EXPRESSION PROFILING AND FUNCTIONAL VALIDATION OF RICE INSERTIONAL MUTANTS *OsAPC6* AND *Ossl*

A THESIS

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

of

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in

BIOTECHNOLOGY

by

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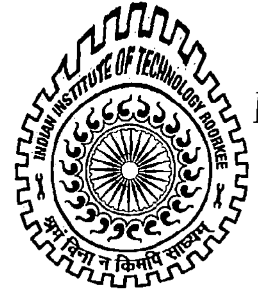

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **EXPRESSION PROFILING AND FUNCTIONAL VALIDATION OF RICE INSERTIONAL MUTANTS *OsAPC6* AND *Ossl*** in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during the period from January 2009 to January 2012 under the supervision of Dr. R. Prasad, Associate Professor and Dr. H. S. Dhaliwal, Professor (Rtd.), Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee, India.

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

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ABSTRACT

ABSTRACT

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 has been a model monocot for functional genomics research due to its small genome size (430Mb) relative to other cereals, its ease of transformation, high density genetic maps, physical maps, large-scale analysis of expressed sequence tags (ESTs) and extensive synteny shared with other cereals, availability of large number of flanking sequence tags (FSTs) and economic importance.

The delicately curved, long grained, highly aromatic rice which elongates and cooks soft and fluffy is categorized as Basmati which enjoys privileged treatment both in domestic and international markets commanding premier market price. Traditional Basmati varieties grown in India are low yielding, tall, and lodge even under low nitrogen fertilizer dose. The completion of the rice genome sequence provided a new platform for its functional genomics. Assigning functions to these genes will help greatly to improve both quality and quantity of rice. For assigning functions to the predicted genes various approaches such as insertional mutagenesis, serial analysis of gene expression (SAGE) and RNAi have been used.

A dwarf *OsAPC6* with reduced seed set and insensitivity to gibberellic acid (GA) and another seedling lethal (*Ossl*) insertional mutant were isolated using independent *Agrobacterium mediated* transformation of Basmati 370. Thermal asymmetric interlaced PCR (TAIL-PCR) approach indicated the position of insertion at chromosome 3 and 11 of *OsAPC6* and *Ossl*, respectively. Southern hybridization confirmed the single copy T-DNA insertion in the *OsAPC6* mutant. In the present study, further characterization of these mutants was taken up using confocal laser scanning microscopy, microarray and RNAi.

The role of *APC6* in cell division and the reason behind reduced seed set (45%) in the *OsAPC6* mutant was elucidated using confocal laser scanning microscopic technique. Meiotic analysis at metaphase, anaphase and early telophase I of the pollen mother cells of the mutant showed normal chromosome pairing with twelve bivalents,

normal movement of homologous chromosomes to poles during anaphase, normal telophase and normal pollen tetrads. On an average 92-98% pollen grains were stainable and hence viable in most of the florets. The results of normal meiotic chromosome pairing and high pollen stainability similar to that of the wild type Basmati 370 indicated that microsporogenesis and male gametophyte development were normal in *OsAPC6* mutant. The embryo sac development was studied using CLSM and found that the fertilization was normal as zygote was formed and the degenerating antipodal cells were also observed. On further analysis of immature embryo sacs it was found that female gametophytes of wild type Basmati 370 had normal mitotic division while in the mutant gametophyte a cell at the micropylar end with brighter nuclei and a dividing cell at the chalazal end with two nuclei were observed. The chalazal end cell was probably undergoing second mitosis whereas in the micropylar end cell the division was delayed or arrested. The female gametophytes were arrested at three-nucleate or seven nucleate stages, indicating that loss-of-function of *APC6* impairs megagametogenesis after first or second mitotic division. There was, however, no endosperm development in a high proportion of female gametophytes of the *OsAPC6* mutant due to abnormal polar nuclei formation. The reduced seed set upto 40-45% in *OsAPC6* could be attributed to the absence of endosperm. Further, the RT-PCR, *hpt* and hygromycin resistance results showed that the mutant phenotype was due to knock-down of the *APC6* gene and mutant dwarf plants with reduced seed set could be maintained only in heterozygous condition.

The expression profiling of the *OsAPC6* and *Ossl* mutants using the microarray was studied. The effects of T-DNA insertion on the entire transcriptome of the *OsAPC6* and *Ossl* mutants have been elucidated. The transcriptome profiles of a T-DNA insertional mutant *OsAPC6* and wild type Basmati 370 was studied using microarray. The average hybridization and percentage of probe sets detected in *OsAPC6* mutant was found to be 42.6% of the total 57,381 probe sets. A total of 92 genes were found to have differential expression out of which 81 genes (88.04%) were found to be up-regulated, while only 11 genes were down-regulated. Gene Ontology studies of these genes revealed that 89% belonged to molecular function (MF), 69.5% were of biological process (BP) and 20.6% came under the category of cellular component (CC). The expression profile of *OsAPC6* mutant revealed that the genes encoding

enzymes involved in GA biosynthesis were highly up-regulated. These results indicated that the biosynthesis of GA in the mutant plant is normal, or even higher than the wild type. An increase in the chlorophyll content was further estimated in mutant as compared to the wild type. In the microarray study a large number of genes encoding the chloroplast precursor were found to be up-regulated. The APC6 protein encoding gene (LOC_Os03g13370) was found to be down-regulated in the mutant. The sensing of GA is mediated by the gibberellin insensitive dwarf 1 (GID1) receptor protein, and the complex between GA and GID1 allows the capture of the growth-repressing DELLA protein slender rice 1 (SLR1) which forms a GA-GID1-SLR1 complex. The SLR1 protein in this tripartite which acts as a repressor is then targeted by SCF^{GID2} ubiquitin complex for proteolytic degradation. This results in release of repressive state of the GA action to signal transducing state. In our microarray data, the genes encoding for GID1 and SLR1 were found to be up-regulated, indicating that the signaling is normal till this tripartite formation. A pathway for GA signaling where APC/C plays a role in SLR1 degradation instead of SCF^{GID2} complex was speculated. Since the APC6 is a part of holo-enzyme APC/C, which has a role in ubiquitin-mediated proteolytic degradation, mutation in APC6 probably somehow does not allow the degradation of SLR1 protein, thereby leading to GA-insensitive dwarf phenotype of *OsAPC6* mutant.

Another T-DNA insertional mutant of Basmati 370, *Ossl* showed 5-15% of albino seedlings during germination. These albino seedlings died soon after germination while among non-albino 10-20% died slowly without tillering after transplantation. The transcriptome expression analysis using microarray showed average hybridization and percentage of probe sets detected in *Ossl* mutant was found to be 42.4% and a total of 86 genes were found to have differential expression out of which 52.6% genes were found to be up-regulated. Gene Ontology studies of these genes revealed that 53.4% belong to molecular function (MF), 76.7% were of biological process (BP) and 24.4% come under the category of cellular component (CC). The expression analysis of the seedling lethal mutant revealed some genes related to the development of chlorophyll including lipoxygenase gene which is a chloroplast precursor which was found to be down-regulated by more than 10 folds.

The domain architecture of APC6 protein using bioinformatics studies of this protein among different organisms. ClustalW analysis and multiple alignment was done to study the conservation of this protein. *In silico* homology modeling of Basmati APC6 protein was done to locate the tetratrico peptide repeats (TPR) motifs in APC6. The phylogenetic analysis and multiple alignment results of APC6 protein of Basmati 370 and other organisms revealed a high conservation of this protein from prokaryotes to eukaryotes. These results also indicated the presence of TPR domains involved in protein-protein interaction. The TPR domains are present in a number of proteins that are functionally unrelated, and mediate a variety of different protein-protein interactions and were discovered in the first identified APC subunits. They are also involved in chaperone, cell-cycle, transcription, and protein transport complexes.

RNAi was used to silence the *APC6* gene using *in planta* and biolistic transformation. The RNAi construct was prepared by using Gateway® cloning technique in pANDA cloning vector. The target gene was PCR amplified and cloned in pENTR/D-TOPO entry vector. The ligated product was then transformed in *E. coli DH5α* and the selection of recombinant colonies was done using kanamycin in growth medium as the entry vector contained kanamycin resistance gene. The insertion of target gene was confirmed by PCR using target gene specific primers. The plasmid DNA containing the target gene was inserted in destination vector pANDA using LR clonase from invitrogen. The ligated product was again transformed in *E. coli DH5a* and the selection of recombinant colony was done using kanamycin in growth medium as the destination vector has kanamycin resistance gene. The insertion was confirmed by PCR using gene specific and GUS gene primer pairs. The LR cloned product was transformed in *Agrobacterium tumefaciens* strain *LBA4404* containing rifampicin resistance gene. The transformation was further confirmed using colony PCR, using gene specific primer pairs which gave a product length of 177 bp. The construct was transformed to rice plants by two different approaches. The embryogenic rice calli were infected with pANDA vector containing the target gene by biolistics. On the other side, via *in planta* transformation two days old germinated rice seeds were infected with *Agrobacterium* culture containing RNAi construct. Fertile transgenic plants have been obtained which will be used for selection of RNAi transformants using G148 antibiotic and other molecular analysis.

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CONTENTS

I	*ABSTRACT*	*i-iv*
II	*ACKNOWLEDGEMENTS*	*v-vii*
III	*CONTENTS*	*viii-xi*
IV	*LIST OF TABLES*	*xii*
V	*LIST OF FIGURES*	*xiii-xiv*
VI	*ABBREVIATIONS USED*	*xv-xvi*
1.	**INTRODUCTION**	*1-5*
2.	**REVIEW OF LITERATURE**	*6-42*
	2.1 Rice as major food crop	6
	2.2 Rice as a model crop plant	6
	2.3 Basmati rice	7
	2.4 Functional genomics and insertional mutagenesis	8
	2.5 Ubiquitin proteasome pathway	10
	2.5.1 26 S proteasome	11
	2.5.2 E3 ubiquitin ligase	12
	2.5.3 Anaphase promoting complex	13
	2.6 Cell cycle and cyclins	16
	2.7 Role of APC/C in cell cycle progression	16
	2.8 Female gametophyte development	18
	2.9 Confocal laser scanning microscopy	19
	2.10 Gibberellic acid responses	22
	2.10.1 Gibberellic acid metabolism	22
	2.10.2 Gibberellic acid signal transduction	23
	2.10.3 Gibberellic acid mutants	24
	2.10.4 Role of DELLA protein in gibberellic acid signaling	30
	2.11 Microarray a tool for expression analysis at transcriptome level	32
	2.12 Genetic transformation of rice	34
	2.12.1 *Agrobacterium*-mediated transformation	34
	2.12.2 Biolistic-mediated transformation	36
	2.12.3 *In planta* transformation	38
	2.13 Functional validation of gene using RNAi	38
	2.14 Bioinformatics as a tool for protein interaction prediction	41

3. FEMALE STERILITY IN RICE INSERTIONAL MUTANT *OsAPC6* ***43-59***

3.1 Introduction 43

3.2 Materials and methods 45

3.2.1 Plant material 45

3.2.2 Cytological analysis 46

3.2.3 Pollen viability 46

3.2.4 Fixation of material for CLSM 46

3.2.5 Staining and mounting of material 46

3.2.6 Scanning of embryo sac 47

3.2.7 Reverse Transcriptase PCR 47

3.3 Results 48

3.3.1 Chromosome pairing and pollen viability in *OsAPC6* *48*

3.3.2 Female fertility 49

3.3.3 Female gametophyte development 52

3.3.4 Expression of *APC6* in the wild type and mutant plants 55

3.4 Discussion 57

4. TRANSCRIPTOMIC EXPRESSION ANALYSIS OF *OsAPC6* AND *Ossl* MUTANTS ***60-82***

4.1 Introduction 60

4.2 Materials and methods 62

4.2.1 Plant material 62

4.2.2 RNA isolation and quantification 62

4.2.3 aRNA preparation and hybridization 62

4.2.4 Gibberellic acid response 63

4.2.5 Chlorophyll estimation 64

4.2.6 Quantitative Real-time PCR analysis 64

4.3 Results 65

4.3.1 *OsAPC6* mutant 65

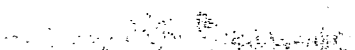
4.3.1.1 Phenotypic analysis of *OsAPC6* mutant 65

4.3.1.2 Gibberellic acid response on shoot elongation 66

4.3.1.3 Genome wide expression analysis 66
4.3.1.4 Genes related to chlorophyll content 72
4.3.1.5 Genes related to gibberellic acid response 72
4.3.1.6 Validation of microarray data 72
4.3.2 *Ossl* mutant 72
4.3.2.1 Phenotypic analysis of *Ossl* mutant 72
4.3.2.2 Genome wide expression analysis 73
4.3.2.3 Differentially regulated genes in *Ossl* 75
4.3.2.4 Validation of microarray data 78
4.5 Discussion 79

5. *IN SILICO* ANALYSIS AND MODELING OF APC6 PROTEIN ***83-97***
5.1 Introduction 83
5.2 Materials and methods 84
5.2.1 Sequence and phylogenetic analysis 84
5.2.2 Comparative molecular modeling 85
5.2.3 Molecular dynamics simulations 86
5.3 Results 87
5.3.1 Phylogenetic analysis 87
5.3.2 APC6 three-dimensional structure analysis 94
5.4 Discussion 96

6. PREPARATION AND TRANSFORMATION OF RNAi CONSTRUCT FOR *APC6* GENE ***98-110***
6.1 Introduction 98
6.2 Materials and methods 98
6.2.1 Plant material 98
6.2.2 RNA isolation and cDNA synthesis 98
6.2.3 Target gene amplification and its recovery for cloning 99
6.2.4 Cloning and transformation of target gene in Entry clone 100
6.2.5 LR recombination reaction and transformation to *E. coli* 101



6.2.6 Plasmid DNA isolation and confirmation of LR reaction 101

6.2.7 Transformation of recombinant LR clone in *Agrobacterium* 102

6.2.8 Verification of positive clones 102

6.2.9 *In planta* transformation 102

6.2.10 Screening of transformed plants 103

6.2.11 Callus induction for biolistic transformation 103

6.2.12 Particle gun-mediated genetic transformation and regeneration 103

6.2.13 Confirmation of transgenics 104

6.3 Results and Discussion 104

6.3.1 Cloning of target gene into entry clone 104

6.3.2 Cloning and confirmation of target gene into destination vector 105

6.3.3 Confirmation of RNAi construct in *Agrobacterium* 107

6.3.4 Results of *in planta* transformation 107

6.3.4 Regeneration and particle bombardment of rice calli 108

7. CONCLUSIONS AND FUTURE SCOPE ***111-112***

8. REFERENCES ***113-151***

9. APPENDICES ***152-160***

9.1 Appendix 1 Tables of microarray data 152

9.2 Appendix 2 Miame database files 155

9.3 Appendix 3 Composition of media used 157

9.4 Appendix 4 Publications 159

LIST OF TABLES

Table No.	Title	Page No.
2.1	Components of APC/C complex in rice	14
3.1	Female sterility observed in mature panicles of mutant *OsAPC6* and wild type Basmati 370 plants	49
3.2	CLSM analysis of megagametophyte development in fixed florets from panicles of *OsAPC6* mutant plants	51
3.3	Hygromycin resistance in well-developed seeds of four plants of *OsAPC6* mutant and the wild type Basmati 370	52
4.1	Different categories of genes showing differential expression in *OsAPC6* mutant in comparison to Basmati 370	69
5.1	TPR Domains in characterized APC6 protein	90

LIST OF FIGURES

Fig. No.	Title	Page No.
2.1	Functional genomics approaches	9
2.2	The 26S proteasome	12
2.3	The APC complex and its subunits	15
2.4	Rice floret and female gametophyte	19
2.5	Information flow in a generic CLSM	20
2.6	Gibberellic acid biosynthesis pathway	23
2.7	Mechanism of RNA interference (RNAi)	40
3.1	Chromosome pairing and pollen grain stainability in *OsAPC6* mutant	48
3.2	Female fertility in rice panicles showing reduced seed set in mutant	50
3.3	Female gametophyte at different stages of development in wild type Basmati 370 and *OsAPC6* mutant	53
3.4	Megagametogenesis in *OsAPC6* mutant	54
3.5	RT-PCR results and *Hpt* amplification in wild type and mutant plants	56
3.6	T-DNA insertion in the *APC6* gene and position of primer pair A and B used for RT-PCR	56
4.1	Phenotype of *OsAPC6* mutant and gene structure	65
4.2	Expression analysis of *OsAPC6* mutant using microarray	67
4.3	Histogram showing differentially expressed genes in the mutant as compared to the wild type	69
4.4	Phenotype of Seedling Lethal (*Ossl*) mutant	73
4.5	Scatter plot of *Ossl* using ArrayStar	74
4.6	Scatter plot and Heat Map for *Ossl* mutant showing the differentially expressed genes as compared to wild type Basmati 370	75
4.7	Histogram of functionally categorized genes for *Ossl* mutant showing the differentially expressed genes as compared to wild type Basmati 370	75
4.8	qRT data to show the validation of the differential regulation of genes in Microarray data	79

4.9	Proposed GA signaling pathway for rice.	82
5.1	CDS and amino acid sequence of *Oryza* APC6 protein (Source_Gramene Database)	87
5.2	Phylogenetic tree analysis of APC6 protein	88
5.3	Multiple sequence alignment of amino acid sequences for APC6 protein in different organisms using Clustal W software	89
5.4	*In silico* analysis of APC6 proteins of different organisms	91
5.5	TPR Domains of *Oryza sativa* APC6 (shown in red and blue colour) as predicted from alignment of *Saccharomyces pombe* Cut9 protein and *Oryza sativa* APC6 sequences.	92
5.6	Alignment result of *Oryza sativa* APC6 sequence and *Saccharomyces pombe* Cut9 sequence using BlastP	93
5.7	Cartoon representation of molecular model of *OsAPC6*	94
5.8	Superimposition of *Oryza sativa* APC6 (red color) with *Saccharomyces pombe* APC6 (Blue color) developed in PyMOL	95
6.1	APC6 CDS sequence showing red colored region where gene specific primers were designed.	100
6.2	Entry vector map obtained from Invitrogen	100
6.3	Destination vector map for LR cloning	101
6.4	Schematic from PCR gene cloning to *Agrobacterium* expression clones using the Gateway® cloning technology	105
6.5	Confirmation of APC6 cloning using PCR	106
6.6	*In planta* transformation in rice seedlings	108
6.7	Calli development and regeneration of PR-118 rice seeds	109
6.8	Biolistic transformation and GUS assay results	110

ABBREVIATIONS USED

%	Percentage
µ	Micron
µg	Microgram
µm	Micrometer
µL	Microlitre
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
Cdc genes	Cell division cycle genes
cDNA	Complementary DNA
CLSM	Confocal laser scanning microscope
cM	Centi Morgan
cm	Centimeter
cv.	Cultivated Variety
Cys	Cystine
DNase	Deoxyribonuclease
DEPC	Diethyl Pyrocarbonate
dNTPs	Nucleotide Triphosphates
Ds	Dissociation Element
EDTA	Ethylene diamine tetraacetic acid
Fig.	Figure
G	Gram
GA	Gibberellic Acid
h	Hour
hpt	Hygromycin Phosphotransferase
IRGSP	International Rice Genome Sequencing Project
Kb	Kilobase pairs (10^3 bp)
kDa	Kilo Dalton

L	Litre
Lys	Lysine
M	Molarity
MD	Molecular dynamics
Mb	Megabase pairs (10^6 bp)
Mg	Milligram
min	Minute
mL	Millilitre
mm	Millimeter
mM	Millimole
NCBI	National Centre for Biotechnology Information
NPT	Neomycin Phosphotransferase
°C	°Centigrade
ORF	Open Reading Frame
OsAPC6	*Oryza sativa* Anaphase promoting complex 6 mutant
***Os*APC6**	*Oryza sativa* Anaphase promoting complex 6 protein
Ossl	*Oryza sativa* seedling lethal
PCR	Polymerase Chain Reaction
ppm	Parts per million
qRT-PCR	Quantitative real time PCR
RNAi	RNA interference
RT-PCR	Reverse transcriptase-PCR
RiceGE	Rice Functional Genomic Express Database
Sec	Second
SPT	Streptomycin Phosphotransferase
SSC	Sodium Citrate
SSRs	Simple Sequence Repeats
TAE	Tris Acetate
TAIL-PCR	Thermal asymmetric interlace-PCR
TPR	Tetratrico peptide repeat

Chapter I

Introduction

Introduction

Rice is an important crop and a primary source of food for more than half of the world's population. It is planted in about 154 million hectares annually on about 11% of the world's cultivated land with a production of 600 million tonnes. Rice occupies a pivotal role in Indian agriculture. It is the staple food for more than 70 percent Indians and a source of livelihood for 120-150 million rural households.

Basmati, the aromatic rice praised for its unique quality, is a connoisseur's delight, a nature's gift to Indian sub-continent. The word Basmati originated from the Hindi word, '*bas*' meaning smell and '*mati*' meaning full of aroma. Basmati rice has a typical flavor caused by the compound 2-acetyl-1-pyrroline (Buttery *et al.*, 1983). Basmati which is a tall, low-yielding variety with beautiful, long slender and lustrous grains, possessing exquisite scent, sweet ambrosial taste, soft texture and high kernel elongation commonly suffers from the problem of lodging due to its very tall stature. This causes considerable yield losses and a reduction in grain quality. Dwarf stature is one of the most valuable traits in plant breeding because semi-dwarf cultivars are more-resistant to damage by wind and rain (lodging-resistant).

Rice was the first crop plant to be fully sequenced (IRGSP, 2005). The *japonica* genome is 389 Mb in size and approximately 370 Mb, or >97% of the genome, have been assembled as reference molecules with the release of the 'build 3.0 pseudomolecules' by the International Rice Genome Sequencing Project (Sasaki *et al.*, 2005). Using these non-overlapping genome sequences as templates for annotation, 57,888 genes now have been predicted by the annotation team of The Institute for Genomic Research (TIGR). In addition, the rice cDNA project has generated sequence data for 1,75,642 full-length cDNAs clustered into 28,469 non-redundant clones (Kikuchi *et al.*, 2003), 15232 transposable elements and 41478 non-transposable elements (Jung *et al.*, 2008).

