati 370 may be caused by the loss-of-function of dwarfing gene in *OsGAI/Sd* which may be responsible for cell elongation in wild type Basmati 370. The dwarfing gene may affect cell division rate. The decrease in the cell length of internode tissues in *OsGAI/Sd* shows a primary defect in cell-elongation in the stems and leaves. In the internodes, the diameter of cells in parenchyma was reduced from ~120μm in Basmati 370 to ~60μm in *OsGAI/Sd.* The length of the first, second and third internodes in Basmati 370 was 60, 30 and 15 cm while it was 40, 15 and 6 cm, respectively, in *OsGAI/Sd.* The number of cells in a particular transect in each internode in *OsGAI/Sd* was estimated to be reduced by ~35, 48, and 70%, relative to Basmati 370. The reduction of cell numbers per internode suggests reduced cell division in the *OsGAI/Sd* mutant. Sato *et al.* (1999) also observed 50, 54 and 30% reduction in the number of cells in second, third and fourth internode in *OSH15* mutant in rice due to decreased frequency of cell-division in the intercalary meristem.

High seed sterility (40-45%) was also found in the *OsGAI/Sd* mutant. In the dwarf mutant, the viable pollen grains took more time to mature as compared to Basmati 370. It might be due to a defect in GA signaling, as the GA signal is necessary for production of

viable pollen grains (Huang *et al.*, 2003). In GA-related dwarf mutants, male sterility is also caused by impaired outgrowth of stamens. Short stamens limit the chances of proper pollination on the stigma. *ga1* mutants in *Arabidopsis* (a GA signaling mutant) were male-sterile dwarfs (Koornneef and Van Der Veen, 1980) which could be rescued by mutations in DELLA growth repressor proteins (Tyler *et al.*, 2004). Shorter internodes, small seed size, reduced cell size and high tillering ability in the dwarf mutant indicated the pleiotropic effect of the inactivated gene due to T-DNA insertion. Most dwarfing genes exhibit their pleiotropic effects for tillering, grain size, panicle type and other characters. The dwarf mutant can be used for breeding dwarf Basmati because of its favorable effects for lodging resistance and fertilizer response.

Pleiotropic effect of the loss-of-function of dwarfing gene was also reflected in 3:1 segregation ratio for dwarf *vs.* tall and GA-insensitive *vs.* GA-sensitive F_2 plants obtained from the crosses of *OsGAI/Sd* mutant with its wild type Basmati 370. This indicated that the dwarfing gene had pleiotropic effect on GA-sensitivity of the plants in such a way that the dwarf F_2 plants showed GA-insensitive phenotype.

Plants with height ranging from 50 cm to 140 cm and 8 to 30 percent increase in height after GA_3 spray were found among the selfed seeds of homozygous dwarf mutant *OsGAI/Sd* plants. All the dwarf and tall plants were *hpt* positive during PCR amplification. About 55-60% of the plants carrying the *hpt* gene showed variable dwarfness and GA-insensitivity. This may be due the presence of incomplete penetrance and variable expressivity of the mutant. Penetrance is the ability of a gene to express itself in the individuals carrying it in a genotype while expressivity is the extent of expression in various individuals that carry it. Expressivity is thus dependent on

penetrance (Kral, 1999). In the homozygous *OsGAI/Sd* insertional mutant, confirmed on hygromycin resistance and *hpt* amplification, there was a maximum penetrance of 60% while the expressivity among the progenies ranged from severe-dwarf, dwarf, semi-dwarf to tall individual plants which showed 2-8%, 8-10%, 10-30% and more than 30% increase in plant height after GA_3 spray, respectively. Jessup *et al.* (2002) found penetrance-expressivity effects between apomictic and sexual genotypes in Buffelgrass. Park *et al.* (2007) found variable expressivity and complete penetrance in the outgrowth of vestigial leaf-like structures and bracts in *Ds*-insertional mutant of *OSH6* (*Oryza sativa* Homeobox 6) gene in rice.

The F_2 mapping population was developed by crossing dwarf mutant (*OsGAI/Sd*) mutant with distantly related fine rice variety PR106. The F_1 of *OsGAI/Sd* X PR106 and *OsGAI/Sd* X Basmati 370 crosses had height equal to that of *OsGAI/Sd* and the plants showed only up to 10% increase in plant height after GA_3 treatment, indicating that dwarfing gene in *OsGAI/Sd* is dominant. Amplification of *hpt* gene in F_2 mapping population of *OsGAI/Sd* X PR106 cross shows a good fit to 3:1 segregation ratio, suggesting a single copy T-DNA insertion in the dwarf mutant. Ivandic *et al.* (1999) observed 12:3:1 ratio of GA-insensitive: low responding GA-sensitive: high responding GA-sensitive F_2 plants and suggested a digenic inheritance of one dominant and second semi-dominant gene for GA-insensitivity in barley.

Bulk Segregation Analysis and mapping of dwarfing gene in *OsGAI/Sd* mutant, located it on chromosome 3 and linkage with SSR markers RM14645 and RM14667, which were mapped at distance of 1.21cM and 6.49cM, respectively from the dwarfing gene. Ivandic *et al.* (1999) used RFLP and microsatellite markers for a gibberellic acid-

insensitive dwarfing gene (*Dwf2*) on chromosome 4HS in barley. BSA has been used as a screen for qualitative trait loci (QTL) for various traits in rice like cooked kernel elongation (Ahn *et al.*, 1993), partial resistance to blast disease (Wang *et al.*, 1994), sheath blight (Li *et al.*, 1995), aroma (Amarawathi *et al.*, 2008), seed dormancy (Gu *et al.*, 2004) etc. BSA is a widely used tool to find the linkage of PCR based DNA markers, including, simple sequence repeats (SSRs) to target genes controlling important economic traits such as disease resistance, grain yield etc. in several crops like wheat (Balyan *et al.*, 2005; Kumar *et al.*, 2007; Singh *et al.*, 2006c), millets (Lakshmi *et al.*, 2002) and rice (Parida *et al.*, 2006; Sharma *et al.*, 2005, 2005a; Singh *et al.*, 2001).

In order to confirm the mapping results from BSA, TAIL-PCR and genome walking approaches were used as reverse genetics tool in dwarf mutant *OsGAI/Sd* by isolating the flanking region of T-DNA right border. To carry out genome walking and TAIL-PCR, nested primers were designed for T-DNA right and left borders. Since no results were obtained by using left border specific primers in both the approaches, it was concluded that LB might be lost or modified in the course of transformation. This might be due to rearrangements of host DNA that frequently occur at the T-DNA insertion point (Brunaud *et al.*, 2002). According to Tinland (1996), a majority of the T-DNA insertions occur at the right-border at specific loci in dicot species. Sha *et al.* (2004) found that the T-DNA nick positions in tagged rice plants were between 25 bp before the right border repeat to 4 bp after the right border repeat. Muskette *et al.* (2003) and Krysan *et al.*, (2002) reported that TAIL-PCR was more successful when using the LB primers in *Arabidopsis*. The T-DNA transfer is in the right to left direction and many times, left border is found to have deletions during transformation. Eamens *et al.* (2004) reported

deletions of 2-84 bp inside the 30% of RB FSTs and 1-97 bp inside the 70% of LB FSTs in rice. Using three T-DNA right border nested primers and an arbitrary degenerate (either AD1 or AD2 or AD3) primer in consecutive primary, secondary and tertiary reactions in TAIL-PCR, different length of amplification in the T-DNA flanking sequence was obtained in tertiary reaction. Sequencing of the amplified products (obtained by using three different AD primers in tertiary reaction) with T-DNA specific RB3 primer showed significant alignment with the *Oryza sativa* cv. Nipponbare with only single hit at chromosome 3 spanning 7178921 to 7187583 bp position. The T-DNA insertion was present in the second exon of the candidate gene Os03g0236900. The candidate gene encoding sixth subunit of APC/C holo-enzyme contains 16 exons and 15 introns. The 8.6 Kb length of the candidate gene encodes 728 amino acid protein containing a conserved tetratricopeptide repeat (TPR) domain.

To confirm the T-DNA insertion at 7180174th bp position on chromosome 3, two sets of nested gene specific forward and reverse primers flanking the insertion site were designed in such a way that they should amplify about 980 bp sequences in the wild type Basmati 370. Amplification of expected size was obtained in *OsGAI/Sd* but not in Basmati 370 by using different combinations of gene specific and T-DNA specific primers, confirming the insertion on chromosome 3. But, PCR with gene-specific primers gave amplification only in Basmati 370 and not in *OsGAI/Sd* because of the insertion of approximately 10Kb fragment of the T-DNA cassette cannot be amplified by the *Taq* polymerase used for the this PCR. The dwarf F_2 plants cosegregated with the PCR amplification with DTF1+RB2 and *hpt* gene in the F_2 population. This also supported the single copy T-DNA insertion in the dwarf mutant in the present study.

Genome walking was carried out by using T-DNA right border nested primers along with adaptor primer and digesting the genomic DNA of the dwarf mutant with four different enzymes i.e. *EcoRV*, *DraI*, *RsaI* and *HpaI*. But no specific DNA sequences were amplified even up to tertiary reactions. From the T-DNA flanking sequence obtained from TAIL-PCR, it was concluded that the genome walking strategy did not work for dwarf mutant mainly because of the two reasons: (i) location of the restriction site was very far away (for *RsaI* site) upstream of the gene specific primer, and (ii) there were no restriction sites available for the *EcoRV*, *DraI* and *HpaI* to digest the genomic region upstream of the insertion site in dwarf mutant even upto 2 Kb of T-DNA flanking region. However, the restriction sites for blunt end tetra cutter such as *DpnI, HaeIII, PhoI* and *AluI* were present in this region. Hence, use of restriction enzymes *DpnI, HaeIII, PhoI* or *AluI* could have been better for digesting the dwarf mutant DNA for genome walking strategy.