Rice is an excellent model plant for genomics, second only to *Arabidopsis* (Izawa, 1996). It has the smallest genome (Khush, 1997) among cereals. While maize and wheat have one gene every 100 and 500 kbp, respectively (Goff, 1999), rice has an average of approximately one gene every 15 kilobase pairs (kbp). Other factors that aid in the use of rice as a model plant species include the fact that it can be regenerated from protoplasts and can also be transformed by exogenous DNA,

employing methods like *Agrobacterium tumefaciens*-mediated transformation (Hiei *et al.*, 1994) or particle bombardment, making it an easy target for genetic manipulation among cereals (Tyagi and Mohanty, 2000), the availability of genome resources such as well-defined genetic maps (Causse *et al.*, 1994; Harushima *et al.*, 1998), an extensive collection of expressed sequence tags (ESTs) (Kurata *et al.*, 1994; Yamamoto and Sasaki, 1997) and the TIGR Rice Gene Index.

After completing the rice genome sequencing the major focus was on elucidating the function of the entire genome. Several reverse genetics approaches like insertional mutagenesis, fast neutron mutagenesis, RNA interference (RNAi), SAGE and virus induced genes silencing (VIGS) etc. are exploited for the purpose (Gilchrist and Haughn, 2010).

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 Feldman, 1997) have been widely used for revealing gene function in rice (Chin *et al.*, 1999). 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). 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.

Keeping in view the various aspects of insertional mutagenesis for functional genomics in rice, the present investigation was carried out to characterize a T-DNA insertional dwarf mutant *OsAPC6* and *Ossl* of Basmati 370. Both the 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 Seedling lethal (*Ossl*) mutant where the insertion was in chromosome 11, showed 5-15% of albino plants which died soon after germination

while among non-albino 10-20% died slowly without tillering after transplantation. In the dwarf mutant, the T-DNA insertion was found to be at the second exon of a gene on chromosome 3 which encodes one of the subunit of Anaphase promoting complex/cyclosome (APC/C) (Kumar *et al.*, 2010).

Microarrays are one of the important breakthroughs in experimental molecular biology, which allow monitoring of gene expression of tens of thousands of genes in parallel. The first arrays made on impervious supports were made by Maskos (1993). In 1995, Brown and his colleagues at Stanford University published the first paper on DNA microarrays (Schena *et al.*, 1995). Microarrays have enabled a number of studies to be performed on a nearly genome-wide scale, including the monitoring of steady-state gene expression (Cheadle *et al.*, 2005), comparative studies such as locating regions of copy number changes in cancer (Nannini *et al.*, 2009), mapping the binding sites of transcriptional regulators (Chip-on-Chip) (Wang, 2005), and surveying long-range DNA interactions (Wilhelm and Landry, 2009).

The dwarf mutant *OsAPC6* was insensitive to exogenous GA. The GA signaling involves the key components DELLA protein, a membrane bound GA receptor GID1and an E3 ligase enzyme SCF^{GID2} (Sasaki *et al.*, 2003; Ueguchi-Tanaka *et al.*, 2005). The sensing of GA is mediated by the GID1 protein, and the complex between GA and GID1 allows the capture of the nuclear growth-repressing DELLA proteins. The N-terminal region of SLR1 interacts with the GID1-GA complex, effectively stabilizing it. This tripartite structure functions as the 'ubiquitination chaperone', stimulating its recognition by the SCF complex. The GID1-GA-DELLA complex is subsequently polyubiquitinated and the DELLA proteins are finally degraded by E3 ubiquitin-ligase SCF^{GID2} (Gao *et al.*, 2011).

The APC/C complex is also essential for the ubiquitin-dependent degradation of cell cycle regulatory proteins. APC/C regulates mitosis including metaphase-anaphase transition and mitotic exit and maintains G1 phase. APC/C has 11 core subunits but the function of some of these subunits has been characterized (Gieffers *et al.*, 2000). The functions of APC/C in cell-cycle control started to emerge during the early 1990s through two different approaches: (1) the establishment of in vitro systems for cyclin-B ubiquitylation using Xenopus and clam oocyte extracts; and (2) a genetic screen in budding yeast that allowed the isolation of mutants unable to degrade the mitotic

cyclin Clb2. In addition to the mitotic cyclins, many other important cell-cycle proteins have been proved to be targets of APC/C degradation, including the anaphase inhibitors called securins (Pellman and Christman, 2001), the DNA-replication inhibitor geminin (McGarry and Kirschner, 1998), CDC6 (which is involved in the initiation of DNA replication), chromokinesin Xkid (which is involved in chromosome alignment during metaphase), the mitotic-spindle-associated protein Ase1p (Juang *et al.*, 1997), different protein kinases (e.g. polo kinase), Hsl1, Nek2A and Aurora-A (Littlepage and Ruderman, 2002).

Confocal microscope which exploits the noninvasive method of image collection uses light rather than physical means to section the specimen. The invention of the confocal microscope is usually attributed to Marvin Minsky, who built a working microscope in 1955 with the goal of imaging neural networks in unstained preparations of living brains (Minsky, 1988). Later in 1987, White and his colleagues (1987) developed a confocal laser scanning microscope to tackle a fundamental problem of imaging specific macromolecules in immunofluorescently labeled embryos. CLSM is widely used in biological disciplines from cell biology and genetics to microbiology and developmental biology. Zeng and coworkers (2007) studied the abnormal embryo sacs formed in hybrid of *japonica* and *indica* rice. In another autotetraploid rice hybrid the sterility in rice embryo sac and defect during megagametogenesis and in megasporocyte was studied (Hu *et al.*, 2010). In IR36-Shuang rice, Dai Xi-mei observed double-ovule and double-embryo development using CLSM (Dai Xi-mei, 2009).

RNAi is a precise method for investigating gene functions as transgene-induced RNAi has been effective at silencing one or more genes in a wide range of plants, this technology also bears potential as a powerful functional genomics tool across the plant kingdom (Fire *et al.*, 1998; Agrawal *et al.*, 2003). The high-throughput reverse-genetic screens have been based on using RNAi to interrupt the expression of targeted genes. In plants, RNA interference, also known as post-transcriptional gene silencing (PTGS) or co-suppression is thought to be a key defense against viruses, as well as a way of regulating endogenous genes (Myers *et al.*, 2003).

The candidate gene for *OsAPC6* has been cloned (Kumar *et al.*, 2010) and the reason for its female sterility due to abnormal megagametophyte development has been

established (Awasthi *et al.*, 2012) but the validation of the gene has not been done. The insertion site of *Ossl* on chromosome 11 has been known at a place where there is no ORFs or microRNA. The present investigation has been undertaken with following broad objectives:

- To study the cause of female sterility in *OsAPC6* mutant using confocal laser scanning microscopy (CLSM).
- To study the expression profile of the insertional mutants *OsAPC6* and *Ossl* of Basmati rice.
- To predict a comparative molecular model of Basmati APC6 protein highlighting its functional domains.
- To develop RNAi construct of the *APC6* gene and transform the rice using biolistic and *in planta* technique.

INSTITUTE OF TECHNOLOGY

Chapter II

Review of Literature

2.1 Rice as major food crop

Rice remains the most important staple food on the planet since it feeds roughly half the population on a daily basis. 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 is the monocotyledonous cereal crop and a member of *Poaceae* family. These crops provide the bulk of the calorific intake of the world's population. Rice has become an important model flowering plant for studying many aspects of plant biology. Rice occupies a pivotal role in Indian agriculture. In India rice is cultivated round the year in one or the other part of the country and area under rice cultivation in India during 2008-09 was 45.60 million hectare with production of 96.43 million tonnes (Datanet, 2011). At the current rate of population growth, rice requirement in India by 2020 would be around 110 million tonnes. The intervention of biotechnology and genetic engineering will be very crucial for enhancing productivity and stabilization of production for ensuring food security.

2.2. Rice as a model crop plant

After complete sequencing of the rice genome (IRGSP, 2005) and annotation of a majority of approximate 35,000 rice genes (Sundaram, 2010), the crop has been catapulted to the centre stage of plant genome research along with *Arabidopsis* and has become the model genome among cereals. Each of the 12 rice chromosomes has a characteristic karyotype. The rice chromosomes range in size from 45 Mb (chromosome 1) to 24 Mb (chromosome 10) and the gene density varies from 8.7 (chromosome 3) to 11.6 (chromosome 12) genes per kilobase pair. The nuclear organizing region is located at the end of the short arm of chromosome 9 (Wu *et al.*, 2004). It has been a model monocot for functional genomics research due to its small genome size (420Mb) relative to other cereals which is 16 and 40 times smaller than the maize and wheat (Gill *et al.*, 2004; Li *et al.*, 2004; Kalavacharla *et al.*, 2006) genomes, respectively, its ease of transformation and its economic importance. 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.*, 2007). Rice seems to encode more genes that have a redundant function as compared with *Arabidopsis thaliana* because the duplicated genomic segments estimated to cover 27-65.7% of its genome (Yu *et al.*, 2005; Ouyang *et al.*, 2007; Singh *et al.*, 2007). Rice belonging to

genus *Oryza*, has two cultivated and twenty one wild species (Vaughan, 2003). Of the two cultivated species, the Asian rice *Oryza sativa* (L.) originated from *Oryza nivara* and *Oryza rufipogon* (Griff.) and is grown worldwide, whereas the African rice *Oryza glaberrima* (Steud.), grown on a limited scale, originated in West Africa from *Oryza barthii* (A. Chev.) (syn. *Oryza breviligulata*) (Khush, 1997; Vaughan, 2003). The Asian rice species *Oryza sativa*, which is spread in large parts of the world, is more diverse than *Oryza glaberrima*. *O. glaberrima* is a valuable source of useful genes for combating several biotic and abiotic stresses. *O. sativa* is broadly divided into *indica* and *japonica* subspecies. Among the twenty one wild species in genus *Oryza*, nine are tetraploid and remaining wild species as well as cultivated species are diploid.

2.3 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, 2006). 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 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) (Peng *et al.*, 1999) 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 and production.

2.4 Functional genomics and insertional mutagenesis

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). Reverse genetics is a powerful tool that establishes a direct link between the biochemical function of a gene product and its role in *in vivo* (Gilchrist and Haughn, 2010). 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 (Koornneeff *et al.*, 1982; Sundaresan, 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; Wisman *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 Feldman, 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 (Bouchez 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).

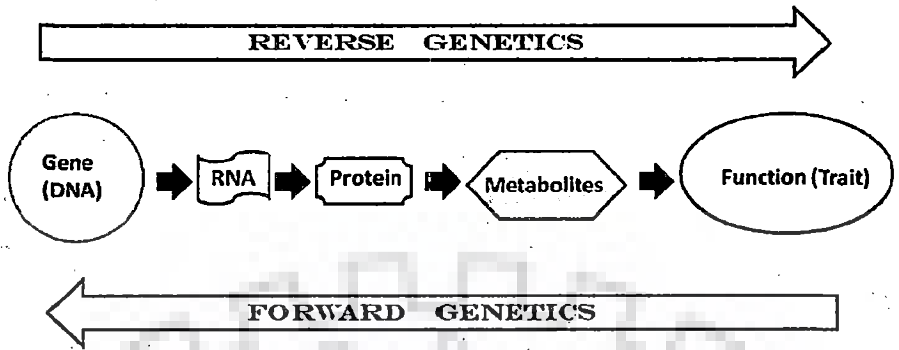


Figure 2.1 Functional genomics approaches.

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 Sundaresan, 2001).

To isolate genes from several plant species like maize and snapdragon, mobile genetic elements *activator (Ac)* and *dissociation (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 200 bp 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* (McClintock, 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).

2.5 Ubiquitin proteasome pathway

In eukaryotic cells, most proteins in the cytosol and nucleus are degraded via the ubiquitin-proteasome pathway. The importance of Ubiquitin-proteasome pathway was formally acknowledged in 2004 when Nobel Prize was awarded to Aaron Ciechanover, Avram Hershko and Irwin Rose for the discovery of ubiquitin-mediated protein degradation. In plants, regulated protein degradation by the ubiquitin/26S proteasome contributes significantly to development by affecting a wide range of processes, including embryogenesis, hormone signaling and senescence (Moon *et al.*, 2004). For degradation of the substrate protein by 26S proteasome polyubiquitination is found to be necessary (Wilkinson, 2000; Doherty *et al.*, 2002). Ubiquitin is a highly conserved 76 amino acid protein. Ubiquitination of cellular proteins has been implicated in nearly all cellular processes.

Ubiquitin conjugates covalently to the Lys residues within the substrate protein through a multistep enzymatic process that involves at least three enzyme activities. An ubiquitin-activating enzyme (E1) forms a high-energy bond with ubiquitin, which is then transesterified to an ubiquitin-conjugating enzyme (E2). The E2 either transfers ubiquitin directly to the E3 in the case of HECT E3s or binds the E3 and transfers the ubiquitin to the substrate. In either case, the E3 enzyme specifies the substrate. This process is then repeated several times to attach multiple ubiquitin molecules to the substrate, which is sufficient to target it for degradation by a large ATP-dependent multicatalytic protease (Voges *et al*., 1999). Possible involvement of ubiquitin dependent proteolysis in specific plant processes (e.g. auxin signaling or the repression of photomorphogenesis in the dark) have recently been reviewed (Hellmann and Estelle, 2002). Among the different components of this pathway, the E3s seem to be the most interesting class of enzymes, because they are the main players of substrate specificity.

2.5.1 26S proteasome

The 26S proteasome is a multisubunit complex which is implicated in the degradation of abnormal and damaged proteins (Coux *et al*., 1996), of cell-cycle regulators (King *et al*., 1996; Hershko and Ciechanover, 1998; Townsley and Ruderman, 1998), of oncogens and tumor suppressors (Lee and Goldberg, 1998), and in the processing of antigens (Monaco and Nandi, 1995; Pamer and Cresswell, 1998), in the regulation of neural progenitor proliferation, cell specification, neuronal differentiation, maturation and migration (Tuoc and Stoykova, 2010) and the activation or degradation of transcription factors. It consists of a cylindrical 26S core protease capped on each end by a 19S regulatory particle (Groll and Huber, 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 (Fig. 2.2) (Voges *et al*., 1999; Yang *et al*., 2004).

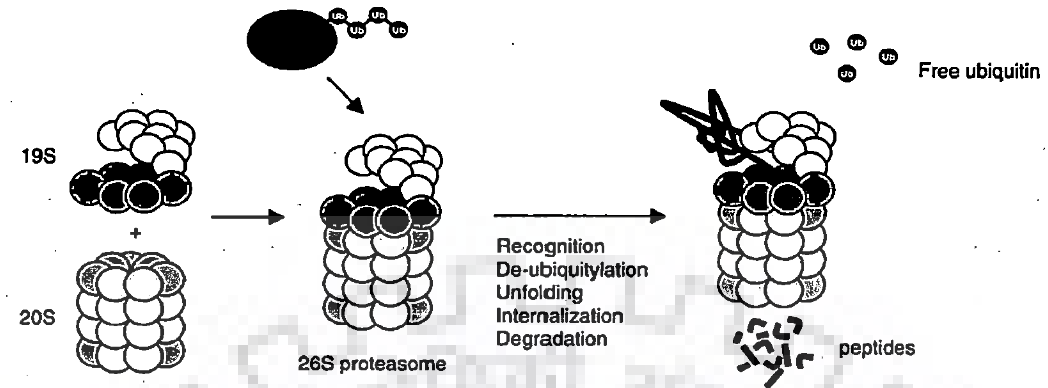


Figure 2.2 The 26S proteasome consists of a 20S core and 19S lid. The 20S part contains the catalytic domain, which harbors catalytic activities. The 19S component recognizes polyubiquitylated proteins and subsequently unfolds and removes ubiquitin from substrates. (Marteijn et al.,2006).

2.5.2 E3 ubiquitin ligase

Among the different components of the ubiquitin-proteasome pathway, the E3s seem to be the most interesting class of enzymes, because they are the main players of substrate specificity. RING E3s (including the APC/C and SCF) are proposed to function as scaffolds, bringing substrates and ubiquitin-conjugated E2 into close proximity to allow the spontaneous transfer of ubiquitin, without the participation of E3 catalytic residues (Zheng *et al*., 2002; Passmore and Barford, 2004). The E3 ubiquitin ligases comprise a large and diverse family of proteins or protein complexes containing either a HECT domain or a RING/U-box domain. HECT E3s are large proteins, typically 100-400 kD. The HECT domain is a 350-amino acid motif and contains both a ubiquitin binding site and a Ub E2 binding site (Pickart, 2001). The RING (for Really Interesting New Gene) proteins are characterized by the presence of a zinc binding motif, or RING finger. The RING domain E3s can be further divided into single subunit RING/U-box E3s, such as Constitutive Photomorphogenesis1 (COP1), Seven in absentia in *Arabidopsis thaliana* 5 (SINAT5), and Arm Repeat-Containing1 (ARC1), and multisubunit RING E3s, which include the SCF (Skp-Cullin-F-box), CUL3-BTB (Broad-complex, Tramtrack, Bric-a-Brac), and APC (Anaphase Promoting Complex) complexes. With the exception of the ECS (for Elongin C-SOCS box-CUL2), all multisubunit E3s are present in plants. All of these complexes contain a cullin (or cullin-like) protein and a RING-finger protein (Moon *et al*., 2004). Among these cullin-RING ligases the most widely studied 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).

2.5.3 Anaphase promoting complex/cyclosome (APC/C)

The key component of the proteolytic system is a multi-subunit ubiquitin ligase, the anaphase-promoting complex (APC/C) that provides a platform and specificity for the ubiquitination reactions (Deak *et al.*, 2007). APC/C is 1.5 MDa protein complex that is found in the nucleus of interphase cells, and that spreads throughout the cytoplasm and associates with parts of the spindle apparatus during mitosis (Peters, 2006). The APC/C is essential for the ubiquitin-dependent degradation of cell cycle regulatory proteins. The complex multisubunit structure of APC/C facilitates its intimate involvement in the formation of substrate-ubiquitin conjugates, and thus determines substrate specificity of the whole process. The functions of APC/C in cell-cycle control started to emerge during the early 1990s through two different approaches: (1) the establishment of *in vitro* systems for cyclin-B ubiquitylation using Xenopus and clam oocyte extracts (King *et al.*, 1995; Sudakin *et al.*, 1995) and (2) a genetic screen in budding yeast that allowed the isolation of mutants unable to degrade the mitotic cyclin Clb2 (Irniger *et al.*, 1995) . In addition to the mitotic cyclins, many other important cell-cycle proteins have been proved to be targets of APC/C degradation, including the anaphase inhibitors called securins (Pellman and Christman, 2001), the DNA-replication inhibitor geminin (McGarry and Kirschner, 1998), CDC6 (which is involved in the initiation of DNA replication) (Petersen *et al.*, 2000), chromokinesin Xkid (which is involved in chromosome alignment during metaphase) (Funabiki and Murray, 2000), the mitotic-spindle-associated protein Ase1p (Juang *et al.*, 1997), different protein kinases (e.g. polo kinase) (Shirayama *et al.*, 1998), Hsl1(Burton and Solomon, 2000), Nek2A (Hames *et al.*, 2001) and Aurora-A (Littlepage and Ruderman, 2002). Most of these APC/C targets carry a short peptide motif of nine amino acids called the destruction box (D box) or the KEN box (Pfleger and Kirschner, 2000). Phosphorylation of APC/C subunits during the cell cycle both activates and inhibits its activity (Harper *et al.*, 2002). To be active, APC/C also requires crucial factors, the Cdc20/Fizzy and Cdh1/Fizzy-related proteins (hereafter called Cdc20/FZ andCdh1/FZR, respectively), which are similarWD40-repeat proteins (Morgan, 1999). These activator proteins are directly involved in the mechanisms of D-box and KEN-box recognition, and are believed to assume most of APC/C's substrate selectivity (Vodermaier, 2001; Harper *et al.*, 2002). However, some

proteins like Mad2 and Emi1 have been reported in fungi and animals which act as inhibitors in APC/C activity (Capron *et al.*, 2003a).

The anaphase-promoting complex/cyclosome (APC/C) is a highly conserved complex consisting of thirteen subunits in yeast (Passmore and Barford, 2004) while eleven subunits have been identified in plants (Capron *et al.*, 2003a; Gieffers *et al.*, 2001). The three-dimensional structure of the human complex has been solved at a resolution of 24 Å (Gieffers *et al.*, 2001). However, little is known about the function of individual APC/C subunits.

All vertebrate APC/C subunits have counterparts in plants and are enlisted in Table 1 (Peters, 2002) and Apc9 seems to be unique to budding yeast while APC7 have been identified only in vertebrates (Harper *et al.*, 2002). Two of the 11 components have homology to subunits in the SCF: APC2 and APC11 (Tang *et al.*, 2001b). Whereas APC2 is a distant member of the cullin protein family that functions as a scaffold in SCF (SKP1, Cullin/CDC53, F-box protein) assembly(Gieffers *et al.*, 2000), APC11 is similar to the RING-H2 finger protein Rbx1, which plays a key function in the ubiquitylation reaction (Gmachl *et al.*, 2000). The largest APC/C subunit (APC1), which also shares some homology with two subunits of the 26S proteasome, the TPR-repeat-containing APC/C subunits (APC3, APC6, APC7 and APC8), the Doc-domain protein APC10/DOC1 and the RING-H2 domain protein APC11 are some other subunits identified in plants (Fig. 2.3).

Table1: Components of the APC/C complex in rice (from Lima *et al.* 2010)

APC subunits	Access Number	Probe id	Protein motif
APC 1	TC286185	-	Rpn1/2 repeats
APC2	LOC_Os04g40830	Os.52729.1.S1_at	Cullin Domain
APC3/CDC27	LOC_Os06g41750	OsAffx.15881.1.S1_at	TPR repeats
APC4	LOC_Os02g54490	OsAffx.3070.1.S1_at	WD-40 repeats
APC5	LOC_Os12g43120	OsAffx.32165.1.S1_at	TPR repeats
APC6/CDC16	LOC_Os03g13370	OsAffx.25081.1.S1_at	TPR repeats
APC7	LOC_Os05g05720	Os.49925.2.S1_x_at	TPR repeats
APC8/CDC23	LOC_Os02g43920	Os.22624.2.S1_at	TPR repeats
APC10	LOC_Os05g50360	Os.2307.1.S1_at	Doc domain
APC11	LOC_Os03g19059	Os.18502.1.S1_at	RING-H2 domain
APC13	TC311476	-	-

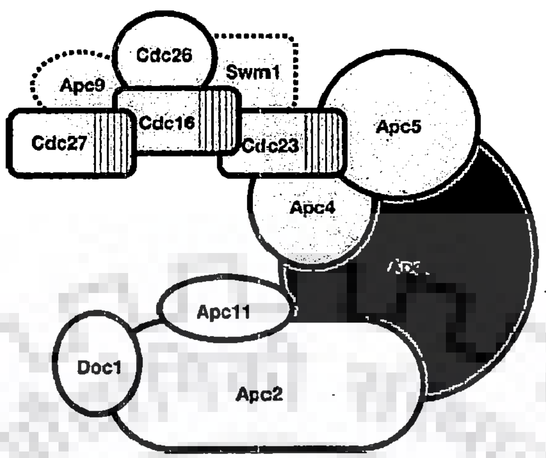


Figure 2.3 *The APC is composed of two subcomplexes. Cdc23, Apc5, and Apc4 bind cooperatively to Apc1, which in turn binds to the more peripheral Cdc16, Cdc27, Cdc26, and likely Swm1 and Apc9. The catalytic subcomplex is composed of Doc1, Apc11, and Apc2, and depends on Apc2 for interaction with Apc1 (Thornton et al., 2006).*

The TPR motifs are involved in protein-protein interaction (Blatch and Lassle, 1999), whereas the Doc domain might also bind ligands other than proteins (Harper *et al.*, 2002). Apc10 was suggested as the processivity factor for the APC/C (Carroll and Morgan, 2002). The Apc3 (also known as Cdc27 or Mks), Apc6 (also known as Cdc16), and the Apc8 (also known as Cdc23) subunits constitute a group of structurally related proteins within the APC/C, all of which contain nine to ten copies of the tetratrico peptide repeat (TPR) motifs in tandem arrays. The TPRs are repeats of 34 amino acid structural motifs with a consensus sequence restricted only to eight residues. There is no invariant residue even within the consensus but amino acids at these positions are conserved in terms of size, hydrophobicity and spacing (Lamb *et al.*, 1995; Blatch and Lassle, 1999). The first X-ray structure of the TPR containing protein phosphatase-5 revealed that each motif forms two- helices in an antiparallel, helix-turn-helix configuration (Das *et al.*, 1998). The neighboring motifs are packed in a parallel fashion resulting in the formation of a superhelical structure. TPR motifs are present in functionally divergent proteins and thought to mediate protein-protein interactions and the assembly of multiprotein complexes. TPR containing proteins are involved in a diverse spectrum of cellular functions with the majority of them participating in cell cycle control, transcription and splicing events, protein transport especially protein import, regulatory phosphate turnover and protein folding (Blatch and Lassle, 1999).

2.6 Cell cycle and cyclins

Tim Hunt discovered a protein in rapidly dividing sea urchin embryos that was synthesized during interphase but suddenly destroyed during cell division. The cyclic expression pattern of this protein suggested that proteolysis is a key to its regulation. Due to its cyclic expression pattern, this protein was called cyclin (Evans *et al.*, 1983). In the three decades that have passed since then, these ideas have turned into one of the central dogmas of the eukaryotic cell division cycle. The Hunt's cyclin is referred as cyclin B today and is known that it and several related "mitotic cyclins" are central regulatory elements of an enzymatic switch that triggers entry into mitosis. Progression through mitosis depends on the periodic accumulation and destruction of cyclins. Cyclin B accumulates and activates the cyclin-dependent kinase 1 (Cdk1) in mitosis to form mitosis-promoting factor (MPF). Cyclin-dependent kinase 1 (Cdk1) initiates mitosis and later activates the anaphase promoting complex/cyclosome (APC/C) to destroy cyclins. MPF drives chromosome reorganization and formation of the mitotic spindle. Later in mitosis, MPF down regulates its own activity by initiating the ubiquitination and destruction of cyclins by the anaphase-promoting complex/ cyclosome (APC/C), an E3 ubiquitin ligase (Murray, 2004).

2.7 Role of APC/C in cell cycle progression

The APC/C is a multisubunit complex that functions as an E3 ligase which ubiquitinates substrates, thereby marking them for destruction by the 26S proteasome (Peters, 2006). The recognition of substrates during mitosis is mediated primarily by the APC/C associated coactivator Cdc20 (Visintin *et al.*, 1997). Degradation of the APC/C substrates securin and cyclin B triggers the metaphase-anaphase transition and initiates mitotic exit, respectively (Murray and Kirschner, 1989; CohenFix *et al.*, 1996).

Successful progression through the cell cycle is dependent on an ordered sequence of cell division events, such as DNA replication, spindle assembly, nuclear division, and cytokinesis. To coordinate these events, eukaryotic cells have evolved a highly regulated oscillator that is driven by waves of cyclin dependent kinase (Cdk) activity. The Cdk activity and the control the cell-cycle clock is regulated by gradual accumulation and abrupt destruction of cyclins which are the activating subunits of Cdks (Murray, 2004). Likewise, many other proteins undergo proteolysis at different cell-cycle transitions. In all cases, the route to destruction leads via the ubiquitin-proteasome system, which uses the highly conserved polypeptide

ubiquitin as a tag that marks target proteins for degradation by the 26S proteasome. Ubiquitination requires the generation of polyubiquitin chains on substrate proteins through the combined action of ubiquitin-carrying enzymes (UBCs or E2s) and ubiquitin-protein ligases (or E3s) that bring substrates and UBCs together (Pickart, 2001). The specificity of ubiquitin dependent proteolysis is achieved at the level of substrate ubiquitination, which gives E3 enzymes key roles in several cellular processes, especially in the cell cycle. Two related E3 complexes are most intimately dedicated to basic cell-cycle control, namely the anaphase-promoting complex or cyclosome (APC/C), and the SCF (Skp1/Cullin/F-box protein) related complexes.

APC/C regulates mitosis including metaphase-anaphase transition and mitotic exit and maintains G1 phase (Vodermaier, 2004; Nakayama and Nakayama, 2006). APC/C is a large (1.5 MDa complex) composed of at least 11 core subunits. It relies on two WD-40 repeat-containing adaptor proteins, Cdc20/fizzy (fzy)/p55CDC and Hct1/srw1/fizzy related (fzr)/Cdh1, to engage with its substrates. Destruction box (RXXLXXXXN/D/E) and KEN box are motifs frequently found in APC's substrates, but other motifs are also possible for recognition by APC^{Cdc20} or APC^{Cdh1} (Harper *et. al.*, 2002). The consensus sequence of destruction box can be found in many proteins. However, not all of these proteins are APC's substrates. Moreover, some substrates only have an RxxL motif and yet are recognized by APC, indicating the last amino acid in the consensus is not stringently conserved. APC^{Cdc20} initiates the metaphase-anaphase transition through mediating the ubiquitination and degradation of cyclin B1 and securin. To prevent premature separation of sister chromatids and mitotic exit, APC^{Cdc20} is inhibited by Mad2 and BubR1 through the spindle assembly checkpoint mechanism (Fang *et al.*, 1998; Hwang *et al.*, 1998; Wu *et al.*, 2000;Tang *et al.*, 2001a). Only when the sister chromatids are aligned at the metaphase plate and have established bivalent spindle attachment, the inhibition of APC^{Cdc20} can be released. In contrast to APC^{Cdc20}, APC^{Cdh1} is inactive in early mitosis (Harper *et al.*, 2002) when it is inhibited by phosphorylation (Kramer *et al.*, 2000) and binding of Nup90/Rae1 complex (Jeganathan *et al.*, 2005; Jeganathan *et al.*, 2006). APC^{Cdh1} only becomes active from late mitosis to G1. The difference in the timing of activation between APC^{Cdc20} and APC^{Cdh1} suggests a functional division between the two E3 ubiquitin ligases in mitosis.

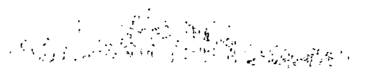
2.8 Female gametophyte development

The plant life cycle is characterized by the alternation of generations between a diploid sporophyte and a haploid gametophyte. In contrast to lower plant species, in which the gametophyte is the dominant (Cove and Knight, 1993), gametophytes of angiosperms are smaller and less complex than the sporophyte and are formed within specialized organs of the flower. The male gametophyte (pollen or microgametophyte) develops within the anther, whereas the female gametophyte (embryo sac or megagametophyte) is a product of the ovule. The ovule is the source of the megagametophyte and the progenitor of the seed. Specification of the megasporocyte, production of a functional megaspore (megasporogenesis), formation of the embryo sac (megagametogenesis), and embryogenesis all occur within the ovule.

The process of embryo sac development can be divided into two stages: megasporogenesis and megagametogenesis. In general, during megasporogenesis, the megasporocyte undergoes meiosis and four megaspore nuclei are produced. Subsequent mitotic divisions, nuclear migration, and cytokinesis during megagametogenesis produce the mature embryo sac.

In approximately 70% of the species including rice, *Arabidopsis* and maize, Polygonum form of embryo sac is found (Mansfield *et al.*, 1990; Russell, 1993). This type of embryo sac originates from a single chalazally located megaspore that undergoes three successive mitotic divisions. During the first meiotic division, the spindle is oriented parallel to the micropylar-chalazal axis of the nucellus. Wall formation occurs perpendicular to this axis, creating a dyad of megaspores. After the second meiotic division, another transverse wall is made, resulting in a linear arrangement of four megaspores. The megaspore closest to the chalaza enlarges before undergoing mitosis. The three nonfunctional megaspores degenerate and are eventually crushed by the expanding functional megaspore.

The female gametophyte is generated from the functional megaspore via a process termed megagametogenesis. The functional megaspore at the chalazal end enlarges prior to the first of three free nuclear divisions. After the first mitosis, the two nuclei migrate to opposite poles and the smaller vacuoles coalesce into a large central vacuole. Cass *et al.* (1985) suggested that formation of this central vacuole plays an important role in positioning the nuclei before subsequent mitotic divisions. Each of the two nuclei then divides two more times, resulting in an eight-celled coenocytic megagametophyte. Wall formation, nuclear migration and differentiation follow further, forming a mature seven-celled and eight-nucleated embryo sac (Fig. 2.4) (Hamada *et al.*, 2003; Lersten, 2004). Three cells at the opposite of micropylar end



form antipodal cells, three cells at the micropylar end form the egg apparatus consisting of an egg cell and two synergids. During cellularization one nucleus from each pole migrates towards the centre forming the polar nuclei or the central cell.

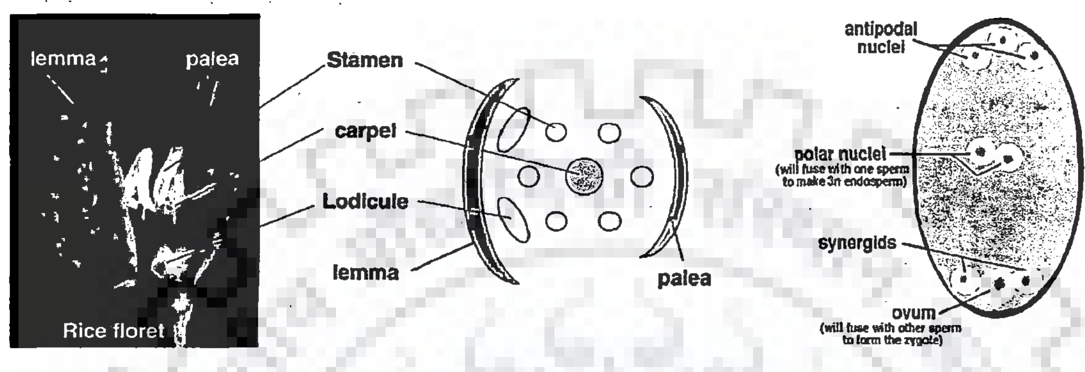


Figure 2.4 Rice floret and female gametophyte (Shimamoto and Kyozuka, 2002)

2.9 Confocal laser scanning microscopy

The invention of the confocal microscope is usually attributed to Marvin Minsky, who built a working microscope in 1955 with the goal of imaging neural networks in unstained preparations of living brains (Minsky, 1988). Later in 1987, White and his colleagues (1987) developed a confocal laser scanning microscope to tackle a fundamental problem of imaging specific macromolecules in immunofluorescently labeled embryos. This technological advance allowed them to follow changes in the cytoskeleton in cells of early embryos at a higher resolution.

Modern confocal microscopy which is built around a conventional light microscope can be considered as a completely integrated electronic system, where the optical microscopy is linked to one or more electronic detectors, a computer and several laser systems of various wavelengths, coupled to a beam scanning assembly (Fig. 2.5). Three-dimensional (Z-series), four-dimensional images and X-Z imaging can be taken using CLSM (Paddock, 1999). At the same time, it offers several advantages over conventional widefield microscopy, including control depth, elimination or reduction of background information away from the focal plane, exploits the noninvasive method of image collection capable to collect series of optical sections which uses light rather than physical means to section from thick specimens. The series of images are collected by increments in the fine focus mechanism, using a step motor,

to perform sequential image acquisitions in each step. The illumination is achieved by scanning one or more focused beams of light, usually from a laser, across the specimen (Amos and White, 2003). Unstained preparations can also be viewed with the CLSM using reflected (backscattered) light imaging. This method of imaging has the many advantages like it prevents photobleaching, especially for living samples, it provides a slight increase in both lateral and axial resolution and eliminates the "out-of-focus" flare from thick fluorescently labeled specimens. It has facilitated the imaging of living specimens, enabled the automated collection of three dimensional (3D) data in the form of Z-series, and improved the images of multi-labeled specimens. The advantage of confocal microscopy lies within its great number of applications and its relative ease for producing extremely high-quality images from specimens prepared for the light microscope. CLSM microscopy uses laser scanning and has an advantage of detecting 3D images without sectioning them whereas the differential interface contrast (DIC) microscope uses the polarized light beam to detect objects. DIC requires sectioning of the material and gives black and white image.

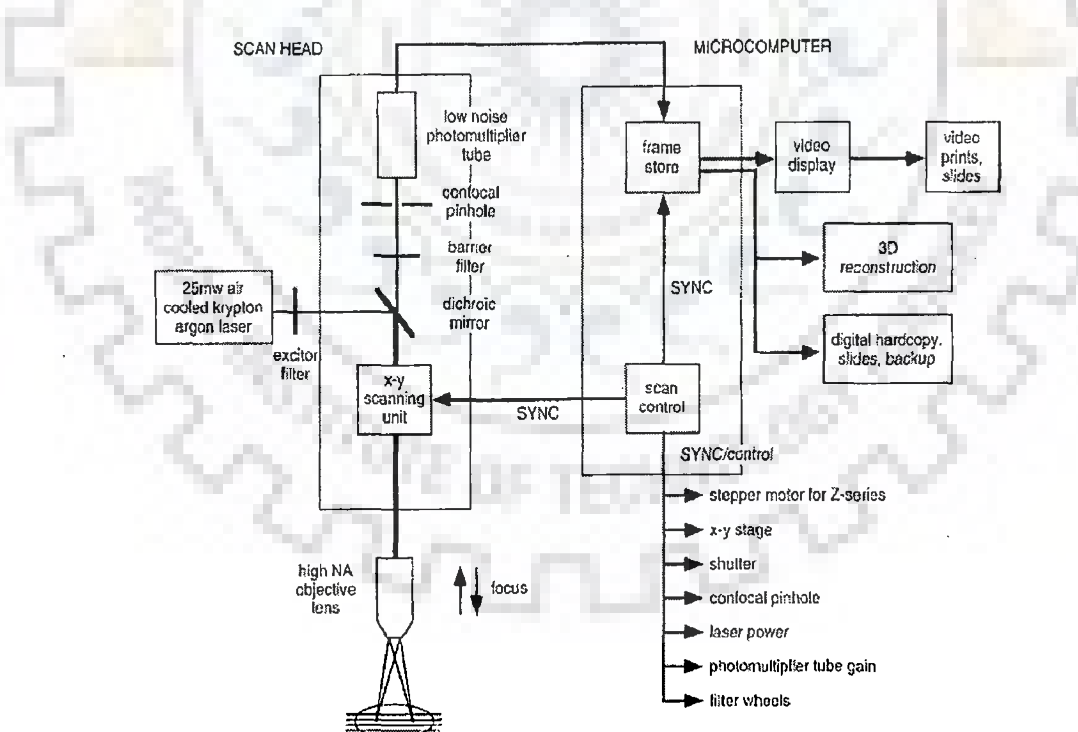


Figure 2.5 *Information flow in a generic CLSM (Paddock, 1999).*

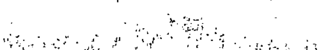
The introduction of fluorescent probe technology has revolutionized the confocal microscopy field and many fluorescent probes are available which are designed to recognize specific biological macromolecule (for example, a protein, nucleic acid), or to localize within a specific structural region, such as the cytoskeleton (Phalloidin-Rodamine) (Deng *et al.*, 1995), mitochondria (Mitotracker: Green, Orange and Red, 3,3'-dihexyloxacarbocyanine iodide (DiOC6)), golgi apparatus (BODIPY-Brefeldin), endoplasmic reticulum (Diaz-Munoz *et al.*, 2008), and nucleus (DAPI, TO-PRO, SYTOX, Hoechst 33342) (Diaz-Munoz *et al.*, 2008; Kahn *et al.*, 2010). Other fluorescent molecules are employed to monitor dynamic processes and localized environmental variables, including concentrations of ions (calcium, sodium and potassium) (Saldaña *et al.*, 2009), pH, reactive oxygen species (Kahn *et al.*, 2010), and membrane potential (Hibbs, 2004). Recently, the development of nanoprobes, nanosensor and nanoparticles has provided a new avenue for research in confocal microscopy. These molecules have been developed for imaging and dynamic monitoring *in vivo* of the molecular or ionic components, constructs, forces and dynamics, all in real time, during biological/chemical/physical processes. With their biocompatible small size and inert matrix, the nano molecules have been successfully applied for non-invasive real-time measurements of analyses and fields in cells and rodents, with spatial, temporal, physical and chemical resolution (Diaz-Munoz *et al.*, 2008; Saldaña *et al.*, 2009).

Confocal microscopy has been exploited to study various developmental abnormalities in plants. Analysis of female meiosis (megasporogenesis) and embryo sac development (megagametogenesis) in angiosperms is technically challenging because the cells are enclosed within the nucellus and ovule tissues of the female flower. Zeng and coworkers (2007) studied the abnormal embryo sacs formed in hybrid of *japonica* and *indica* rice. In another autotetraploid rice hybrid the sterility in rice embryo sac and defect during megagametogenesis and in megasporocyte was studied (Hu *et al.*, 2010). In IR36-Shuang rice, Dai Xi-mei observed double-ovule and double-embryo development using CLSM (Dai Xi-mei, 2009). Similarly abnormal female gametophyte development was studied in *nomega* mutant of *Arabidopsis* (Kwee and Sundaresan, 2003) and female sterility due to abnormal endosperm development in Basmati rice using CLSM technique (Awasthi *et al.*, 2012). In maize meiotic mutant *elongate1,* which produces functional diploid instead of haploid embryo sacs, defect in meiosis II was observed using confocal microscopy (Barrell and Grossniklaus, 2005).

2.10 Gibberellic Acid response

2.10.1 Gibberellic acid biosynthesis

Gibberellins (GAs) are tetracyclic diterpenoid plant hormones that promote a number of plant growth responses, including seed germination, stem elongation, leaf expansion and flowering. GA also plays role in the regulation of various growth and developmental processes which are associated with dwarf phenotype in higher plants. To date, more than 100 GA molecules have been identified in plants. However, only a very small number of them, such as GA_1, GA_3 and GA_4, are considered to be bioactive hormones, whereas the others are precursors (e.g., GA_9, GA_{12} and GA_{20}) or degradation products (e.g., GA_8, GA_{34}, etc.) (Yamaguchi, 2008). Bioactive GAs are synthesized from trans-geranylgeranyl diphosphate (GGDP) (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 (GA_{20ox}) and GA3-oxidase (GA_{3ox}) (Fig. 2.6). The bioactive GA_1/GA_4 and their immediate precursors GA_{20}/GA_9 are inactivated by a third 2ODD, GA 2-oxidase (GA_{2ox}). In some species, GA_9 and GA_{20} are also converted to GA_7 and GA_3, respectively via 2, 3-didehydro GA_9 and GA_5 (Albone *et al.*, 1990). The genes encoding the seven GA metabolic enzymes (CPS, KS, KO, KAO, GA_{20ox}, GA_{3ox}, and GA_{2ox}) have been isolated from various plants. Any mutation in the genes, except GA_{2ox}, 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.