Total RNA was isolated from roots and shoots of 10 days old seedlings of Basmati 370 and *OsGAI/Sd*. The transcript of *OsGAI/Sd* gene was detected in shoots as well as in roots. RT-PCR using reverse transcribed mRNA and primers pair designed on exon region, the expression of *OsGAI/Sd* wild type gene was found in Basmati 370 shoots and roots and not in *OsGAI/Sd*. RT-PCR has been used to evaluate the expression of tagged dwarfing gene in various crops, including rice (Sakamato *et al.*, 2001; Jeong *et al.*, 2002) and *Arabidopsis* (Fu *et al.*, 2001; Fridborg *et al.*, 1999; Dill *et al.*, 2004).

T-DNA flanking sequences obtained from TAIL-PCR in B-3-1 and B-2-2-3 (*OsGAS/Sd*) were matched on chromosome 11 and 3, respectively when aligned with the total genomic sequence of Nipponbare in NCBI database. But both the insertions were

found in the junk region where no gene has been identified in *japonica* and *indica* cultivars. However, the height of *OsGAS/Sd* was approximately 110 cm and the seed length/breadth ratio was less than that of Basmati 370. It can be possible that the wild type gene might control these traits in Basmati 370 but is still uncharacterized. The absence of any distinct phenotype in B-3-1 is evident from its insertion (at 15587862th bp position of chromosome 11) in non-coding region without any gene knockout.

Conserved TPR domain, present in APC/C sixth subunit, plays a role in chaperone, cell-cycle, transcription, protein transport complexes and protein-protein interactions with another TPR protein or a non-TPR protein (Goebl and Yanagida, 1991; Lamb *et al.*, 1995). The number of TPR motifs varies among proteins and 5-6 tandem repeats of TPR domain generate a right-handed helical structure with an amphipathic channel that is thought to accommodate an alpha-helix of a target protein (Scheufler *et al.*, 2000). Examples of TPR-proteins include, Cdc16 (or APC6), Cdc26 (or APC8) and Cdc27 (or APC3) components of the cyclosome/APC, the Pex5p/Pas10p receptor for peroxisomal targeting signals, the Tom70p co-receptor for mitochondrial targeting signals, Ser/Thr phosphatase 5C and the p110 subunit of O-GlcNAc transferase. The TPR domain is also found in *Arabidopsis* GA-signaling mutant SPY (first GA-signaling gene to be cloned) (Jacobsen *et al.*, 1996). The SPY protein contains 10 TPRs and it functions as a negative regulator of GA-response. Orthologous of SPY have been isolated in barley (cv. Himalay; HvSPY), in petunia (PhSPY) and in tomato (LeSPY) (Robertson *et al.*, 1998; Izhaki *et al.*, 2002). The TPR domain is important for function of SPY in *Arabidopsis*, due to its role in protein interaction for GA-signaling (Tseng *et al.*, 2001).

Multiple sequence alignment of different TPR domain containing amino acid sequences PhSPY, LeSPY, AtSPY, HvSPY and APC6 (in rice) was performed using ClustalW multiple sequence alignment (http://www.ebi.ac.uk/). The alignment score was highest between PhSPY and LeSPY. APC6 showed highest alignment with LeSPY. Among 523 hits of orthologous genes in different species, the *Arabidopsis thaliana* NCBI gene entry AT1G78770 (Anaphase Promoting Complex component 6) showed highest identity score of 0.724 with the candidate gene. In rooted phylogenetic tree, amino-acid sequence encoded by the candidate gene was clustered with TPR-domain containing *Oryza sativa* ABF94847, *Schizosaccharomyces pombe* NP_59330 and *Drosophila melanogaster* NP_477397 genes. Among 67 paralogous genes, searched through database (KEGG), the candidate gene showed highest identity (0.400 score) with NCBI gene ID Os03g0618300 (without TPR domain), located at 23252738 -23250755 bp position on chromosome 3, which encodes an isopencillin N-synthase family protein.