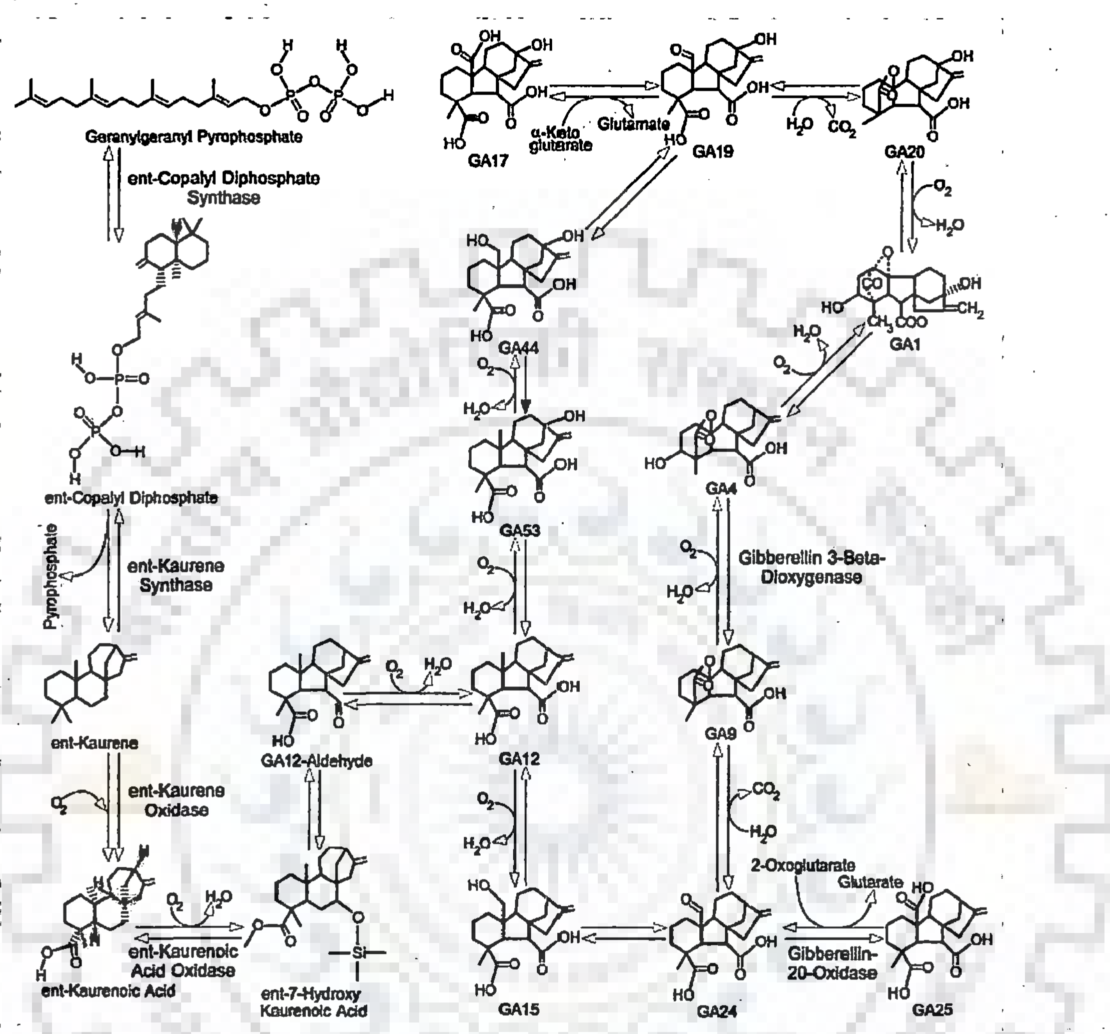


Figure 2.6 *Gibberellic acid biosynthesis pathway.*

2.10.2 Gibberellic acid signal transduction

GAs in addition to their effect on cell expansion, play a role in many other plant processes, including seed germination, root development, shoot growth, flowering time, sex determination, and chlorophyll content (Dellaporta and Calderon-Urrea, 1993; Blazquez *et al.*, 1997; Cho and Kende, 1997; Ogas *et al.*, 1997). Each of these processes represents a potential target for improving the agronomic properties of crop plants, adding further impetus to studies of GA perception and signal transduction. 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." Derepression of the repressed state is considered to be the key step of GA action in the GA signaling pathway. 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.

2.10.3 Gibberellic acid mutants

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 components, 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.

Positively acting components of GA signaling in different plants

The *d1* mutant exhibits phenotypes characteristic of GA deficiency, including semidwarfism and dark green coloration of leaves, but it has increased levels of active GA. *DWARF1* (*D1*) gene in rice encodes a prototypical heterotrimeric G protein α-subunit (Gα) (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 α-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 GA_{20} oxidase, is up-regulated and GA levels are elevated in the stunted internodes of *d1* (Ueguchi-Tanaka *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.

PHOTOPERIOD RESPONSIVE 1 (*PHOR1*) was identified in a screen for genes whose mRNA levels are increased in the leaves of potato during growth under short days (Amador *et al.*, 2001). Antisense inhibited expression of *PHOR1* gene caused semidwarf phenotype, reduced response to GA, and increased levels of endogenous GAs. Whereas its overexpression caused an overgrowth phenotype and an enhanced response to applied GA. GA treatment promoted nuclear localization of a PHOR1-GFP fusion protein in tobacco BY2 cells, and GA biosynthesis inhibitors caused the fusion protein to be localized to the cytosol. Analysis of deletion mutants has identified two domains that are important in the GA-regulated localization of PHOR1. Deletion of a conserved Cys-Pro-Ile motif (CPI) caused PHOR1-GFP to be localized constitutively to the nucleus, suggesting that CPI is a GA-inhibited cytosolic retention signal. PHOR1 also contains seven armadillo repeats. Deletion experiments suggest that the PHOR1 armadillo repeats function as a nuclear localization signal that can be overcome by the action of the CPI. These data suggest that in the absence of GA signaling, CPI retains PHOR1 in the cytosol, in which it is inactive; during GA signaling, CPI is inhibited, allowing the armadillo repeats to localize PHOR1 to the nucleus and stimulate the transcription of genes encoding products with a positive role in the GA response.

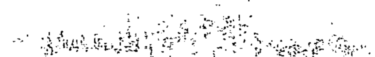
GAMYB is a GA-induced MYB transcription factor that was identified by its ability to activate the α-amylase promoter of barley. Three GAMYB proteins from *Arabidopsis* have been shown to substitute functionally for barley GAMYB in aleurone cells (Gocal *et al.*, 2001). One of these proteins, AtMYB33, is implicated in the induction of flowering by GA. Expression of AtMYB33 occurs in the shoot apex during the induction of flowering and is induced by GA. Expression of the floral meristem gene *LEAFY* (*LFY*) is induced by GA, and a specific promoter element, GOF9, has been shown to confer GA responsiveness (Blazquez and Weigel, 2000). AtGAMYB33 binds to GOF9, suggesting that, during the induction of flowering, GA-induced AtGAMYB33 binds to GOF9 and stimulates *LFY* expression. Because AtGAMYBs are expressed in seed and vegetative tissues, they also may participate in GA responses other than flowering. GA regulation of another *Arabidopsis MYB* gene, *GLABROUS1* (*GL1*), may play a role in the initiation and branching of trichomes (Perazza *et al.*, 1998). The *ga1* mutation causes *Arabidopsis* to have fewer trichomes, and treatment with

GA reversed this effect (Chien and Sussex, 1996; Perazza *et al.*, 1998). Because *GL1* mRNA is less abundant in *ga1* and GA treatment increased the expression of a reporter gene that is driven by the *GL1* promoter (Perazza *et al.*, 1998), GA-induced expression of *GL1* may promote both the initiation and branching of trichomes.

The *Arabidopsis SLY1* (*SLEEPY1*) gene positively regulates gibberellin (GA) signaling. Positional cloning of *SLY1* revealed that it encodes a putative F-box protein that regulates GA responses (McGinnis *et al.*, 2003). The DELLA domain protein RGA is a potential substrate of SLY1, because *sly1* mutations cause a significant increase in RGA protein accumulation even after GA treatment. RGA (repressor of *ga1-3*) is a repressor of GA response that appears to undergo GA-stimulated protein degradation. The predicted SLY1 amino acid sequence is highly conserved among plants, indicating a key role in GA response.

Another link between GA signaling and root development has been established recently with the identification of the *Arabidopsis* PICKLE (PKL) gene (Ogas *et al.*, 1997). The PICKLE (PKL) protein of *Arabidopsis* contains domains that are the hallmarks of CH3 chromatin-remodeling factors, and several of the phenotypes of *pkl* mutants suggest that it is involved in GA action (Ogas *et al.*, 1997; Ogas *et al.*, 1999). Mutations in this gene block the transition between embryonic and adult developmental programs in the primary root and also affect many aspects of shoot development that are influenced by GA (Ogas *et al.*, 1997). Loss-of-function *pkl* mutants are GA-insensitive dwarfs that have increased amounts of GAs.

The *gid1* mutant show very severe dwarf phenotype in which *GA20ox* gene is highly expressed and consequently the level of GA1 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 GA3 and over accumulates bioactive GA because GA signaling inhibits biosynthesis and promotes catabolism of these GAs. GA1, 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 GA3 (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).

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 GA1 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 636 bp 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 (Li and Johnston, 1997; Skowyra *et al.*, 1997; Deshaies, 1999).

Negatively acting components of GA signaling in different plants

The RGA/GAI family is a subset of the larger GRAS family (Pysh *et al.*, 1999). In addition to sharing a number of motifs with all members of the GRAS family, the N terminus of all RGA/GAI proteins contains the DELLA domain, which is absent from other GRAS proteins (Peng and Harberd, 1997; Silverstone *et al.*, 2001). Mutations affecting RGA/GAI proteins have been identified in *Arabidopsis* (*rga* and *gai*), barley (*sln1*), maize (*d8*), rice (*slr1*), and wheat (*reduced height* [*rht*]) (Peng *et al.*, 1997; Silverstone *et al.*, 1998; Peng *et al.*, 1999; Ogawa *et al.*, 2000; Ikeda *et al.*, 2001; Chandler *et al.*, 2002; Gubler *et al.*, 2002; Multani *et al.,* 2003). These mutations fall into two classes: semidominant mutations in *Arabidopsis*, maize, wheat and barley causing dwarfism; and recessive loss-of-function mutations in

Arabidopsis, barley, and rice causing increased growth. The phenotypes of the recessive loss-of-function alleles indicate that these proteins are negative regulators of the response pathway. *RGA* was identified in a screen for mutations suppressing the vegetative dwarfism of the GA-deficient *ga1* mutant (Silverstone *et al.*, 1997). Loss-of-function *rga* alleles partially suppress most of the phenotypes of *ga1* plants, including delayed abaxial trichome initiation, dwarfism of the rosette leaves, delayed flowering, dwarfism of the internodes of the floral shoot, and reduced apical dominance. The *GAI* gene of *Arabidopsis* was identified as a semidominant mutation, *gai-1*, that greatly reduces GA responsiveness during vegetative development (Koorneef *et al.*, 1985). The *gai-1* mutant contains increased amounts of bioactive GAs, suggesting that RGA/GAI proteins are involved in the feedback regulation of GA biosynthesis (Talon *et al.*, 1990).

Overexpression of *SPY* in *Arabidopsis* (Swain *et al.*, 2001) and petunia (Izhaki *et al.*, 2001) under the control of the 35S promoter of *Cauliflower mosaic virus* produced phenotypes consistent with reduced GA activity. The expression of *Arabidopsis SPY* using a *SPY::GUS* reporter gene (Swain *et al.*, 2002) and petunia *SPY* by reverse transcriptase-mediated polymerase chain reaction (Izhaki *et al.*, 2001) have found that they are expressed constitutively at all stages of development and that their expression is not regulated by GA. *Arabidopsis* SPY is found in both the cytosol and the nucleus (Swain *et al.*, 2002). In wild type background *spy* causes several phenotypes that are observed when the wild type was treated repeatedly with GA3, including more erect rosette leaves with a pale green color, early flowering, and reduced seed set (Jacobsen and Olszewski, 1993). The SPY protein has overall similarity with UDP-GlcNAc protein transferase (OGT) from animals (Roos and Hanover, 2000) and has been shown to have OGT activity (Thornton *et al.*, 1999.). Based on these results, SPY is believed to be a negative regulator of the GA response pathway.

The *SHORT INTERNODES* (*SHI*) gene is expressed in young organs that are not undergoing rapid elongation growth (Fridborg *et al.*, 2001), suggesting that SHI prevents young organs from initiating inappropriate elongation growth in response to GA. An overexpression of *SHI* caused by an activation tag *Ds* transposon produces a semidwarf phenotype that is not reversed by GA treatment and that increases the concentration of endogenous GAs (Fridborg *et al.*, 1999). SHI proteins have a RING finger-class zinc finger motif, which is known to mediate protein-protein interactions involved in proteolysis or transcriptional regulation (Freemont, 2000; Peng *et al.*, 2000). Because SHI is not expressed normally in cells that are

undergoing rapid expansion, the semidwarfing effect of the *SHI* overexpression allele is likely attributable to ectopic expression. Expression of SHI in barley aleurone cells reduced the GA induction of α-amylase expression (Fridborg *et al.*, 2001), indicating that overexpression of SHI can negatively regulate GA responses in a heterologous species.

Many genes are associated with a semidwarf growth habit of wheat (Ellis *et al.*, 2005) which is known as *Reduced height* (*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.

The *slr1* mutant has 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 GA3 (Ikeda *et al.*, 2001; 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 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 belongs to the GRAS family (Pysh *et al.*, 1999). 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 localized 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 localized in nuclei but disappear following the application of GA3 (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; Wen and Chang, 2002).

2.10.4 Role of DELLA protein in GA signaling

The DELLA proteins, recognized by their highly conserved 'DELLA' domain, belong to the plant-specific GRAS family. The GRAS proteins (named from the first three members of the family isolated, i.e. GA insensitive, repressor of GA1-3, and Scarecrow) are putative transcriptional regulators. The *Arabidopsis thaliana* DELLA proteins include GAI, RGA, RGL1,RGL2, and RGL3 (Jiang and Fu, 2007), all acting as repressors of GA signaling (Dill and Sun, 2001; Silverstone *et al.*, 2001; Itoh *et al.*, 2002; Fu and Harberd, 2003; Solanke and Sharma, 2008). DELLA proteins are negative regulators of gibberellin (GA) signaling that act immediately downstream of the GA receptor. Localized 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.*, 2004). The GA signals seem 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.*, 2002). But light promotes accumulation of DELLA proteins by reducing GA levels (Achard *et al.*, 2007).

The key components of GA signaling in both *Arabidopsis thaliana* and rice, which have been identified using mutation analysis, include the DELLA proteins, the GA receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1), and the F-box proteins SLEEY1 (SLY1) and SNEEZY (SNZ) in *Arabidopsis thaliana* and GIBBERELLIN INSENSITIVE DWARF2 (GID2) in rice (Peng *et al.*, 1997; McGinnis *et al.*, 2003; Sasaki *et al.*, 2003; Ueguchi-Tanaka *et al.*, 2005; Ariizumi *et al.*, 2011). The sensing of GA is mediated by the GID1 protein, and the complex between GA and GID1 allows the capture of the nuclear growth-repressing DELLA proteins; the tripartite GID1-GA-DELLA structure is subsequently polyubiquitinated and the DELLA proteins are finally degraded by E3 ubiquitin-ligase $^{SCFSLY1/GID2/SNZ}$. (McGinnis *et al.*, 2003; Sasaki *et al.*, 2003; Dill *et al.*, 2004; Fu *et al.*, 2004; Griffiths *et al.*, 2006; Itoh *et al.*, 2008; Shimada *et al.*, 2008; Ariizumi *et al.*, 2011). Thus, the DELLA proteins act to restrain plant growth, while GA promotes it by targeting them for destruction (Davière *et al.*, 2008; Gao *et al.*, 2008). The strength of the interaction between GID1 and the DELLA is increased to a remarkable degree once GID1 has become bound with GA (Ueguchi-Tanaka *et al.*, 2005; Griffiths *et al.*, 2006; Willige *et al.*, 2007). However, $GID1^{P99S}$ could interact with rice DELLA protein even in the absent of GA. These experiments suggest that GA does not appear to be essential for the normal interaction between GID1 and DELLA (Yamamoto *et al.*, 2010). Previously, it has been demonstrated that the DELLA domain is essential for GA perception, since deletions within the conserved DELLA motifs (e.g. *gai*) render the mutant proteins immune to GA-induced degradation, thereby resulting in a GA-insensitive dwarf phenotype (Peng *et al.*, 1997; Dill and Sun, 2001). In fact, the interaction between GID1 and the DELLA proteins also requires a stable interaction between the DELLA protein's GRAS domain and GID1 (Hirano *et al.*, 2010). Loss-of-function mutants, such as *slr1* in rice and *sln1* in barley, are taller and reach flowering earlier than their respective wild-type plants (Fu *et al.*, 2002; Itoh *et al.*, 2002). Both gain-of-function mutants (which generally involve mutation events within the DELLA domain) and transgenic plants engineered to overexpress DELLA proteins tend to be dwarfed in stature and flower late (Peng and Harberd, 1997).

The DELLA proteins also, however, interact with other hormonal and environmental signaling molecules, and so are involved in many aspects of plant growth, development, and adaptation to stresses (Fu and Harberd, 2003; Arnaud *et al.*, 2010; Hou *et al.*, 2010). GA–DELLA regulates root growth in *Arabidopsis thaliana* via its effect on cell expansion. As an example,

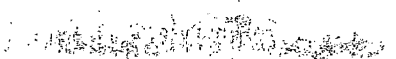
the GA-deficient *ga1-3* mutant produces short roots, but this characteristic is not observed when GAI and RGA are absent (Fu and Harberd, 2003; Benková and Hejátko, 2009). The DELLA proteins also promote mitotic activity, and thus exert some influence over cell proliferation (Achard *et al.*, 2009). Mutations within the DELLA domain render these proteins resistant to degradation which results in a GA-insensitive dwarf phenotype (Peng *et al.*, 1999; Dill and Sun, 2001). 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.

2.11 Microarray a tool for expression analysis at transcriptome level.

With the advent of transcriptional profiling technique the study of expression and action of not only a single gene but the entire transcriptome as a whole is made possible (Brady *et al.*, 2006). The transcriptome or expression profile involves the measurement of level of expression of all mRNA species. It is an important technique as it is the set of expressed genes and interplay between the products encoded by them that determine the phenotype of a cell (Somasundaram *et al.*, 2002). Thus, gene expression profiling holds tremendous promise for dissecting the regulatory mechanisms and transcriptional networks that underlie biological processes.

Microarray is a robotic instrumentation for miniaturization and detection of nucleic acid abundance in RNA populations derived from multiple samples (Alba *et al.*, 2004). It harnesses the ability of nucleic acids with complementary sequences to hybridize to each other under suitable conditions. It can measure tens of thousands of different mRNA transcripts in parallel (Schena *et al.*, 1995), is sensitive to low abundance transcripts and can monitor the expression of the entire genome in a single experiment (Gill *et al.*, 2002; Wang *et al.*, 2003).

There are three major steps involved in a typical microarray experiment: preparation of microarrays; preparation of fluorescently labeled cDNA probes and hybridization; and finally scanning, image and data analysis. In microarray analysis, the differential gene expression is analyzed by co-hybridizing fluorescently labeled cDNA probes prepared from two different RNA sources. The quality of RNA, proper removal of unincorporated fluorescently labeled nucleotides, proper hybridization and post-hybridization washing conditions are some of the



important factors that affect the outcome of a microarray experiment. The way an array is designed and printed also have an impact on the experimental data. In addition to the probes representing the genes under investigation, printing various control probes (both positive and negative) can aid spotfinding and normalization as well as provide a measure for array quality and consistency (Russell *et al.*, 2009).

A number of pre-existing tools allow the lists of genes to be organized and overlaid on pre-existing biological pathways were developed for microbial or animal systems, limiting their usefulness in interpreting microarray data from plant systems as irrelevant pathways are imported and plant-specific pathways and processes are absent (Thimm *et al.*, 2004). One of the first plant-specific programs for microarray interpretation was created by combining a database of microbial and animal pathways (www.metacyc.org) with the annotated *Arabidopsis* genome, allowing the exploration of plant biochemical pathways (Mueller *et al.*, 2003; Thimm *et al.*, 2004). The concept was later extended by Thimm *et al.*, (2004) in the creation of the MapMan tool that was designed specifically for use with the Affymetrix 22K *Arabidopsis* array, though this tool can be expanded to other species as genomic annotations become available.

The first arrays made on impervious supports were made by Maskos (1993). In 1995, Brown and his colleagues at Stanford University published the first paper on DNA microarrays (Schena *et ul.*, 1995), describing them as a high-capacity system developed to monitor RNA levels of numerous genes simultaneously, using two-color immunofluorescence. Later on many papers were published on describing the use of gene expression microarrays as a discovery platform for functional genomics (Schena, 1996; Schena *et al.*, 1998) microarray application for gene expression and its potential for revolutionizing drug discovery and diagnostics (Service, 1998). The use of microarrays for gene expression application (Marcelo B, 1997; Watson *et al.*, 1998; Brown and Botstein, 1999; Duggan *et al.*, 1999), oligonucleotide arrays for resequencing and mutation analysis (Hacia, 1999), expression profiling in cancer (Khan *et al.*, 1999), microchips as a specific genetic tool in psychiatry (Watson *et al.*, 2000) and impact of microarray technology on the modern pathology laboratory (Becich, 2000) emphasized the role of microarrays in medicine. More specialized work on microarray and gene expression includes study of aging process in mice (Weindruch *et al.*, 2001), genome-wide expression analysis for plant cell-modulated genes (Breyne and Zabeau, 2001), DNA microarray analyses of host pathogen interaction (Diehn and Relman,

2001), use of microarrays for molecular diagnosis of mycobacteria (Soini and Musser, 2001) and microarrays for environmental health applications (Medlin, 2001).

Microarrays have been in use for roughly 15 years, and in that time have revolutionized biological research (Fu *et al.*, 2009). Microarrays have enabled a number of studies to be performed on a nearly genome-wide scale, including the monitoring of steady-state gene expression (Cheadle *et al.*, 2005), comparative studies such as locating regions of copy number changes in cancer (Nannini *et al.*, 2009), mapping the binding sites of transcriptional regulators (Chip-on-Chip) (Wang, 2005), and surveying long-range DNA interactions (Wilhelm and Landry, 2009). Analysis of transcriptional activity showing a correlation between transcriptional activity and chromosomal organization in rice (Jiao *et al.*, 2005), study on correlated concurrence of differential expressed genes and yield related quantitative trait loci, providing a potential group of heterosis related genes in superhybrid rice LYP9 (Wei *et al.*, 2009), transcriptomic expression profiling showing the existence of multiple low-temperature regulatory pathways in *Arabidopsis* (Fowler and Thomashow, 2002) and analysis of expression profiling of sHsp genes revealing the differential expression of these genes under stress and at different stages in the life cycle of rice plant (Sarkar *et al.*, 2009) has been studied. This myriad of studies has vastly improved our understanding of genetic interactions and control.

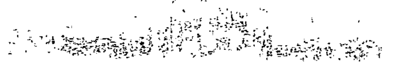
2.12 Genetic transformation of rice

Plant genetic transformation is a technique by which functional genes are inserted in a genome and can be defined as delivery, integration and expression of genes into plant cell, which ultimately regenerate into whole plants.