The APC/C is an ubiquitin-protein ligase (E3) which controls important transition in mitosis by ubiquitinating regulatory proteins and thereby targeting them for proteolysis by the 26S proteasome (Irniger *et al.*, 1995). To control the exit from mitosis and to prevent premature entry into S-phase, the proteolytic events triggered by the APC are required to release sister chromatid cohesion in anaphase. APC/C is a highly conserved complex consisting of thirteen subunits (Gieffers *et al.*, 2001; Capron *et al.*, 2003) of which, two of them (APC2 and APC11) have homology to subunits in the SCF (an another E3 ligase protein; the name is derived from the first letter of three of its four subunits: SKP1, Cullin, and the F-box protein): APC2 to cullin and APC11 to RING protein (Tang *et al.*, 2001). In plants, the genes APC2, APC/NOMEGA, and

APC/HOBBIT (HBT) have been recently characterized. The Cdc16 (or APC6), Cdc23 (or APC8) and Cdc27 (or APC3) subunits of the APC each contain 8-10 copies of the 34-amino-acid TPR motif. Mutations in TPR-containing subunits cause cell-cycle arrest at the metaphase-to-anaphase transition. APC1 is the largest subunit of APC (Passmore, 2004). Cdc16 (or APC6) creates linkage with an APC subunit Doc1 (or APC10) to cause the holo-enzyme to partially close around a substrate (Vodermaier *et al.*, 2003).

Several dwarf mutants in rice have been reported which are related to gibberellic acid signal transduction pathway and are gibberellic acid insensitive. The dwarf mutant SLR1 is very much similar to semi-dwarf wheat cultivars containing DELLA-truncated *RHT* genes (Itoh *et al.*, 2002). Report of Ueguchi-Tanaka *et al.* (2005) revealed that GID1 is the receptor protein for GA in rice. In the model of gibberellic acid (GA) signaling proposed so far in rice, DELLA protein SLR1 is the target protein for degradation by SCF^{GID2} E3 complex under high concentration of gibberellic acid in the nucleus. The receptor of gibberellic acid in rice i.e. GID1 binds to SLR1 protein depending on the level of GA in the nucleus. The GID1-SLR1 complex binds with SCF^{GID2}-ubiquitin complex and 26S proteasome to degrade SLR1 protein. The repressed state of GA dependant growth is then released. Any mutation in the components involved in GA-signaling in rice may cause gibberellic acid insensitive phenotype. Different GA signaling mutants like *Gid1, Gid2, d1* and *slr1* have already been reported. In the present study a new model for GA-signaling in rice can be proposed where APC/C instead of SCF^{GID2} may play role in the degradation of SLR1 protein, thus triggering the transcription of GA-biosynthetic genes by different transcription factors. The proposed model can be explained with the help of Fig 5.1. Since the candidate gene product APC6

is the subunit of large holo-enzyme APC/C, mutation caused due to T-DNA insertion in the wild type gene in Basmati 370 in the present study, led to GA-insensitive dwarf phenotype of *OsGAI/Sd* mutant.

To suppress specific gene functions, RNA silencing has been successfully used in *Arabidopsis* (Chuang and Meyerowitz, 2000) and rice (Hayama *et al.*, 2003 ; Moritoh *et al.*, 2005). Miki *et al.* (2005) suggested that the RNA silencing machinery is highly conserved in rice and dsRNA-mediated RNA silencing is a useful tool for the functional analysis of highly conserved multigene families in rice. Miki and Shimamoto, (2004) have recently developed efficient Gateway vectors for RNA silencing of rice genes. RNA silencing can be used to complement existing tagged lines to suppress all of the members of a gene family. Thus, silencing of the gene by RNAi is another approach in Basmati 370 for functional validation of dwarfing gene in *OsGAI/Sd.*

Rice insertion lines have been generated in laboratories world-wide using retrotransposons (*Tos17*), T-DNA, *Ac/Ds* and *En/I* as mutagens. The Flanking Sequence Tags (FSTs) of the insertional line, publicly available at the RiceGE and OryGeneDB database, can be used as alternative means for validation of gene function of dwarfing genes. No FST for the *OsGAI/Sd* was found in RiceGE database, but in OryGeneDB, four FSTs namely DX888666, A20869, A32498, C09262 and CZ552601 were found in the gene region of the candidate gene. Among these, a FST A32498 in an exonic region would be a good candidate for functional validation. No FST was found for B-3-1 in 1 kb up and downstream region from insertion site while in case of *OsGAS/Sd*, two FSTs were found in RiceGE database. The seeds from OryGeneDB and RiceGE for these insertional lines will be procured for phenotypic and molecular analysis.

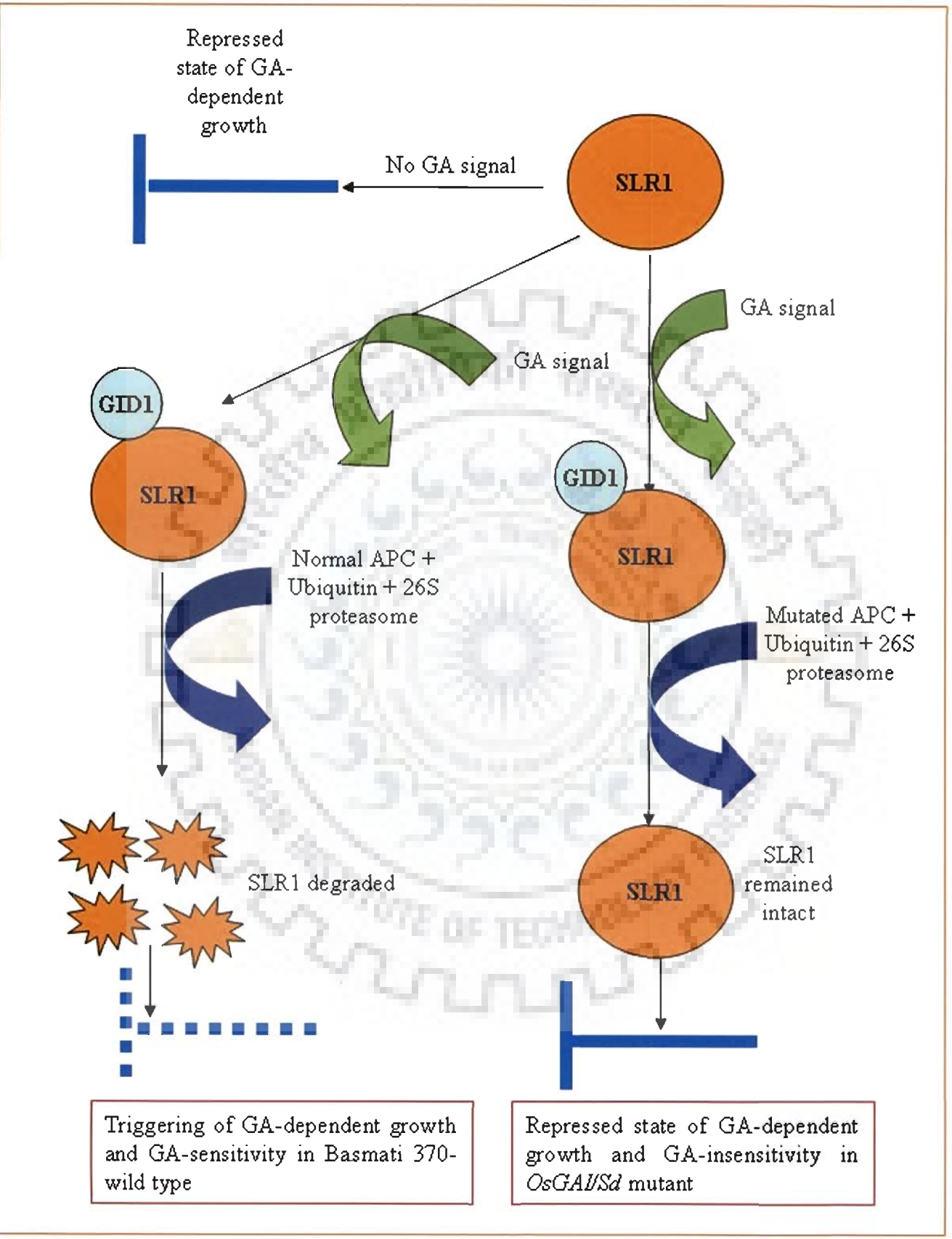

Figure 5.1 Putative model of GA-signaling in *OsGAI/Sd*

Two element *Ac-Ds* system can be another strategy to find additional alleles of the dwarfing genes. In the two component system, a *Ds* element inserted in a parental line can be moved to another position by crossing with a line carrying the *Ac* transposase gene. The advantage of this strategy is that a limited number of lines have to be generated with mapped inserts. Following crossing with the *Ac* line containing the transposase gene, a large number of mutants can be recovered in the same region, thus saturating a chromosome region with insertions. The frequency of transposition in *Ac-Ds* system decreases very rapidly as the distance from donor site increases (Ito *et al.*, 2002). But this property is retained in transgenic plants and thereby allowing the *Ac-Ds* system for closely targeting insertions in the immediate vicinity of donor site (Zhang *et al.*, 2003). Several groups are presently generating collections of maize *Ac-Ds* tagged rice mutants (Enoki *et al.*, 1999; Izawa *et al.*, 1997; Chin *et al.*, 1999). The insertional mutants in the present study have a *Ds* element in the Hm^R*Ds* construct. To mobilize the *Ds* element present in the Hm^R*Ds*, the dwarf and semi-dwarf mutants can be crossed with a transgenic rice line containing *Ac* element. This is likely to give more alleles within the gene due to linked transposition in the same and other genes in the vicinity of T-DNA/*Ds* insertions.