2.12.1 *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens is a tumor-inducer bacterium in plants (Smith and Townsend, 1907). Its oncogenic capacity (ability to produce tumors) is ascribed to the ability of transferring a portion of DNA into the host genome. The first evidence that a strain of this bacterium was able to transfer the T-DNA with a modified portion of DNA was described by Bevan *et al*, (1983), Fraley *et al*, (1983). The T-DNA penetration from the bacteria to the plant is efficient to produce transgenic plants.

The T-DNA region, which is the part of *Agrobacterium* Ti (tumor-inducing) plasmid DNA transferred to the plant cell, is bracketed by two 25-bp border sequences. The *vir* region of the



Ti plasmid is essential for T-DNA transfer and is induced by specific wound substances from plant. *VirA* and *VirG* proteins (Tzfira and Citovsky, 2000) are involved in sensing plant signals. *VirA* acts as a membrane sensor protein and *VirG* promotes activation of all the *Vir* genes. *VirA* functions as protein kinase and phosphotransferase (Tzfira and Citovsky, 2000). *VirG* is expressed both in the presence and absence of plant phenolic compounds (Stachel and Zambryski, 1986). For the purpose of infection, *Agrobacterium* first weakly attaches to plant, and then synthesizes cellulose fibrils, which anchor them to the wounded plant cell surface. *chvA, chvB, pscA* and *att* bacterial genes are required for this process, as a mutation in any of these genes leaves the bacterium not capable to attach to the plant. A plant glycoprotein vitronectin, which is a component of the plant extracellular matrix (ECM), is also thought to be involved in attachment process. The bacterial cell controls the transfer of the T-DNA complex into the plant cell. T-DNA is covered with proteins to prevent it from degradation outside the bacterial cell; it is referred to as T-complex. The T-complex is made up of single stranded T-DNA coated by VirE2 protein (Tinland, 1996) and VirD2 protein attached at the 5' end (Howard and Citovsky, 1990). The VirD1 and VirD2 protein also recognize and cut the left and right T-DNA borders. The T-complex is coated by VirE2 protecting it from nuclease attack when it enters the plant cell (Tinland, 1996). T-complex is exported into plant cell by the type IV secretion system. This system is assembled from proteins encoded by the virD4 gene and the virB operon (Tzfira and Citovsky, 2000). Eleven VirB proteins play a role in the transport of the T-complex across the membrane. VirB1 initiates the assembly, and VirB2 is the main structural protein in the pilus (Zupan *et al.*, 1998).

The T-DNA does not encode functions for transport and integration; it requires only right and left border sequences for integration. This property makes it so useful that any DNA sequence inserted between the T-DNA borders will be transferred into the plant genome, allowing the efficient production of transgenic plants. The insertion of T-DNA in the genome is a random process. Recently, efforts to improve genetic transformation are based on modifying bacterial components. It has been observed that the transfer of T-DNA depends on the age and physiological stage of the plant tissue (Chang and Chan, 1991; Gould *et al.*, 1991). Acetosyringone, a wound induced phenolic compound, has been reported to increase the frequency of *Agrobacterium*-mediated transformation in soyabean and *Arabidopsis* (Chang and Chan, 1991). Hiei *et al.* (1994) achieved the *Agrobacterium*-mediated transformation of different japonica cultivars. Aldemita and Hodges (1996) also transformed two japonica and

two indica rice varieties using *Agrobacterium.* Transformation of pigeonpea (Singh *et al.*, 2004) and maize was reported by several groups (Schlappi and Hohn, 1992). Transgenic plants carrying the *gus* and *nptII* genes were obtained using this technology by the infection of shoot apices (Gould *et al.*, 1991) and the first robust method for maize *Agrobacterium*-mediated transformation was reported in 1996 (Ishida *et al.*, 1996).

2.12.2 Biolistic-mediated transformation

Particle bombardment is an efficient method for delivering DNA into plant cells. The biolistic gun system is an apparatus capable to deliver high-velocity microparticles. This system relies in the acceleration of microparticles coated with DNA, which are forced to penetrate into the target tissue. This procedure introduces genes into the living cells, which can either express them transiently or stably. This method is especially beneficial for those plants which appear to be a poor interaction with *Agrobacterium*, which is a natural vector used for gene transfer to plants (Weising *et al.*, 1988). It also offer other advantages (Gray and Finer, 1993) over *Agrobacterium*-mediated transformation such as the use of more simplified plasmid constructions, elimination of false positives due to *Agrobacterium* persistence in the host tissue and simplified transformation protocols. Historically, plant virologist used high velocity virus particles as microprojectiles to wound the plant cells and facilitate entry of particles or nucleic acids (Mackenzie *et al.*, 1966). After the pioneering work of Sanford *et al.* (1987) and Klein *et al.* (1987) who developed the first particle delivery system, several different types of bombardment devices have been developed, including an electrically triggered discharge gun that used input voltage (McCabe *et al.*, 1988; Christou, 1993) pneumatic particle guns which used compressed nitrogen gas (Oard *et al.*, 1990; Seki *et al.*, 1991) helium, nitrogen and carbon dioxide powered devices (Finer and MCmullen, 1990; Vain *et al.*, 1993) and a microtargeting gun (Sautter, 1993). These devices have been developed toward the same goals more simplicity safety, accuracy, and a lower cost for DNA delivery.

In this method, 1-2 µm tungsten or gold particles coated with the DNA to be used for transformation are accelerated to velocities which enable their entry into plant cells/nuclei. Particle acceleration is achieved by using a device which varies considerably in design and function. The most successful device accelerates particles in one of the two ways.

(1) by using pressurized helium gas

(2) by the electrostatic energy released by a droplet of water exposed to a high voltage.

The main components of a helium pressure device are gas acceleration tube, rupture disc, stopping screen, microcarrier carrying particles coated with DNA and target cells. These components are enclosed in a chamber to enable creation of partial vacuum which facilitates particle acceleration and reduces damages to plant cells. After creation of partial vacuum sufficiently pressurized helium gas is released in the acceleration tube to break the rupture disc. This generates helium shock waves which accelerates the macroprojectile to which DNA coated microprojectiles are attached. The macroprojectile is stopped by a stopping screen, and the microprojectile pass through this screen. Generally a 1000 psi of helium pressure is used for acceleration. The macrocarrier is a 2.5 cm diameter, 0.06mm thick plastic membrane which is used only once. The light mass of macroprojectile offers certain advantages, including rapid acceleration. The microprojectiles vary in diameter from 0.5 to 2.0 µm. The average size of 1.0 µm is commonly used.

Biolistic technique, a means for direct gene transfer has enabled plant biologists to transform soybean (McCabe *et al.*, 1988; Christou, 1993), corn (Gordon-Kamm *et al.*, 1990), rice (Christou *et al.*, 1991), cotton (Finer and MCmullen, 1990) and peanut (Livingstone and Birch, 1995).

Christou *et al.* (Christou *et al.*, 1991) successfully developed transgenic rice plants from both *japonica* and *indica* varieties. Koziel *et al.* (1993) introduced a synthetic gene encoding a truncated version of Cry1Ab protein developed from *Bacillus thuringiensis* (Bt) into an immature embryo of an elite rice cultivar using particle bombardment. Transgenic fertile plants were also obtained using herbicide resistance *bar* (phophinothricin acetyltransferase) gene (Cao *et al.*, 1992) and hygromycin resistance, *hpt* (hygromycin phosphotransferase) gene (Li *et al.*, 1993) as a selective agent. Zhang *et al*, (1996) used embryogenic suspension to transform *indica* rice varieties IR24, IR64 and IR72 by optimizing osmotic conditions for biolistic transformation. Potato proteinase II gene was introduced into rice by biolistic bombardment of cell suspension culture by Duan *et al.* (1996). Procedures for biolistic transformation of rice have been improved by using embryogenic callus or cell suspension aggregates, optimizing the age of tissue at the time of gene transfer, giving an osmotic pre- and post-transformation treatment of 0.6M carbohydrate and by applying an improved selection procedure (Chen *et al.*, 1998; Breitler *et al.*, 2002).

The particle bombardment technique allows transforming those plants, which cannot be transformed by using conventional *Agrobacterium* or electroporation techniques. However,

the conversion frequency of transient to stable transformation events using biolistic gun is challenging. There is a need to identify ways to make the cells competent for stable DNA uptake.

2.12.3 *In planta* transformation

Since the breakthrough of Hiei and coworkers in 1990s, the *Agrobacterium* mediated transformation in rice has become a routine method (Hiei *et al.*, 1994). This method harbours tissue culture practices which are time consuming and require sterile conditions. Also somatic mutation and somaclonal variation frequently occurs in the tissue during *in vitro* culture. But in case of *in planta* transformation, which do not involve *in vitro* culture of plant cells or tissues, these disadvantages are overcome (Supartana *et al.*, 2005). This method had been used in transformation of buckwheat (Kojima *et al.*, 2000), mulberry (Ping *et al.*, 2003) and kenaf (Kojima *et al.*, 2004). For buckwheat the apical meristems of seedlings were inoculated with *Agrobacterium tumefaciens*, whereas in mulberry the meristems of axillary buds were inoculated. *In planta* transformation methods have also been standardized for rice (Supartana *et al.*, 2005). In all the crops, *Agrobacterium* is directed towards either the apical meristem or the meristems of axillary buds. *In planta* transformation protocol has also been standardized for other crops like peanut (Rohini and Sankara Rao, 2000a, Rohini and Sankara Rao, 2001), safflower (Rohini and Sankara Rao, 2000b) and sunflower (Sankara Rao and Rohini, 1999). The strategy essentially involves *in planta* inoculation of embryo axes of germinating seeds and allowing them to grow into seedlings in soilrite.

2.13 Functional validation of gene using RNAi

RNA silencing is a novel gene regulatory mechanism that limits the transcript level by either suppressing transcription (TGS) or by activating a sequence- specific RNA degradation process [PTGS/RNA interference (RNAi)] (Agrawal *et al.*, 2003). RNA interference, most commonly referred to as RNAi, is a naturally occurring mechanism employed by the cell to mediate gene regulation. It has also been extensively used in various species in host antiviral defense (Ratcliff *et al.*, 1999; Fusaro *et al.*, 2006), transposon silencing (Jensen *et al.*, 1999) and has a potential application in plant functional genomics, to suppress gene function (Hannon, 2002). RNAi has developed into one of the most efficient and robust genetic method for unlocking the secrets of genome as other methods have drawbacks over RNAi like

lack of control over transgene copy number and over the transgene integration site. RNAi-based reverse genetics has its own advantages like screening of a large population is not required as silencing is directed against a specific gene, transcripts of multiple genes from a family can be silenced by a single construct (Alvarez *et al.*, 2006) and stable inheritance of the transgenic RNAi gene.

The mechanism of gene silencing was first noted in plants in 1990 by Napoli *et al.* (1990) and later confirmed by Van Blokland (1994), where it was termed co-suppression as it resulted in the repression of both the transgene and endogenous gene. siRNA mediated suppression was confirmed as a mechanism by Fire *et al.* (1998), who showed a similar transient phenomenon occurred when double-stranded RNA was injected into in *C. elegans.* Subsequently, stable silencing has been achieved by the enforced expression of short hairpin RNAs (shRNAs) in different model systems such as *C. elegans*, *Drosophila* and plants (Kennerdell and Carthew, 2000; Smith *et al.*, 2000; Tavernarakis *et al.*, 2000).

Gene silencing using RNA interference (RNAi) utilizes small double stranded RNAs of 23 to 25 nucleotide length to silence the desired complementary mRNA. The short stretches of double stranded RNAs, which bring about the silencing of genes, are referred to as siRNAs (small interfering RNAs). These siRNAs are capable of degrading mRNAs that are complementary to one of the siRNA strands. The mechanisms underlying this functionality have become clearer with the recognition of the role played by the enzyme dicer (an RNase III family member). Following cleavage of the double stranded RNAs (dsRNAs) by dicer, into 23–25 nucleotide lengths (Schwarz *et al.*, 2002; Khvorova *et al.*, 2003; Myers *et al.*, 2003) they associate with a protein assembly called the RNA Induced Silencing Complex (RISC) and subsequently recognize specific mRNAs through base pairing. The 3′ unpaired region of the small RNA, referred to as the 'seed sequence' zipper up with the 5′ region of the binding site on the target RNA so guiding the RISC to its appropriate target. This complex harbours specific catalytic activity (Slicer) that has been suggested to selectively degrade one of the strands (Martinez *et al.*, 2002) leaving the other to be associated with the complex to target further fresh messenger RNAs (Fig. 2.7).

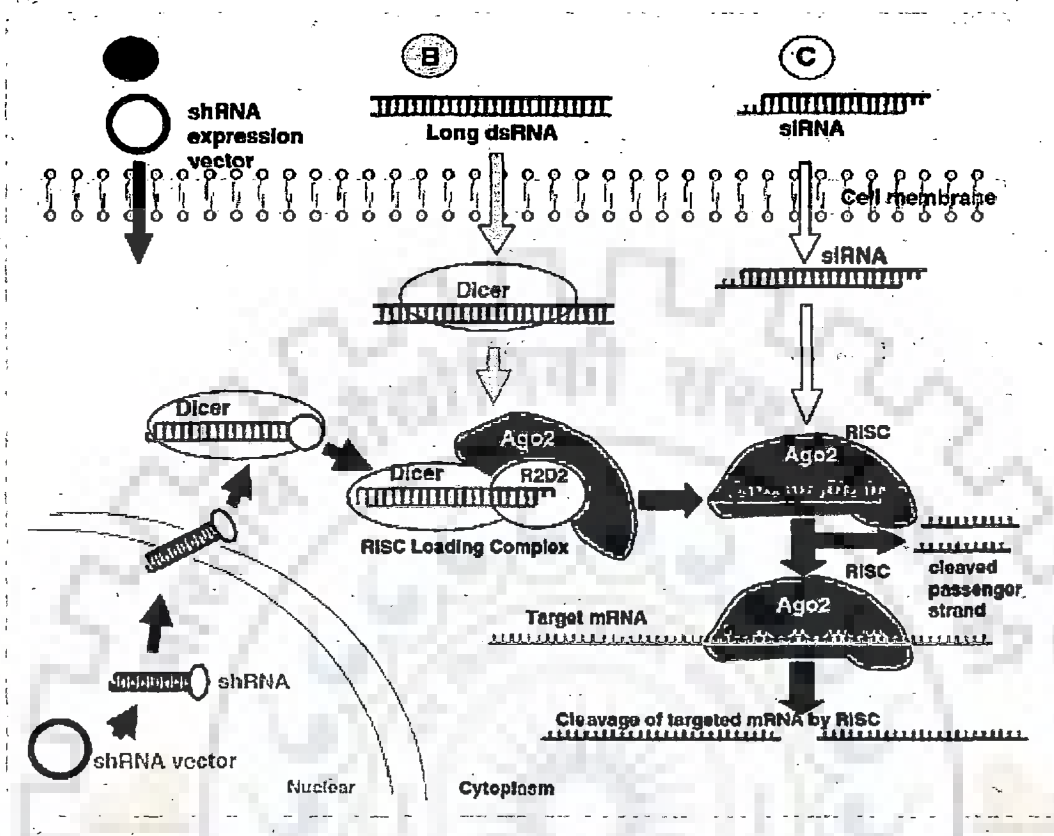


Figure 2.7 Mechanism of RNA interference (RNAi). RLC contains a Dcr-2/R2D2 heterodimer which binds the siRNA containing dinucleotide 3'-overhangs. Core RISC component Ago2 displaces Dcr-2/R2D2. This schematic depicts transfer of duplex siRNA to Ago2 with either concurrent or immediate Ago2-mediated cleavage of the passenger strand. ATP hydrolysis is required for RISC maturation and has been postulated to accelerate release of the cleaved passenger strands as it does for cleaved mRNA. Mature RISC guides strand of the siRNA, and cleave mRNA targets. The pink arrows represent the pathway for a shRNA (A). The green arrows represent the pathway for a long dsRNA (B). The yellow arrows represent the pathway for a siRNA (C) (Chen et al., 2008).

In plants, RNAi is often achieved by a transgene that produces hairpin RNA (hpRNA) with a dsRNA region (Waterhouse and Helliwell, 2003). Conventionally, antisense-mediated gene silencing has been widely used in the analysis of gene function in plants. Although antisense-mediated gene silencing is an RNAi-related phenomenon (Di Serio *et al.*, 2001), hpRNA-induced RNAi has been shown to be much more efficient (Chuang and Meyerowitz, 2000). In an hpRNA-producing vector, the target gene is cloned as an inverted repeat spaced with an

unrelated sequence and is driven by a strong promoter, such as the *35S CaMV* promoter for dicots or the maize ubiquitin 1 promoter for monocots. When an intron is used as the spacer, which is essential for stability of the inverted repeat in *Escherichia coli*, the efficiency becomes very high: almost 100% of transgenic plants show gene silencing (Smith *et al.*, 2000; Wesley *et al.*, 2001). RNAi can be used against a vast range of targets; 3' and 5' untranslated regions (UTRs) as short as 100 nt could be efficient targets of RNAi.

The rice mutant line *LGC-1* (Low Glutelin Content-1) was the first commercially useful cultivar produced by RNAi (Kusaba *et al.*, 2003). In *Arabidopsis* two hypomorphic mutants for *APC6* and *APC10* genes were developed using RNAi strategy, which address the function of APC/C in developing plants and in differentiated cell types, including defects in cotyledon vein patterning and internode elongation leading to a characteristic broomhead-like phenotype. Histological analyses revealed an increased amount of vascular tissue, indicating a role for APC/C in plant vasculature development and organization (Marrocco *et al.*, 2009). Roodbarkelari (2010) used RNAi silenced *APC10* and *APC11* mutant lines and found role of Cullin 4-RING finger-ligase in the control of endoreplication cycles in *Arabidopsis* trichomes. RNAi has 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; Isshiki *et al.*, 2001; Lee *et al.*, 2004; Miki and Shimamoto, 2004; Ogita *et al.*, 2004). 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).

2.14 Bioinformatics a tool for protein interaction prediction

Bioinformatics refers to the application of computer science and information technology in the field of biology and medicine. It involves availability of sequence and structural information, data mining, modeling and simulation studies by implementing computational algorithm and software tools. The field of bioinformatics is playing an increasingly large role in the study of fundamental biomedical problems, designing molecular models and predicting the structure and function of genes and proteins (Baxevanis and Ouelette, 2001). *In silico* domain analysis of the pectinase sequences was done by Yadav *et al.* (2009) which provided insight into

possible functions associated with the existing active site of the enzyme, which might be a target for genetic manipulation for enhanced activity of the enzyme. Prasad *et al.*, (2010) used *in silico* approach to identify in a genome-wide context for Carboxylate Clamp type Tetratricopeptide Repeat Proteins in *Arabidopsis* with potential to interact with Hsp90/Hsp70. A comparative assessment of different protein-protein interaction data sets to identify biases between these methods had been described (Mering *et al.*, 2002).

Chapter III

FEMALE STERILITY IN RICE INSERTIONAL MUTANT OsAPC6

3.1 Introduction

Rice (*Oryza sativa* L., 2n=24) is the second most important cereal and the staple food for more than one-third of the world's population. After the complete genome sequencing of *Oryza sativa* L. (IRGSP, 2005), it is highly imperative to functionally characterize the annotated genes. Several approaches of reverse genetics have been used for functional genomics (Parinov and Sundaresan, 2000; Krishnan *et al.*, 2009) and a large number of insertional mutants have been generated (Gilchrist and Haughn, 2010) in *Arabidopsis* and rice for gene annotation (Filleur *et al.*, 2001; Kumar *et al.*, 2010; Puri *et al.*, 2010).

In about 70% angiosperms including *Arabidopsis* and rice, embryo sac development is of polygonum type (Maheshwari, 1950; Reiser and Fischer, 1993) having two stages of development, megasporogenesis followed by megagametogenesis, occurring inside the ovule. An archesporial cell from nucellus undergoes one mitotic division to produce a megasporocyte and a somatic cell. The megasporocyte (or megaspore mother cell) undergoes meiosis to form four megaspores out of which the megaspore closest to the chalaza enlarges while the three nonfunctional megaspores degenerate and are eventually crushed by the expanding functional megaspore (Reiser and Fischer, 1993). This chalazal megaspore undergoes three successive mitotic divisions to form seven-celled coenocytic megagametophyte. Wall formation, nuclear migration and differentiation follow further, forming a mature seven-celled and eight-nucleated embryo sac (Lersten, 2004). Three cells opposite to the micropylar end form antipodal cells, three cells at the micropylar end form the egg apparatus consisting of an egg cell and two synergids. During cellularization one nucleus from each pole migrates towards the centre forming the polar nuclei or the central cell. In angiosperms, double fertilization occurs where one sperm cell fuses with egg cell forming zygote while the second with central cell resulting in the formation of endosperm (Raghavan, 2003).

A number of mutants defective in gametogenesis have been isolated and described in model plants (Liu and Qu, 2008). Several mutants in *Arabidopsis* with defective cell cycle progression such as mutations affecting division initiation and regulation during the three rounds of mitotic nuclear divisions have been reported (Moore *et al.*, 1997; Pagnussat *et al.*, 2005). In rice defective male gametogenesis has been reported

in a T-DNA insertional mutant *rip1* where the insertion was in the *rice immature pollen 1* a pollen preferrential gene (Han *et al.*, 2006).

The cell cycle in eukaryotes is regulated by coordinated destruction of cell cycle regulatory proteins through ubiquitin mediated 26S proteasome. The key component of the proteolytic system is a multi-subunit ubiquitin ligase and anaphase-promoting complex/cyclosome (APC/C) that provides a platform and specificity for the ubiquitination reactions (Pal *et al.*, 2007). The APC/C is essential for the ubiquitin-dependent degradation of cell cycle regulatory proteins. APC/C regulates mitosis including metaphase-anaphase transition and mitotic exit and maintains G1 phase. The structure of APC is conserved among eukaryotes and phylogenetic analyses indicate that some APC subunits have experienced gene duplication events in plants, in contrast to animals (Lima *et al.*, 2010). In general, plant APC/C has 11 core subunits, where APC2 which is a distant member of the cullin protein family that functions as a scaffold in SCF (SKP1, Cullin/CDC53, F-box protein) assembly (Gieffers *et al.*, 2000). APC11 is similar to the RING-H2 finger protein Rbx1, which plays a key function in the ubiquitylation reaction (Gmachl *et al.*, 2000). The largest APC/C subunit (APC1) also shares some homology with two subunits of the 26S proteasome. The TPR-repeat-containing APC/C subunits (APC3, APC6, APC7 and APC8), the Doc-domain protein APC10/DOC1 and the RING-H2 domain protein APC11 are some other subunits identified in plants. The function of some of these subunits has been characterized. Initial evidence supporting a particular role for the APC/C in plants came from the characterization of the *CCS52* gene, a functional homolog of the yeast CDH1 activator (Cebolla *et al.*, 1999) and its role in cell division comes from characterization of cohesin mutants (Bai *et al.*, 1999; Bhatt *et al.*, 1999). In *Arabidopsis*, expression of several subunits of APC has been detected suggesting that in plants the complex might be regulated by subunit availability and that different subunits could play unique regulatory roles (Eloy *et al.*, 2006). Mutations in *HOBBIT/CDC27b* gene in *Arabidopsis* strongly affects cell division and differentiation after embryogenesis (Blilou *et al.*, 2002). The CDC27a subunit of APC in *Arabidopsis* was ectopically expressed in *Nicotiana tabacum* and was found that the overexpression of *AtCDC27a* exhibits increased growth rate and organ size indicating multiple roles during plant development (Rojas *et al.*, 2009). Defects in

vascular development due to reduced levels of APC6 and APC10 subunits has been reported in *Arabidopsis* (Marrocco *et al.*, 2009). In yeast specific mutation in either CDC16 or CDC23 results in stabilization of cyclin B leading to anaphase arrest (Irniger *et al.*, 1995). Importantly, *cdc16* and *cdc23* mutants are not only defective in exit from mitosis but are also defective for separation of sister chromatids at the metaphase-to-anaphase transition (Harper, 2002). In another mutant *MtCDC16* of *Medicago truncatula* reduced number of lateral roots, a 4-fold increase in nodules and reduced sensitivity to auxin was observed as a result of partial suppression of CDC16 subunit of APC indicating a potential function of CDC16 in auxin signaling (Kuppusamy *et al.*, 2009).

A superfine quality *indica* rice cultivar Basmati 370 was transformed through *Agrobacterium tumefaciens* strain EHA101 (Dhaliwal *et al.*, 2001) and a T-DNA insertional mutant *OsAPC6* of Basmati 370 was isolated (Kumar *et al.*, 2010). Single copy insertion was confirmed using Southern hybridization and TAIL-PCR was conducted to find the position of insertion. The T-DNA flanking region sequenced through thermal asymmetric interlaced polymerase chain reaction showed a single hit on chromosome 3 of *japonica* rice cultivar Nipponbare in the second exonic region of a gene which encodes for one of the subunit *APC6* of anaphase promoting complex. The insertion led to knock-down of the *APC6* gene. The mutant with dark green leaves and normal pollen viability had reduced plant height (nearly 50%) and seed set (upto 40-45%) and insensitivity to exogeneous GA_3. The present article deals with the confocal laser scanning microscopic (CLSM) studies of female gametophyte development in the rice mutant *OsAPC6* with high female sterility.

3.2 Materials and methods

3.2.1 Plant material

Initial generation of T-DNA insertion lines and segregation analysis had already been described in our previous articles by Dhaliwal *et al.* (2001) and Kumar *et al.* (2010). From the selfed progeny of *OsAPC6* mutant, dwarf plants having partially sterile panicles were taken to investigate the cause of sterility in the *OsAPC6* mutant. The isogenic wild type Basmati 370 panicles having normal fertility was taken as

control. All the plant material was sown at the green house at the Indian Institute of Technology, Roorkee, Uttarakhand, India.

3.2.2 Cytological analysis

For meiotic analysis, panicles from the mutant plants were fixed in Carnoy's solution (6 ethanol: 3 chloroform: 1 acetic acid) for 24 h and transferred to 70% ethanol. Anthers at various stages of meiotic division were squashed in 2% acetocarmine, and pollen mother cells (PMCs) were scored for chromosome number and pairing. Photographs were taken with a digital camera (Canon PC1049, No. 6934108049).

3.2.3 Pollen Viability

Pollen grains from the mutant and wild type Basmati 370 plants were collected from freshly dehiscing florets and anthers. Pollen viability in the mutant was recorded after I_2-KI staining. The stained pollen grains were observed by the Zeiss Aristoplus (Germany) microscope. Well developed and round pollen grains with deep blue coloured starch grains were classified as fertile while the smaller grains with pale yellowish colour as sterile.

3.2.4 Fixation of material for CLSM

Rice panicles were collected at different developmental stages from Basmati 370 and its *OsAPC6* mutant. Florets with open glumes with mature embryo sacs ready for fertilization were collected at noon. All the samples were fixed in FAA (formaldehyde, acetic acid and 50% ethanol in 5:6:89 ratio) for at least 24 h, then washed with 50% ethanol and stored in 70% ethanol at 4°C.

3.2.5 Staining and mounting of material

The ovaries were dissected from the florets in 70% ethanol under a binocular dissecting microscope, and hydrated sequentially in 50% ethanol, 30% ethanol and distilled water. The staining procedure for ovaries was according to Zeng *et al.* (2007) but the florescent dye used here was Eosin Y ($C_{20}H_6Br_4Na_2O_5$, FW 691.9, a tissue stain). The ovaries were pretreated in 2% aluminium potassium sulphate for 20 min to allow the dye to enter the embryo sac more readily. The ovaries were then

stained with 1g/L Eosin Y solution (dissolved with 1.6 mL glacial acetic acid) for 10–12 h at room temperature. The samples were post-treated in 2% aluminium potassium sulphate for 20 min in order to remove excess dye from the ovary walls. The samples were rinsed with distilled water three times, and dehydrated with a series of ethanol solutions (30%, 50%, 70%, 90% and 100%). Subsequently, the dehydrated samples were transferred to a mixture of absolute ethanol and methyl salicylate (1:1) for 1 h, and then cleared in pure methyl salicylate solution for at least 1 h. The mounting was done in 80% glycerol and slides prepared were sealed with nail paint.

3.2.6 Scanning of embryo sac

The mounted ovaries were scanned under Leica TCS SP5 (AOBS-Acousto optical beam Splitter based) Laser Scanning Confocal Microscope (CLSM). CLSM was equipped with Ar Laser and AOBS filter set for illumination. The excitation wavelength was 514nm and the emission wavelength was detected to be between 525 to 600 nm. The images were collected at 0.2 to 0.4 μm optical sections using 20x and 40x .The images were collected using LAS AF version 2.3.5. build 5379 software (Leica). Analysis and processing of the images was done using LAS AF version 2.0.2. build 2038 software (Leica).

3. 2.7 Reverse Transcriptase PCR

The seeds of the mutants and wild type Basmati 370 were grown till three-leaved seedling stage. The total RNA was isolated from these seedlings using the Spectrum™ Plant Total RNA Kit obtained from Sigma Aldrich. RNA concentration was measured spectrophotometrically and quality of RNA was determined using denaturing formaldehyde gel. mRNA was reverse transcribed using the superscriptTM II RT Kit (invitrogen) and 2 μL of it was used for further PCR amplification using gene specific primers. Normalization of the signals obtained for the gene of interest was done using a housekeeping gene Elongation factor 1α (Ef1α) as a reference gene. A set of gene specific primers based on the coding sequence of the candidate gene was used for RT-PCR. The sequence of the primer was as follows:
Forward-APC6F: 5'- AGCGCGTTTGCTGGTCTTGCTT-3' (T_m=60.43°C)
Reverse-APC6R: 5'- GTCCGAGCAGTGATTTGACAGCTGG-3' (T_m=60.06°C)

3.3 Results

3.3.1 Chromosome pairing and pollen viability in OsAPC6

Meiotic analysis at metaphase, anaphase and early telophase I of the pollen mother cells of the mutant from the fixed florets showed normal chromosome pairing with twelve bivalents, normal movement of homologous chromosomes to poles during anaphase, normal telophase without any micronuclei (Fig. 3.1a-c) and normal pollen tetrads. On an average 92-98% pollen grains were stainable and hence viable in most of the florets. The results of normal meiotic chromosome pairing and high pollen stainability similar to that of the wild parent Basmati 370 indicated that microsporogenesis and male gametophyte development were normal in *OsAPC6* mutant.

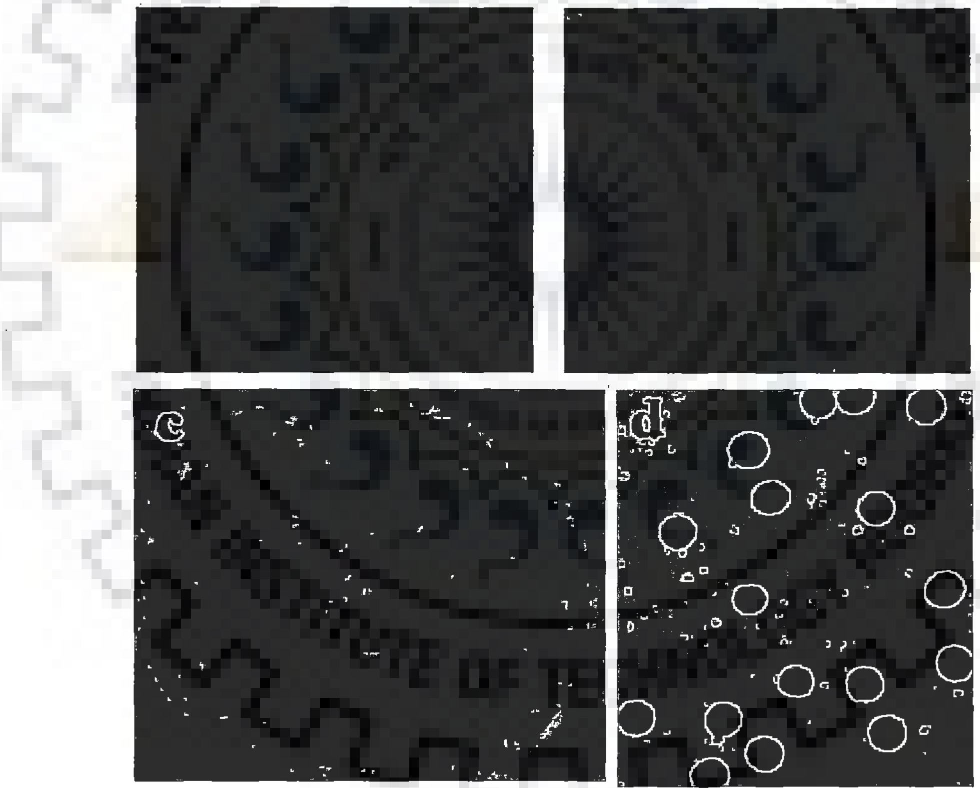


***Figure 3.1** Chromosome pairing and pollen grain stainability in OsAPC6 mutant. **a.** bivalent chromosomes (2n=24) at metaphase I, **b.** Chromosomes arranged at the opposite poles during anaphase I **c.** early telophase I where chromosomes has moved to opposite poles **d.** Nearly normal pollen viability.*

3.2 Female fertility

There was reduced seed set upto 40-45% in the mature panicles of *OsAPC6* as compared to wild type parent Basmati 370 (Table 3.1).

Table 3.1: Female sterility observed in mature panicles of mutant *OsAPC6* and wild type Basmati 370 plants.

Mutant plant	Fertile seeds	Sterile seeds	Percentage sterility	χ^2 value at df =1	
				Observed χ^2 at fertile: sterile 1:1 ratio	Level of significance
OsAPC6-1	170	118	40.9	9.38	$p \leq 0.005$
OsAPC6-2	166	128	43.3	4.90	$p \leq 0.05$
OsAPC6-4	121	91	42.9	4.24	$p \leq 0.05$
OsAPC6-5	115	85	42.5	4.50	$p \leq 0.05$
Basmati 370	221	05	97.7	-	-

The sterility in the mutant was expected to be 50% if the penetrance of the mutation is complete. But there was a decrease in this ratio which was found to be significant at probability $p \leq 0.05$ when tested for χ^2. Significantly less than 50% sterility in the mutant plants indicates incomplete penetrance of *OsAPC6* in causing female sterility due to which some of the female gametophyte with the lethal mutation develops normally. The sterile florets with empty hulls stayed green while the fertile florets had well filled grains with brown hulls (Fig. 3.2).

Furthermore, the panicles were more compact due to dwarfness and gibberellic acid insensitivity in *OsAPC6* as reported previously by Kumar *et al.* (Kumar *et al.*, 2010).

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***Figure 3.2** Female fertility in panicles of **a**. wild type Basmati 370, **b**. OsAPC6 dwarf mutant (with reduced seed set and height) at mature stage. The sterile florets have green coloured hulls.*

The panicles of these mutant plants were fixed in the field conditions at different stages of development and female gametophyte development was observed using CLSM.The abnormal gametophyte development was observed in about 30-35% floret which was significant at $p \leq 0.05$ (Table 3.2).

Table 3.2: CLSM analysis of megagametophyte development in fixed florets from panicles of *OsAPC6* mutant plants

Florets per panicle	Abnormal florets	Normal florets	Percentage abnormality	χ^2 value at df =1	
				Observed χ^2 at normal: abnormal 1:1 ratio	Level of significance
93	30	63	32.2	11.6	$p \leq 0.05$
88	28	60	31.8	11.6	$p \leq 0.05$
105	36	69	34.9	11.6	$p \leq 0.05$
103	36	67	34.9	11.6	$p \leq 0.05$
105	33	72	31.4	9.3	$p \leq 0.05$
94	31	63	32.9	14.4	$p \leq 0.05$

The reduced percentage of abnormality in fixed panicles and mature panicles was observed which could be attributed to the difference in development stages of the florets in a fixed panicle. The germination of seeds at 80 ppm hygromycin in the four plants of *OsAPC6* mutant and wild type Basmati 370 was observed and the ratio of hygromycin resistant: susceptible seeds in the mutant was found to be greater than 50% for all the four plants. The significance of the hygromycin resistance data was checked using χ^2 and it was found that more than 50% resistance in the mutant was significant at $p \leq 0.05$ and for one *OsAPC6* plant it was significant at $p \leq 0.1$ (Table 3.3), further indicating incomplete penetrance of the mutation.

Table 3.3: Hygromycin resistance in well-developed seeds of four plants of *OsAPC6* mutant and the wild type Basmati 370.

Plant	Resistant seeds	Susceptible seeds	Percentage resistance	χ^2 value at df =1	
				Observed χ^2 at resistant: susceptible 1:1 ratio	Level of significance
***OsAPC6*-1**	69	51	57.5	3.33	$p \leq 0.10$
***OsAPC6*-2**	81	49	62.3	7.87	$p \leq 0.05$
***OsAPC6*-4**	77	53	59.2	4.4	$p \leq 0.05$
***OsAPC6*-5**	78	52	60	3.8	$p \leq 0.05$
Basmati 370	0	50	0.0	-	-

3.3.3 Female gametophyte development

Normal male gametogenesis of the mutant indicated that the sterility could be due to defective megagametogenesis in the mutant. To determine the nature of defect in *OsAPC6* female gametophyte, we analyzed the embryo sac formation at different developmental stages using CLSM. Each floret observed in the mutant and wild type had an ovary where the ovule was bent back on itself at nearly 180 degrees (Fig 3.3a). An embryo sac with seven-cell and eight-nuclei structure within a large embryo sac cavity was also observed in wild type Basmati 370 (Fig 3.3b). A mature and fully receptive embryo sac of the wild type Basmati 370 with three antipodal cells, two polar nuclei and an egg apparatus was observed (Fig. 3.3c). After double fertilization of the egg cell and the polar nuclei with the two respective sperm nucleus, zygote was formed and endosperm formation and the degeneration of the antipodal cells were initiated (Fig. 3.3d). A fully developed syncytial endosperm was formed in the wild type which filled the embryo sac cavity completely (Fig 3.3e). CLSM studies in case of *OsAPC6* mutant showed that the fertilization was normal as zygote was formed and the degenerating antipodal cells were also observed (Fig 3.3 g and h). However from CLSM studies we found that around 31-35 % embryo sacs had abnormal gametophyte development (Table 3.2).

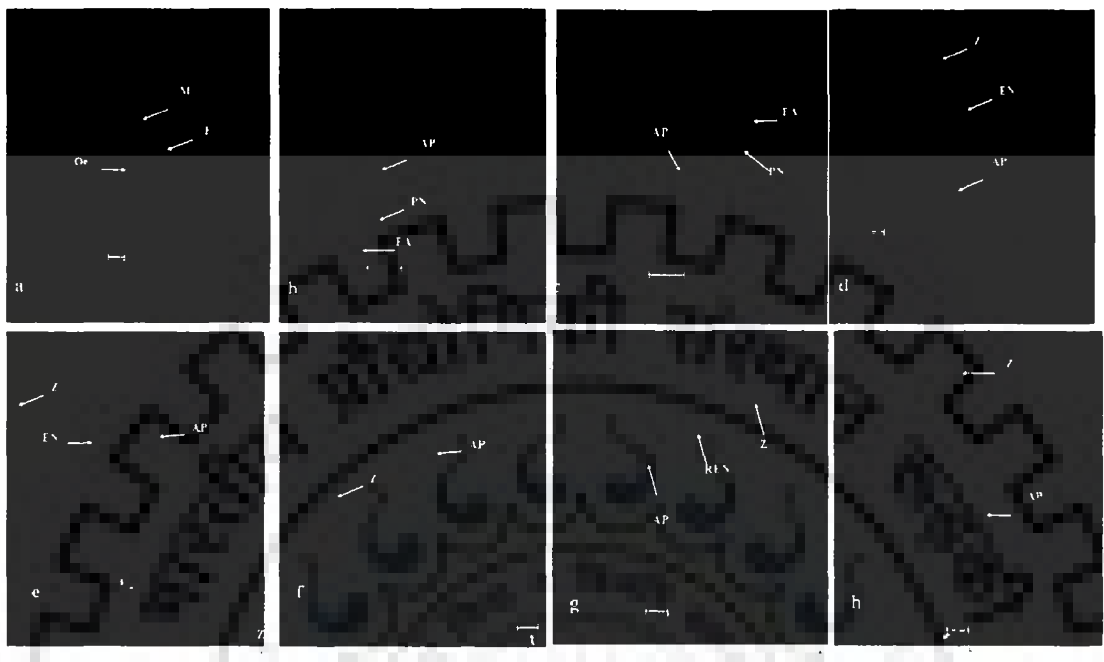

Figure 3.3 *Female gametophyte at different stages of development in wild type (Basmati 370) and mutant (OsAPC6).* ***a.*** *Anatropous ovule,* ***b,c.*** *mature embryo sacs at different stages of development in WT,* ***d.*** *mature embryo sac in WT showing endosperm formed soon after fertilization,* ***e.*** *mature embryo sac in WT showing fully developed endosperm,* ***f.*** *mature embryo sac in mutant with no endosperm,* ***g.*** *mutant embryo sac showing fertilized zygote but reduced endosperm,* ***h.*** *Zygote is developed normally but endosperm is absent in mutant. AP= antipodal cell, EA= egg apparatus, EC= embryo sac cavity, EN= endosperm, F= funiculus, Oe= ovule, M= micropyle, PN= polar nucleus, REN= reduced endosperm, Z= zygote. Bar= 50μm.*

On further analysis of immature embryo sacs we found that female gametophytes of wild type Basmati 370 had normal mitotic division (Fig 3.4 a, b) while in the mutant gametophyte a cell at the micropylar end with brighter nuclei and a dividing cell at the chalazal end with two nuclei was observed (Fig 3.4c). The chalazal end cell was probably undergoing second mitosis whereas in the micropylar end cell the division was delayed or arrested.

In Fig. 3.4 d vacuole formation by the degradation of the nucellar tissue can be observed but towards the central part of the gametophyte single nucleus instead of two polar nuclei was observed.

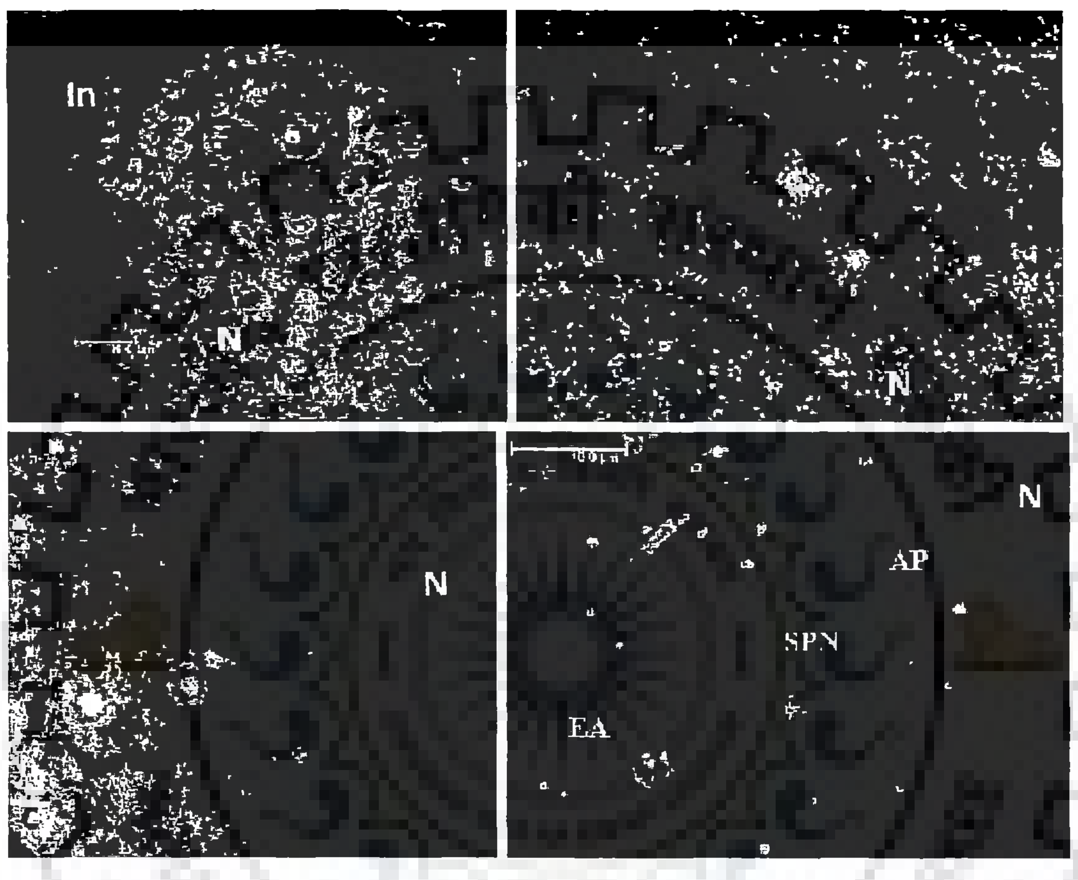

Figure 3.4 *Megagametogenesis in OsAPC6* ***a.*** *a megaspore ready to enter mitotic-phase,* ***b.*** *two nucleus formed after normal first mitotic division* ***c.*** *cell shown by arrowhead did not proceed for second mitosis in mutant,* ***d.*** *after three rounds of mitosisin mutant seven nuclei observed (shown by arrowhead), in the central region of degenerating nucellar tissue single nuclei instead of two nucleus in the secondary polar cell was observed, AP= Antipodal cell, In= integuments, EA= egg apparatus, N= nucellar tissue, SPN= single polar nucleus. Bar= 10μm.*

This could be probably due to the abnormal third mitotic division in one of nuclei formed after second mitosis which led to formation of seven nuclei in place of eight. We found that the female gametophytes were arrested at three-nucleate or seven nucleate stages, indicating that *APC6* loss-of-function impairs megagametogenesis after first or second mitotic division.