***

CHAPTER 6

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5. References

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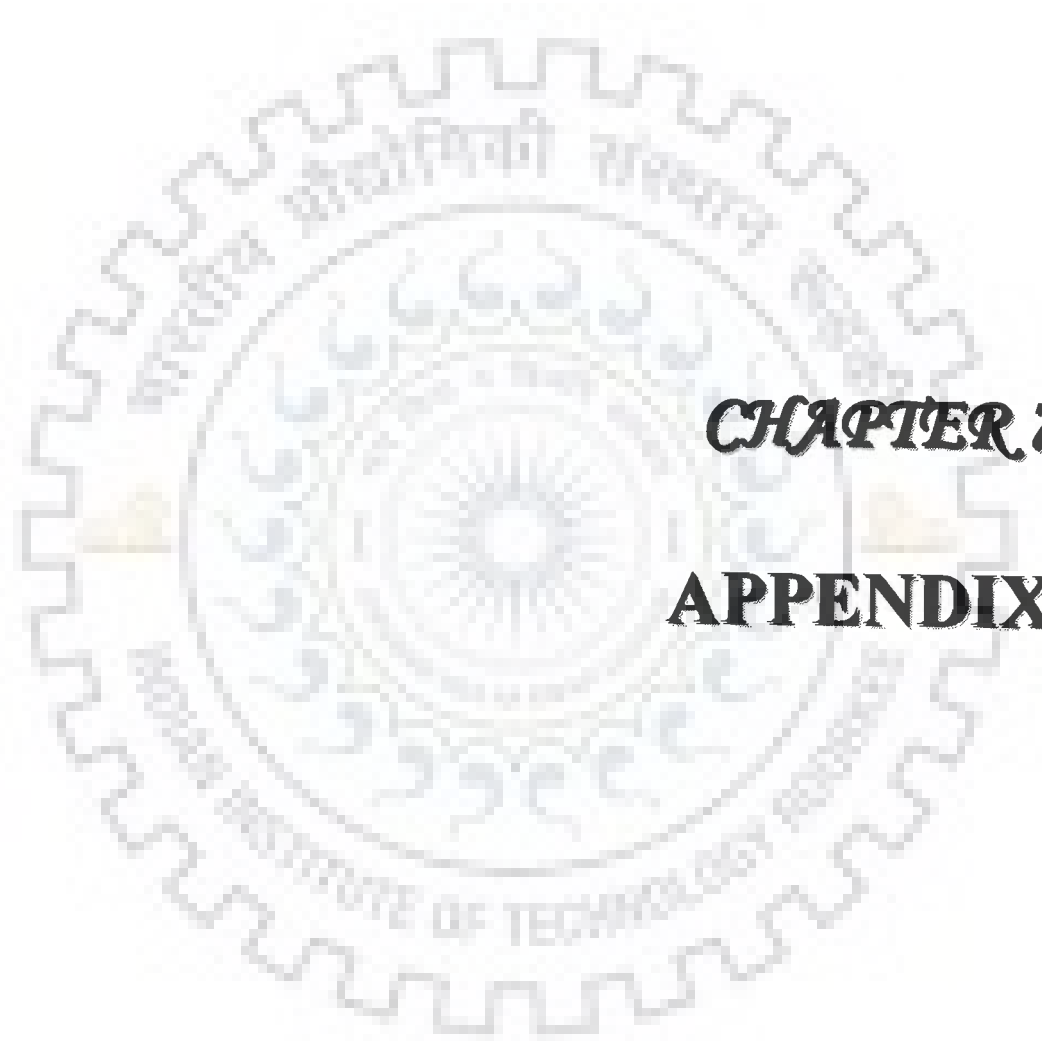

CHAPTER 7

APPENDIX

Appendix I: Phenotypic data of ***OsGAI/Sd*** **X PR106** F_2 mapping population

Plant Number	Plant Height (cm)	GA_3 response	Tiller number	Days to 50% flowering
1	80	13.51	22	117
2	87	17.02	13	112
3	87	2.00	8	114
4	99	31.70	18	98
5	52	34.61	6	106
6	121	39.18	11	111
7	72	33.33	5	100
8	120	38.20	15	118
9	58	25.00	9	120
10	139	27.16	16	111
11	120	42.25	9	115
12	117	45.94	20	112
13	40	20.00	7	108
14	120	12.00	9	101
15	139	6.52	14	111
16	72	16.36	25	101
17	70	3.63	6	106
18	64	12.24	5	119
19	66	17.02	13	111
20	70	33.33	6	108
21	74	8.00	12	100
22	73	15.38	7	112
23	86	80.85	7	117
24	133	16.25	8	117
25	148	35.00	6	111
26	121	27.02	6	111
27	99	25.33	11	108
28	116	34.78	11	111
29	146	62.02	8	108
30	120	24.09	11	111
31	112	45.45	12	101
32	105	56.33	8	108
33	101	27.45	8	122
34	147	142.55	8	123
35	67	19.04	8	142
36	90	25.53	17	111
37	83	13.20	11	111
38	60	22.22	6	111
39	76	6.12	11	116
40	110	37.50	5	142
41	99	28.57	7	130