3.3.4 Expression of APC6 in the wild type and mutant plants

Reverse transcriptase PCR was performed to check the expression of the *APC6* gene in the wild type and the mutant plant mRNA. Normalization of the signals obtained for the gene of interest was done using a housekeeping gene Elongation factor 1α (*Ef1α*) as a reference gene (Fig. 3.5a). The mutant progeny segregating for height consisted of tall and dwarf plants. The expression of *APC6* gene was studied in two dwarf and a homozygous tall plant of the mutant progeny together with the wild type Basmati 370 using two primers. The primer pair A was designed flanking the region of insertion while the second primer pair B was used to amplify the 3'end region of the mRNA as shown in Fig. 3.6. There was reduced expression for the *APC6* gene in the two dwarf plants while the tall plant had similar expression pattern for *APC6* gene as that of the wild type Basmati 370 for both the primer pairs (Fig. 3.5b, c). Further amplification of *hpt* gene was checked and it was found that the dwarf plants were positive for *hpt* while the tall and wild type had no *hpt* amplification (Fig. 3.5d). These results indicate that the mutant dwarf plants with *hpt* selectable marker and the T-DNA insertion had significantly reduced expression of *OsAPC6* transcript. The mutant phenotype was due to knock-down of the *APC6* gene and mutant dwarf plants with reduced seed set could be maintained only in heterozygous condition.

These results indicate that the mutant dwarf plants had *hpt* gene and the T-DNA insertion. The mutant phenotype was due to knock-down of the *APC6* gene and mutant dwarf plants with reduced seed set could be maintained only in heterozygous condition.

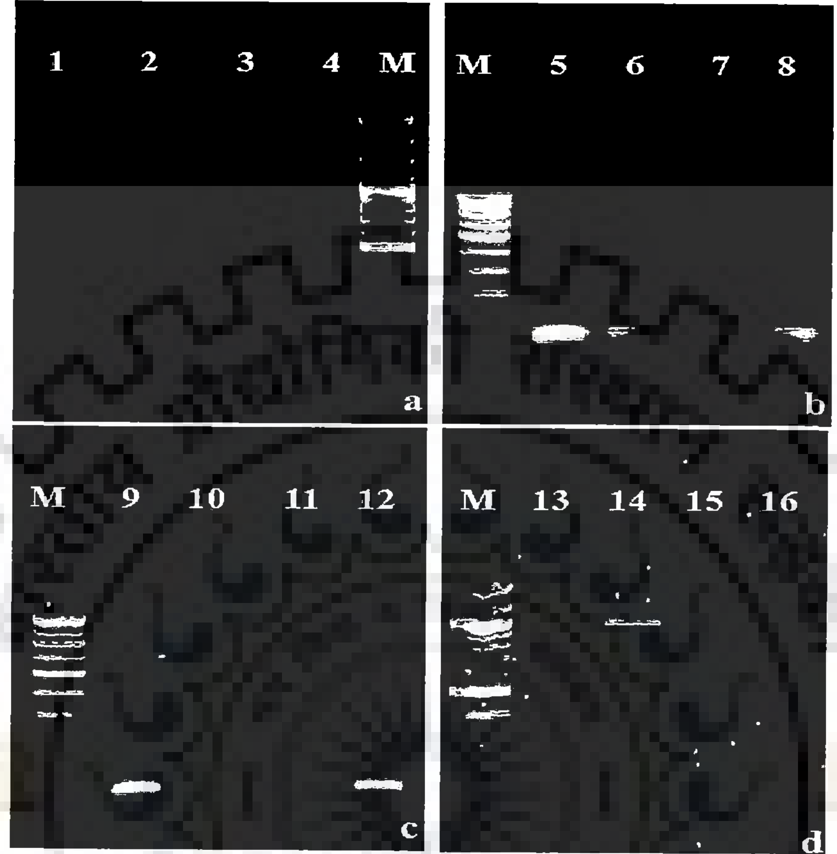

***Figure 3.5** RT-PCR of OsAPC6 and hpt amplification in wild type and mutant plants. **a.** RT-PCR of EF1α (housekeeping gene), **b.** RT-PCR using OsAPC6 gene specific primer, **c.** hpt PCR, Lane 1, 5 and 9 have wild type Basmati 370, Lane 2,3,6,7,10, 11 have dwarf mutant and Lane 4, 8 and 12 have tall mutant, M= 100bp ladder.*

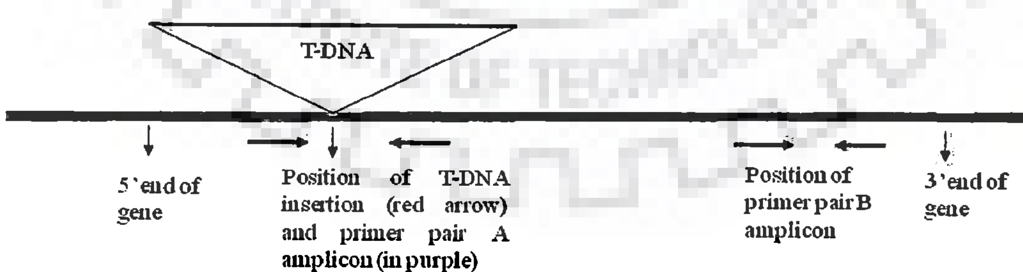

***Figure 3.6** T-DNA insertion in the APC6 gene and position of primer pair A and B used for RT-PCR.*

3.4 Discussion

Normal meiosis and pollen development and slightly higher than 50% fertility and hygromycin resistance in the selfed progeny of the *OsAPC6* mutant plants strongly suggest female gametophyte lethality and incomplete penetrance of the mutant allele. Normal male gametophyte and pollen development have been reported in several mutants like *apc2* and *nomega*, defective in megagametogenesis (Capron *et al*., 2003b; Kwee and Sundaresan, 2003). The expression analysis of the *APC6* gene using RT-PCR showed reduced expression in the mutant indicating that the T-DNA insertion knocked out and suppressed the candidate gene expression. The tall plants without the *hpt* selectable marker used for transformation were found to be normal with almost similar expression of *APC6* gene as that of wild type. All these results unequivocally indicate that the mutant with female gametophyte lethality could be maintained as heterozygous.

The CLSM results showed that the fertilization and the zygote development in both wild type and mutant were normal. There was, however, no endosperm development in a high proportion of female gametophytes of the *OsAPC6* mutant due to abnormal polar nuclei formation. During double fertilization in angiosperms, one sperm nucleus from the pollen grain fuses with the egg cell and the resultant zygote develops into an embryo while the central cell with two polar nuclei fuses with the second sperm nucleus to form a triploid primary endosperm cell which develops into the endosperm (Russell, 1993). Endosperm, as a nutritive source in plants, is generally utilized for embryo development and germination (Mu *et al*., 2010). In *OsAPC6* mutant the fertilization was found to be normal but there was reduced seed set upto 40-45% which could be attributed to the absence of endosperm.

Further megagametogenesis studies showed abnormal second mitosis as one of the nucleus at the micropylar end was found to be more prominent and bright indicating an arrest or delayed division. Also in one of the observed cases there were seven nuclei in the mutant. Normally it is expected to have eight nuclei after three consecutive mitotic divisions but since seven nuclei were observed, indicating that there was an abnormal progression of cell division where one of the nucleus did not either enter third mitotic division or the division was delayed thus the polar nuclei of

the central cell in the mutant were found to be either reduced in number or were completely absent. As it is known that in the Polygonum type embryo sac development, the nuclear migration occurs during cellularization and one nucleus from each of the poles moves towards centre thereby forming two polar nuclei and a central cell (Christensen *et al.*, 1998). In case of *OsAPC6* mutant there was reduction in total number of nuclei at the end of megametogenesis, reduced number or complete absence of the polar nuclei and abnormal development of central cell and endosperm in the mutant.

Abnormal central cell development during megagametogenesis due to arrested or delayed mitotic divisions led to failure of endosperm development and reduced seed set. Several mutations affecting nuclear division, polar nuclear fusion and cell specification associated with megagametogenesis have been described. The majority of the mutants show defects in nuclear division. These include defects in the first division as in female gametophyte 2 (*fem2*), *fem3*, *gametophytic factor* (*gf*), *gametophytic factor 4* (*gfa4*) and *gfa5* of *Arabidopsis* (Drews *et al.*, 1998), in the second or third division as in *cell division cycle 16* (*cdc16*) (Kwee and Sundaresan, 2003) and *prolifera* (*prl*) (Springer *et al.*, 2000) or all the three divisions as in *hadad* (*hdd*) (Moore *et al.*, 1997). Mutation in PRL, a homologue of DNA replication factor Mcm7, causes embryo sac arrest at the four nucleate stage (Springer *et al.*, 2000). A case of slow and arrested cell division in female gametophyte at 2-8 nucleate stage was reported in the *slow walker 2 (swa2)* (Li *et al.*, 2009) and the *slow walker 3 (swa3)* (Liu *et al.*, 2010) mutant of *Arabidopsis* with female sterility. Confocal laser scanning microscopy in another *Arabidopsis* mutant *Gf*, defective in female gametogenesis, showed that the *Gf* mutant female gametophyte was arrested at the uninucleate stage (Christensen *et al.*, 1997). There are several other mutants with of loss-of-function in CYTOKININ INDEPENDENT 1 (Hejátko *et al.*, 2003), DIANA/AGAMOUS-LIKE 61 (Bemer *et al.*, 2008) and Nuclear fusion DEFECTIVE1 (Portereiko *et al.*, 2006) which affect polar nuclei fusion and central cell development in *Arabidopsis*.

The development of female gametophyte involves several rounds of nuclear division during which key proteins like cyclin and securin are needed to be degraded in order to facilitate the metaphase-anaphase transition. This is regulated by ubiquitin

mediated 26S proteolytic system. The key component of this proteolytic system is a multi-subunit ubiquitin E3 ligase, the anaphase-promoting complex (APC/C) that provides a platform and specificity for the ubiquitination reactions (Pal *et al.*, 2007). In yeast several genes have been reported, including *CDC16* and *CDC23*, which, when mutated, blocked mitotic cyclin degradation during G1 (Irniger *et al.*, 1995). In plants, the APC2, APC/NOMEGA, and APC3/HOBBIT (HBT) proteins have been recently characterized. Both *apc2* and *apc6/nomega* mutant plants exhibit defects in gametogenesis (Capron *et al.*, 2003b; Kwee and Sundaresan, 2003). In *Arabidopsis* mutant *apc2* impaired female gametogenesis with cell division arrested at one or two nuclei stage was observed (Capron *et al.*, 2003b). APC2 is a subunit of APC/C complex which plays a role in cell cycle regulation. The embryo sac development in *nomega* mutant of *Arabidopsis* was found to be arrested at the two-nucleate stage leading to female sterility. The NOMEGA gene product has high homology to the CDC16 subunit of APC (Kwee and Sundaresan, 2003). The *nomega* mutant embryo sacs were unable to degrade cyclin B, an important APC/C substrate as the mutation was in the APC6/CDC16 subunit of APC/C complex (Kwee and Sundaresan, 2003). Thus the role of APC/C in plant cell cycle progression is evident.

Since in *OsAPC6* the mutation is found to be in the same gene encoding the Anaphase promoting complex APC6/CDC16 (Kumar *et al.*, 2010) of *Arabidopsis thaliana* (Kwee and Sundaresan, 2003), the abnormal embryo sac development and female sterility could be attributed to this gene. *OsAPC6* had other pleiotropic phenotypic traits such as reduced height, dark green and broad leaves and insensitivity to gibberellic acid which have not been reported in other APC mutants. These pleiotropic effects of the mutant are dominant and hence it was abbreviated as *OsAPC6* (Kumar *et al.*, 2010). Gibberellic acid insensitivity associated with dwarfness has been invariably found to be dominant in wheat, maize (Peng *et al.*, 1999), and rice (Itoh *et al.*, 2002). High female sterility due to abnormal endosperm development in the *OsAPC6* mutant is clearly a lethal pleiotropic effect responsible for its maintenance mostly as heterozygous.

Chapter IV

TRANSCRIPTOMIC EXPRESSION ANALYSIS OF OsAPC6 AND Ossl MUTANTS

4.1 Introduction

Rice is an important cereal for more than a half of the world's population. Rice was the first crop plant with completely sequenced genome (IRGSP, 2005) and is an excellent model plant for genomics studies (Khush, 1997). It contains relatively less repetitive DNA and shares a common synteny with other grass genomes (Moore *et al.*, 1995; Gale and Devos, 1998). In addition, rice has a vast germplasm of cultivated and wild species (Nakagahra *et al.*, 1997; Vaughan, 2003). Traditional Basmati rice cultivars with long, slender and lustrous grains, possessing exquisite scent, soft texture and high kernel elongation are low yielding and susceptible to lodging due to their very tall stature. Introduction of lodging tolerant and fertilizer responsive semi-dwarf rice and wheat cultivars in the mid-1970s ushered in the Green Revolution in developing countries. Semi-dwarf rice mutants like the GA-deficient *sd1* and *d18*, which had mutations in the GA_{20} oxidase and GA_3 oxidase genes, respectively, were used to elucidate the rice GA biosynthetic pathway (Itoh *et al.*, 2001; Ashikari *et al.*, 2002; Sasaki A *et al.*, 2002).

Gibberellins (GAs) which are tetracyclic diterpenoid plant hormones promote a number of plant growth responses, including stem elongation, seed germination, leaf expansion and flowering. GA_3 increases dry matter and leaf-area index in mustard plant (Khan, 1996), and photosynthetic rate in leaves of bean (Khan *et al.*, 2002) and wheat (Ashraf *et al.*, 2002). GA_3 enhances source and sink potential through increasing photosynthetic enzymes, increasing leaf area for higher interception of photosynthetically active radiation and enhancing nutrient use efficiency (Khan *et al.*, 2011).

Genes encoding enzymes involved in the GA biosynthetic pathway have been cloned from a variety of species (Hedden and Phillips, 2000) but much less is known about how the plants perceive GA and transmit the GA signal to cause GA-regulated plant growth. Several positive and negative regulators of GA signal transduction have been identified in several plant species. The positive regulators include *GIBBERELLIN INSENSITIVE DWARF1 (GID1)* (Ueguchi-Tanaka *et al.*, 2005), *GIBBERELLIN INSENSITIVE DWARF2 (GID2)* (Sasaki *et al.*, 2003) in rice, *SLEEPY, (SLY)* (McGinnis *et al.*, 2003), *SNEEZY (SNE)* (Ariizumi *et al.*, 2011) and *PICKLE (PKL)* (Ogas *et al.*, 1997) gene in *Arabidopsis*, whereas the negative regulators include *SLENDER RICE1 (SLR1)* (Ikeda *et al.*, 2001; Itoh *et al.*, 2002), *REPRESSOR OF GA1-3 (RGA 1-3), GIBBERELLIC ACID INSENSITIVE (GAI)* (Peng *et al.*, 1999; Peng and Harberd, 1997; Silverstone *et al.*, 2001), *SPINDLY* (*AtSPY*) (Jacobsen *et al.*, 1996) and

SHORT INTERNODE (*SHI*) (Fridborg *et al.*, 1999) in *Arabidopsis, Reduced height1 (RHT1)* and *Reduced height 2 (RHT2)* (Peng *et al.*, 1999) in wheat, *Slender1 (SLN1)* (Chandler *et al.*, 2002; Gubler *et al.*, 2002) and *SPINDLY (HvSPY)* (Robertson *et al.*, 1998) in barley. The semi-dominant mutation in *Arabidopsis* (*gai* and *rga1-3*), maize (*d8*), wheat (*Rht1* and *Rht2*) 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 semi-dominant mutants have a highly conserved DELLA domain which is the target for proteolysis by the ubiquitin-proteasome mediated pathway. The DELLA proteins from different plant species share a significant homology in amino acid sequence at the N-terminus as well including the highly conserved DELLA domain (Hussain and Peng, 2003).

Although the complete mechanism by which DELLA proteins are degraded by GA has not been described, involvement of the ubiquitin/proteasome-dependent mechanism has been predicted (Dill and Sun, 2001; Itoh *et al.*, 2002). For degradation of the substrate protein by 26S proteasome polyubiquitination is found to be necessary (Wilkinson, 2000). The E3 ubiquitin ligases which help in polyubiquitination comprise HECT domain or a RING/U-box domain. SCF and APC/C complex are two RING E3 ligases (Moon *et al.*, 2004). The anaphase-promoting complex/cyclosome (APC/C) provides a platform and specificity for the ubiquitination reactions (Pal *et al.*, 2007). The APC/C is essential for the ubiquitin-dependent degradation of cell cycle regulatory proteins during metaphase-anaphase transition and mitotic exit and maintains G1 phase (Sudakin *et al.*, 1995; Peters, 2006). Though the role of APC/C in plants hormones signaling has not been yet studied in detail, the SCF complex has been reported to play a major role in signaling of phytohormones including auxin (Gray *et al.*, 2001), jasmonates (Xie *et al.*, 1998; Xu *et al.*, 2002) and gibberellin (Dill *et al.*, 2004; Fu *et al.*, 2004) by degrading the repressors of hormone response. In *Arabidopsis* SCF^{SLY} targets the DELLA proteins RGA and GAI for degradation, which alleviates DELLA-mediated inhibition of GA-regulated growth (McGinnis *et al.*, 2003). Like RGA, in Barley SLN is rapidly degraded in response to GA via the 26S proteasome pathway (Fu *et al.*, 2002). In case of rice the GID1 protein which is a receptor for GA interacts directly with SLR1 in a GA-dependent manner, which forms a GA-GID1-SLR1 complex. The SLR1 which acts as a repressor is then targeted by SCF^{GID2} ubiquitin complex for proteolytic degradation. This results in release of repressive state of the GA action to signal transducing state (Itoh *et al.*, 2002; Ueguchi-Tanaka *et al.*, 2007).

This article deals with the investigations on the response of the *OsAPC6* mutant to exogenous application of GA , effect of T-DNA insertion on the transcriptome of the mutant, conservation of APC6 proteins among different organisms and its possible role in GA signaling.

4.2 Materials and methods

4.2.1 Plant material

The plant material consisted of a T-DNA insertional mutant *OsAPC6* in basmati rice cultivar Basmati 370 along with the wild type parent. The insertional mutant with approximately half the plant height as compared to the wild type Basmati 370, panicle length and reduced seed set was obtained (Dhaliwal *et al.*, 2001). The TAIL-PCR was carried out and the position of insertion was found to be at chromosome 3 on the second exonic region of a gene which encodes for sixth subunit of anaphase promoting complex/cyclosome (APC/C). Southern hybridization confirmed a single copy insertion in the mutant *OsAPC6* (Kumar *et al.*, 2010). The CLSM study of the developing female gametophyte in the mutant indicated that the partial fertility of the mutant was due to failure of normal female gametophyte development (Awasthi *et al.* 2012).

4.2.2 RNA isolation and quantification

The seeds of the mutant and wild type Basmati 370 were grown for 15 days till three-leaved seedling stage. The total RNA was isolated from these seedlings using the Spectrum™ Plant Total RNA Kit (Sigma Aldrich). RNA concentration was measured photometrically (NanoDrop ND-1000 UV-Vis spectrophotometer). To check the quality of RNA optical density (OD) reading of RNA samples were taken at 280nm, 260nm and 230nm wavelengths and the quality was judged on the basis of $OD_{260/280}$ and $OD_{260/230}$. In order to check the resolution and quality of RNA further, denaturing formaldehyde gel was used which was visualized and photographed by using a Gel Documentation system (AlphaInnotech).

4.2.3aRNA preparation and hybridization

The aRNA preparation and labeling was performed by using 3' IVT Express Kit (Affymetrix). An initial concentration of 250ng/µL of RNA was taken for the reverse transcription to synthesize the first strand cDNA at 42°C for two hours. The second strand was synthesized at 16°C for one hour and then at 65°C for 10 min. *In vitro* transcription to synthesize biotin-modified aRNA with IVT labeling from the double stranded cDNA was done by incubating

master mix for 16 hours at 40°C. The purification of the aRNA was done using the magnetic beads and magnetic stand. A set of poly-A RNA control supplied with the GeneChip 3' IVT Express kit was used as a positive control to monitor the entire labeling and hybridization process. The purified aRNA was subjected to fragmentation at 94° C for 35 min. The fragmented aRNA was analyzed on a 1.5% agarose gel. The size distribution was found to be between 35-200nt of aRNA fragments with a peak at 100-120 nt.