Plant Number	Plant Height (cm)	GA_3 response	Tiller number	Days to 50% flowering
42	110	54.21	8	112
43	61	6.81	10	143
44	82	11.11	10	130
45	67	16.66	8	118
46	138	39.02	12	137
47	91	43.63	7	117
48	62	9.09	8	115
49	76	3.77	9	143
50	68	9.30	6	144
51	62	7.14	9	143
52	67	6.00	7	143
53	109	27.20	4	141
54	58	5.00	18	168
55	111	28.98	7	169
56	77	10.00	6	132
57	104	37.80	10	164
58	150	28.75	9	148
59	63	19.56	6	145
60	117	32.85	11	146
61	119	49.42	10	132
62	103	12.06	15	136
63	62	25.64	6	147
64	109	46.15	16	128
65	63	27.90	10	173
66	105	19.23	15	175
67	100	18.75	6	175
68	144	50.66	6	167
69	68	19.14	11	101
70	73	5.12	14	141
71	102	7.93	11	122
72	64	19.44	6	117
73	67	15.21	9	118
74	72	15.55	7	130
75	88	14.00	11	126
76	79	11.32	11	111
77	80	20.93	10	118
78	81	16.80	8	121
79	117	41.25	9	138
80	142	26.37	8	119
81	74	10.20	9	119
82	123	34.92	6	106
83	122	68.57	9	101
84	60	23.33	8	121

Plant Number	Plant Height (cm)	GA_3 response	Tiller number	Days to 50% flowering
85	110	40.00	9	141
86	119	47.50	11	108
87	117	37.87	11	127
88	148	28.94	8	141
89	86	5.00	8	122
90	91	12.76	9	140
91	81	11.36	8	116
92	71	17.50	6	137
93	142	45.94	14	138
94	60	13.88	8	136
95	79	19.56	13	137
96	110	23.18	9	142
97	27	9.09	8	122
98	97	14.70	9	145
99	135	25.33	11	138
100	66	11.76	10	142
101	72	7.50	8	117
102	58	10.86	13	142
103	131	45.45	20	98
104	79	10.41	14	106
105	75	12.5	10	142
106	137	28.94	10	142
107	85	9.61	9	108
108	76	12.50	5	130
109	85	39.72	9	142
110	135	39.47	11	126
111	42	19.23	4	136
112	69	8.69	9	110
113	89	19.56	8	124
114	118	20.45	12	110
115	79	14.28	13	127
116	108	23.50	14	117
117	101	29.16	8	146
118	79	5.76	9	136
119	75	10.20	12	123
120	70	20.93	17	123
121	145	42.85	14	136
122	106	18.18	13	122
123	90	23.52	7	136
124	156	24.17	13	132
125	148	37.36	7	116
126	68	20.51	8	137
127	77	34.88	10	110

Plant Number	Plant Height (cm)	GA_3 response	Tiller number	Days to 50% flowering
128	72	18.91	14	135
129	130	119.14	11	115
130	150	36.66	9	141
131	144	27.16	10	141
132	85	28.16	9	126
133	68	13.04	9	136
134	68	15.38	12	137
135	94	32.60	12	115
136	79	13.63	11	142
137	78	14.63	7	136
138	68	16.27	7	136
139	63	13.04	5	142
140	157	36.14	9	123
141	80	17.64	8	99
142	50	17.64	8	114
143	80	15.21	14	111
144	74	26.47	7	111
145	136	46.15	10	116
146	62	17.14	11	130
147	59	12.50	14	130
148	105	36.48	13	136
149	140	53.33	14	111
150	70	13.63	13	136
151	63	7.31	5	111
152	65	18.91	4	116
153	152	21.05	4	116
154	72	47.56	9	106
155	147	20.00	6	142
156	131	39.50	9	142
157	101	28.10	13	106
158	97	20.60	6	111
159	75	13.89	10	130
160	123	45.70	7	116
161	100	23.10	11	130
162	112	32.10	8	111
163	117	41.30	11	136
164	87	24.60	13	136
OsGAI/Sd	**80**	**9.75**	**15**	**128**
PR106	**110**	**57.01**	**15**	**105**

Appendix II: Phenotypic data of ***OsGAI/Sd*** **X Basmati 370** F_2 population

Plant Number	Plant Height (cm)	GA_3 response	Tiller number	Days to 50% flowering
1	175	31.95	14	124
2	96	21.56	8	124
3	180	32.58	10	122
4	100	3.33	11	121
5	150	68.00	3	124
6	108	12.50	14	123
7	113	8.19	14	125
8	105	4.76	11	122
9	80	12.24	13	122
10	180	34.02	16	121
11	91	11.76	6	125
12	100	6.55	11	122
13	71	8.10	6	125
14	172	16.66	11	122
15	83	27.45	12	152
16	105	13.79	8	152
17	74	7.50	7	151
18	180	26.08	13	124
19	105	2.80	12	122
20	97	4.60	9	120
21	190	22.91	11	122
22	102	5.88	5	124
23	104	9.83	9	122
24	185	56.81	13	122
25	111	4.68	10	120
26	76	22.22	13	126
27	100	11.66	10	123
28	170	32.58	12	121
29	108	20.63	15	121
30	86	17.77	4	132
31	89	25.00	13	136
32	178	56.25	33	113
33	100	12.06	7	122
34	100	6.77	9	121
35	94	3.77	7	123
36	98	5.55	8	123
37	99	3.07	15	119
38	99	3.27	10	121
39	101	7.93	8	121
40	101	6.06	14	121
41	99	6.66	8	123

Plant Number	Plant Height (cm)	GA_3 response	Tiller number	Days to 50% flowering
42	88	4.61	7	123
43	150	21.34	7	134
44	192	18.00	13	122
45	195	68.29	13	121
46	62	37.83	3	130
47	195	64.36	6	120
48	105	12.69	14	118
49	182	92.20	10	119
50	101	5.08	11	120
51	180	84.00	10	127
52	105	10.00	11	126
53	100	11.11	9	123
54	165	101.60	9	125
55	72	4.16	7	120
56	103	30.95	12	122
57	100	9.37	8	121
58	91	3.63	12	118
59	64	9.09	5	137
60	69	9.30	9	127
61	63	6.25	5	128
62	88	6.52	10	122
63	104	4.83	14	121
64	178	64.19	9	128
65	66	14.28	8	130
66	104	34.04	12	123
67	64	8.69	9	95
68	108	1.51	15	122
69	105	11.11	14	121
70	165	49.43	10	123
71	106	56.25	11	125
72	77	30.95	10	130
73	80	21.27	12	125
74	84	22.22	9	125
75	71	13.04	8	128
76	105	17.18	14	152
77	68	11.11	15	123
78	86	9.09	4	130
79	95	13.33	8	125
80	82	6.00	8	122
81	86	4.28	14	120
82	157	27.88	15	135

Plant Number	Plant Height (cm)	GA_3 response	Tiller number	Days to 50% flowering
83	169	19.41	11	122
84	94	3.17	10	120
85	106	1.51	3	144
86	104	6.77	15	126
87	98	1.58	11	122
88	170	11.34	10	121
89	173	40.44	9	122
90	99	6.45	12	122
91	100	6.15	11	122
92	67	8.00	13	132
93	99	5.00	12	123
94	166	18.05	12	123
95	69	2.17	13	133
96	60	20.00	8	135
97	183	13.68	14	152
98	88	6.89	4	129
99	86	5.26	8	130
100	96	3.17	2	123
101	93	2.94	9	124
102	97	3.33	9	125
103	180	31.68	12	122
104	178	35.92	12	122
105	146	46.42	9	124
106	95	5.45	9	123
107	75	5.76	6	124
OsGAI/Sd	**80**	**9.75**	**15**	**128**
Basmati 370	**160**	**31.00**	**13**	**125**

PUBLICATIONS

Publications

Paper and poster presentations:

- Paper Presented and abstract published in National Symposium on 'Basmati Rice Research: Current Trends and future prospects. Sept 6-7, 2005, SVBUA&T, Meerut. **Mankesh Kumar**, G. S. Randhawa and H. S. Dhaliwal (2005). Characterization of a novel *Ds* transposon insertional dwarf mutant (*OsGAI/Sd*) of Basmati 370.

- Paper presented and abstract published in National biotechnology Conference-2006, Current Trends and Future Perspectives, 2-3 September 2006. Department of Biotechnology, IIT Roorkee and Indian Federation of Biotechnologists (IFB-India). 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".

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- Paper presentation and abstract published in AISABBMR: Dec 26-28, University of Bikaner, Bikaner. Deepak Rajpurohit, P.Osman Basha, **Mankesh Kumar**, Anju Bhalla G. S. Randhawa and H. S. Dhaliwal (2006). "Pyramiding of genes for bacterial blight resistance and dwarfism in Type 3 Basmati using molecular markers".

Papers communicated:

- **M Kumar**, D Rajpurohit, P. O. Basha, A. Bhalla, G. S Randhawa and H. S. Dhaliwal. (2008). Genetic control of after-ripening seed dormancy in Basmati rice. In *Seed Science Research.*