Rice Genome Array 49-format array Chip (Affymetrix GeneChip®) was used to hybridize the aRNA samples. After hybridization, the Chip was scanned using a confocal laser scanner (Affymetrix GeneChip Scanner 3000). The images were subsequently analyzed to calculate the relative levels of expression of each gene. The array data set was analyzed using Gene-Chip Operating Software (GCOS 1.2) and Array Star (DNA STAR Inc, Madison, USA) software. For probe level normalization PLIER was performed on Affymetrix raw files (.CEL files). Initial dataset consisted of 57,382 probes. Filtration was performed to remove probes with 'Absent Calls' (Poor quality probes). Transformation and normalization was done in order to facilitate comparison across samples. Biological significance of differentially expressed genes was determined performing functional classification using Gene Ontology after filtering the genes for >2.0 fold change and ≤0.05 p-value. All the raw data files (CEL files; two for wild type Basmati 370 and two for mutant *OsAPC6*) obtained from Gene Chip Operating Software (GCOS; Affymetrix) were subjected for further analysis by using Array Star™ Software.

MIAME compliant Microarray data from this study have been deposited at NCBI Gene Expression Omnibus with the accession number GSE31200.

4.2.4 Gibberellic acid response

Freshly prepared gibberellic acid (GA_3) at 120ppm concentration was used to spray on the leaves of *OsAPC6* mutant and wild type plants during late tillering stage before flowering. Plant height was measured on the day of GA_3 application, prior to spray and 14 days after GA_3 application. The experiment had two sets of plants one set treated with GA_3 and another set without GA_3 application (untreated plants). Final observations of culm elongation after GA_3 spray was taken in both the sets and the response of GA spray were observed.

$$\text{GA3 response (\%)} = \frac{\text{Final height after GA3 spray-Initial height before GA3 spray}}{\text{Initial height before GA3 spray}} \times 100$$

4.2.5 Chlorophyll estimation

The total chlorophyll content of *OsAPC6* 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 (OD663)-0.094(OD644) and
mg chlorophyll b per gram of tissue= 1.77(OD644)-0.280(OD663)

4.2.6 Quantitative Real-time PCR analysis

A part of the samples of total RNA isolated for microarray analysis was also used to validate the functionally important genes which were found to be differentially regulated in microarray results. Nucleotide sequences of differentially regulated genes were downloaded from TIGR rice database (http://rice.plantbiology.msu.edu). Exonic sequences of selected genes were used for primer design using PRIMER3 software (http://frodo.wi.mit.edu/primer3/). The gene encoding ATP synthase (F-GTGCTACAGATGGGTTGATG, R-GACCCAAATTGTCAACAGGC) and Cytochrome P450 (F-GGGAAGCCGTGGAGGACATC, R- TCGAACACCGTGTCGTAGCG) were selected for qRT validation. For gene validation experiments SYBR green based One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA, USA) was used. Thermal cycling conditions comprised of 50°C for 1 h followed by an initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 55- 65°C for 1 min, and extension at 72°C for 1 min on a thermal cycler (Mx3000p, Stratagene). The analysis of dissociation curve was performed by taking a Fluorescence reading at one degree interval between 55 to 95°C, to ensure that only one PCR product was amplified. All experiments were performed in two biological replicates and three technical replicates. The expression data were normalized to EF-1α using the ΔΔCT method described by Livak and Schmittgen (2001).

4.3 Results

4.3.1 *OsAPC6* mutant

4.3.1.1 Phenotypic analysis of OsAPC6 mutant

The T-DNA insertional mutant *OsAPC6* of Basmati 370 had reduced height, dark green leaves and gibberellic acid insensitivity. It had already been shown that the insertion is in the second exonic position of *APC6* gene (Fig. 4.1A) (Kumar *et al.*, 2010) due to which the expression of the gene in *OsAPC6* mutant was reduced and the mutant had partial female sterility due to defective female gametophyte development (Awasthi *et al.*, 2012).The leaves of *OsAPC6* mutant were darker green as compared to the wild type under field conditions (Fig. 4.1B). The Chl a and Chl b contents in *OsAPC6* were found to be 4.48 and 1.04 mg/g of leaf tissues, respectively as compared to 3.86 and 0.895 mg/g of leaf tissues, respectively in the wild type.

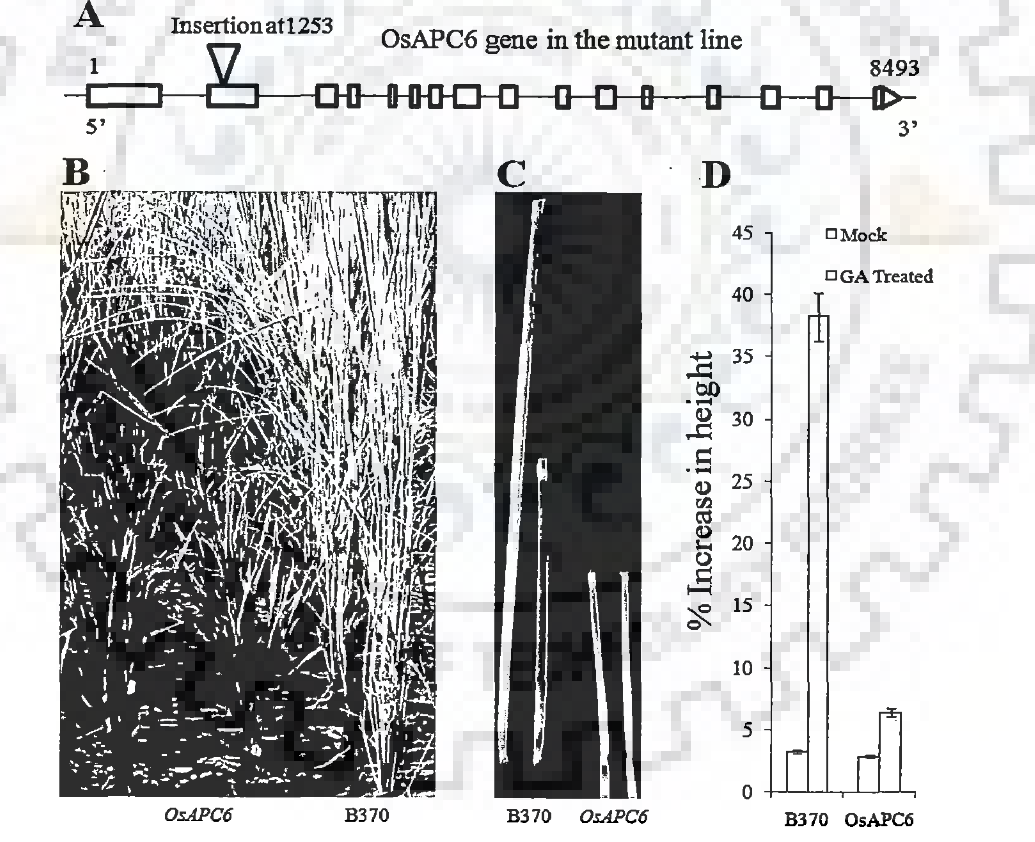

Figure 4.1: *Phenotype of OsAPC6 mutant and gene structure **A**. Gene structure of APC6 showing T-DNA insertion position, **B**. showing pleiotropic effect of OsAPC6 mutant with wild type Basmati 370. The mutant has dark green leaves and reduced height, **C**. Stem elongation assay showing increase in length of second internode in wild type and no major change in mutant, **D**. Graphical representation of height increased after GA spray.*

4.3.1.2 Gibberellic acid response on shoot elongation

Foliar spray of GA_3 (120 ppm) was done to test the GA response on the mutant and wild type plants. The internode length was found to be increased in the wild type while no such major increase in length was observed in the mutant (Fig. 4.1C) after GA_3 spray. Observations on the percent increase in plant height upon the foliar spray of GA showed that the increase in average plant height of wild type was 38.27% whereas the height of mutant *OsAPC6* remained the same with a marginal 6.4% increase (Fig. 4.1D) after exogenous gibberellic acid application.

4.3.1.3 Genome wide expression analysis

The transcriptome profiles of a T-DNA insertional mutant *OsAPC6* and wild type Basmati 370 was studied using microarrays. The average hybridization and percentage of probe sets detected in *OsAPC6* mutant was found to be 42.6% of the total 57,381 probe sets. For expression data analysis logarithmic (Log_{10}) expression values were normalized using PLIER algorithm. At two-fold change and probability $p \leq 0.05$ the differential expression pattern of genes in each case was clustered. A total of 92 genes were found to have differential expression out of which 81 genes (88.04%) were found to be up-regulated, while only 11 genes were down-regulated. Gene Ontology studies of these genes revealed that 89% belong to molecular function (MF), 69.5% were of biological process (BP) and 20.6% come under the category of cellular component (CC). A heat map was prepared individually for both up- and down-regulated set of genes by comparing their expression level in the wild type and mutant (Fig. 4.2A and 4.2B). The majority of genes in the mutant were up-regulated as indicated by the heat map. Further, these differentially regulated genes were assigned into different GO subcategories (Table 4.1). The 18.4% genes involved in catalytic activity were up-regulated while 2.1% were down-regulated in dwarf mutant. Similarly, 14% of the genes involved in signaling were found up-regulated while 3.2% were down-regulated. Interestingly, a high number (11.9% and 9.7%) of the genes for biosynthesis and chloroplast precursors were found up-regulated, respectively, while only 1.08% were found down- regulated in these categories. For binding type activities, 5.4% genes were found up-regulated and 3.2% were down-regulated. Some other genes involved in transcription and transport were found to be differentially regulated (Fig. 4.2C and 2D).

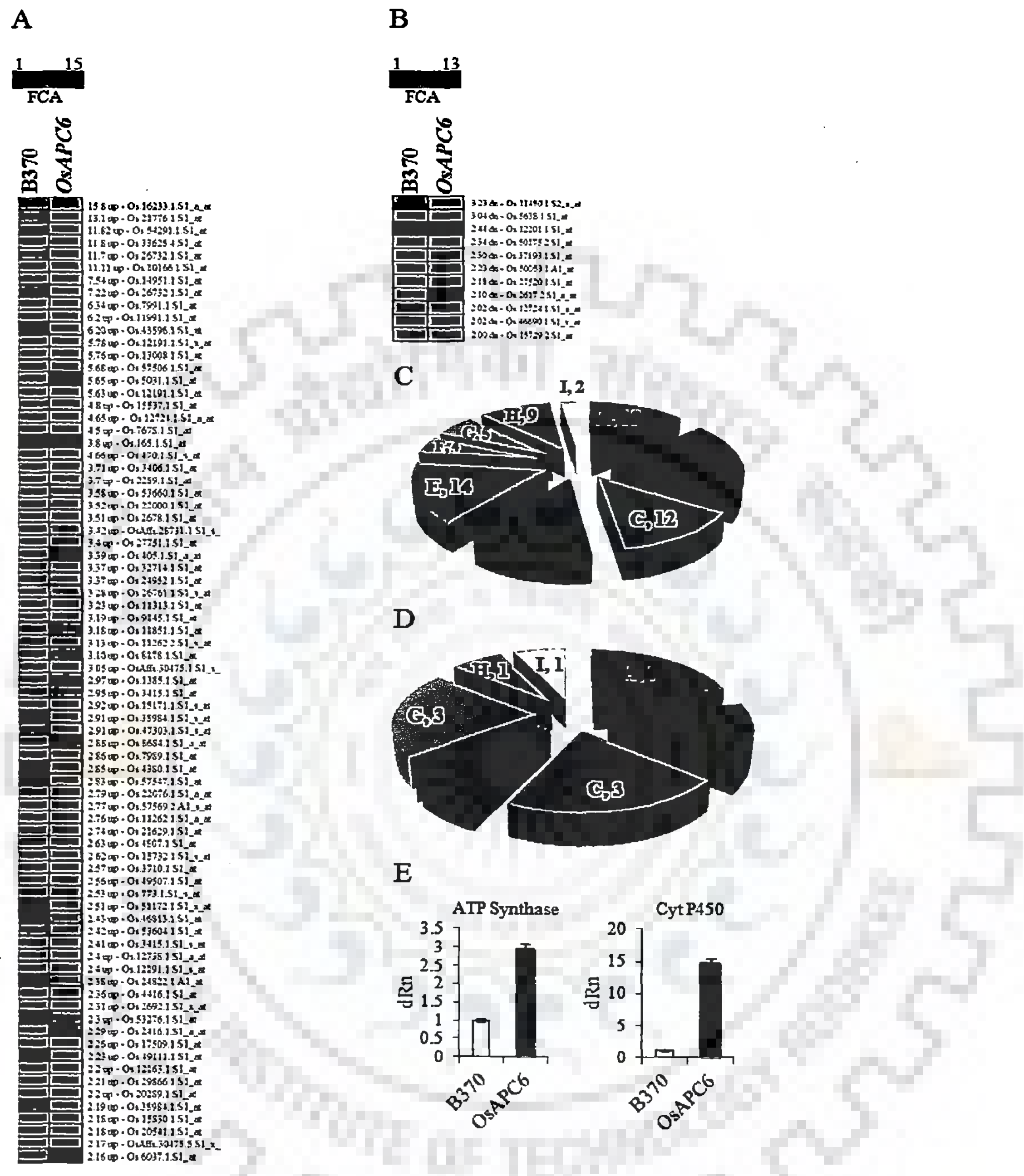

Figure 4.2: *Expression analysis of OsAPC6 mutant using microarray **A**. Heat map of up-regulated genes, **B**. Heat map of down-regulated genes, **C** and **D**. pie-chart representing the up-regulated and down-regulated functionally categorized genes (the functional categories are A: Signal transduction; B: Catalytic activity; C: Transporter activity; D: Biosynthesis; E: Hydrolases; F: Stress-related; G: Binding activity; H: Chloroplast precursors; I: No GO) **E**. Graphical representation of qRT data.*

The *APC6* gene (LOC_Os03g13370) was found to be down-regulated by 0.90 fold in the mutant *OsAPC6*. Some biologically significant genes like encoding multi copper oxidase laccase enzyme (LOC_Os11g42200.1), cytokinin dehydrogenase (LOC_Os01g1110), UDP-glucosyl transferase domain protein involved in lipid glycosylation (LOC_Os01g08080), carboxylesterase involved in plant-pathogen interaction activity (LOC_Os01g70850), pathogenesis-related (LOC_Os12g36830.1, LOC_Os12g36880.1), calmodulin-binding glutamate decarboxylase (LOC_Os03g51080.1, LOC_Os04g37460.1, LOC_Os03g13300.1), WIP4 - Wound-induced protein precursor (LOC_Os11g37960.1), Cupin domain containing stress related protein (LOC_Os08g08970.1) were found to be up-regulated in the mutant. A C2 domain containing protein (LOC_Os01g62430.2) involved in targeting proteins to cell membranes and localization and signaling was found to be up-regulated. Another gene (LOC_Os02g13870.1) encoding for aquaporin (protein located in cell membrane and functions as water channel) was found to have higher expression in the mutant.

A total of 14 encoding for signaling and 15 genes involved in transport were up-regulated in the mutant (Fig. 4.3A and 4.3B). Genes involved in transcription (Os.53660.1.S1_at, Os.24952.1.S1_at) were also found to be up-regulated (Table 4.1). Another gene encoding calmodulin (CaM) binding proteins (LOC_Os03g51080.1) was found to be 4.65 up-regulated. CaM protein contains putative nuclear localization signals and may mediate Ca^{2+} signaling to regulate gene expression in the nucleus. Carboxyl esterase encoding gene which is involved in signaling (LOC_Os01g70850) was found to be 11.8 folds up-regulated.

The genes, PAL gene (LOC_Os04g43760.1) involved in signaling, a DELLA target protein (LOC_Os11g02379), ethylene biosynthesis gene 1-aminocyclopropane-1-carboxylate oxidase (LOC_Os09g27750.1), decarboxylase gene (LOC_Os08g36320.3) and glutamate receptor (Os.15729.2.S1_at) were found to be down regulated.

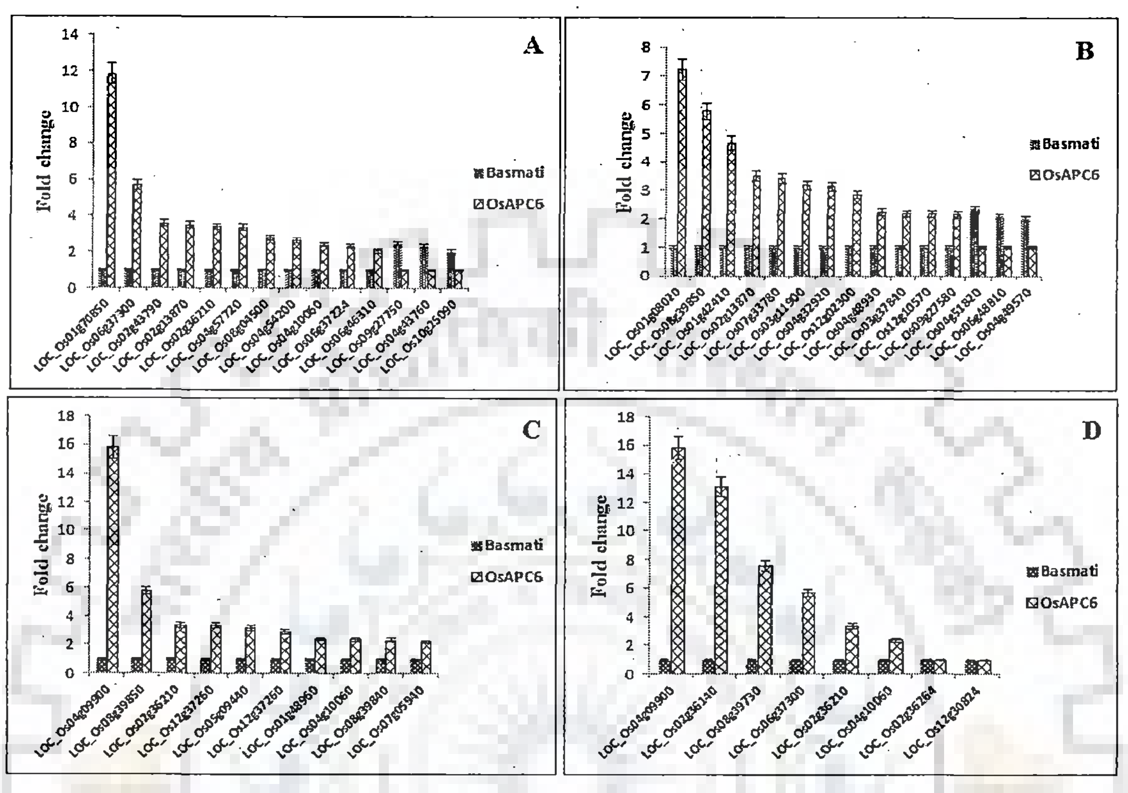

Figure 4.3: *Histogram showing differentially expressed genes in the mutant as compared to the wild type **A**. Genes related to signaling, **B**. Genes related to transport, **C**. Genes encoding chloroplast precursors, **D**. Genes related to gibberellic acid metabolism.*

Table 4.1: Different categories of genes showing differential expression in *OsAPC6* mutant in comparison to Basmati 370

S. No.	Gene Id	Probe Id	FCA	
A.	**Gibberellic acid metabolism genes**			
1.	Os.16233.1.S1_a_at	LOC_Os04g09900	15.8 +	GA Metabolism,
2.	Os.21776.1.S1_at	LOC_Os02g36140	13.1 +	Terpene Synthase
3.	Os.14951.1.S1_at	LOC_Os08g39730	7.54+	Cytochrome P450
4.	Os.57506.1.S1_at	LOC_Os06g37300	5.68 +	Cytochrome P450
5.	Os.27751.1.S1_at	LOC_Os02g36210	3.41+	CPS2 (Ent-Copalyl Diphosphate Synthase 2)
6.	Os.24822.1.A1_at	LOC_Os04g10060	2.38+	Ent-Kaurene Synthase 4
7.	OsAffx.24594.1.S1_at	LOC_Os02g36264	1.03 +	Ent-Kaurene Synthase 6
8.	Os.53402.1.S1_at	LOC_Os12g30824	1.04 +	Ent-Kaurene Synthase 1 0

B. Signal transduction genes

9.	Os.33625.4.S1_at	LOC_Os01g70850	11.8 +	Carboxylesterase Activity
10.	Os.57506.1.S1_at	LOC_Os06g37300.1	5.68 +	Cytochrome P450
11.	Os.53660.1.S1_at	LOC_Os02g43790.1	3.58 +	Ethylene-Responsive Transcription Factor
12.	Os.2678.1.S1_at	LOC_Os02g13870.1	3.51 +	Aquaporin Protein
13	Os.27751.1.S1_at	LOC_Os02g36210.1	3.4 +	Ent-Kaurene Synthase
14	Os.24952.1.S1_at	LOC_Os04g57720.1	3.37 +	Osrr6 Type-A Response Regulator
15	Os.57569.2.A1_s_at	LOC_Os08g04500.1	2.77 +	Terpene Synthase
16	Os.4807.1.S1_at	LOC_Os04g54200.1	2.63 +	Diacylglycerol Kinase
17	Os.24822.1.A1_at	LOC_Os04g10060.1	2.38 +	Ent-Kaurene Synthase
18	Os.53276.1.S1_at	LOC_Os06g37224.1	2.3 +	Cytochrome P450
18	Os.25557.1.S1_at	LOC_Os06g46310.3	2.06 +	Metal Transporter Nramp6
19	Os.12201.1.S1_at	LOC_Os09g27750.1	2.44 -	1-Aminocyclopropane-1-Carboxylate Oxidase
20	Os.37893.1.S1_at	LOC_Os04g43760.1	2.30 -	PAL
21	Os.46690.1.S1_x_at	LOC_Os10g25090.1	2.02 -	Strubbelig-receptor precursor

C. Transcription Factor genes

22	Os.53660.1.S1_at	LOC_Os02g43790.1	3.58 +	Ethylene-Responsive
23	Os.24952.1.S1_at	LOC_Os04g57720.1	3.37 +	Osrr6 Type-A response regulator

D. Genes for Transport proteins

24	Os.26732.1.S1_at	LOC_Os01g08020.1	7.22 +	Boron Transporter Protein
25	Os.13008.1.S1_at	LOC_Os08g39850.1	5.76 +	Lipoxygenase
26	Os.470.1.S1_s_at	LOC_Os01g42410.1	4.66 +	Pleiotropic drug resistance
27	Os.2678.1.S1_at	LOC_Os02g13870.1	3.51 +	Aquaporin Protein
27	OsAffx.28731.1.S1_s_at	LOC_Os07g33780.1	3.42 +	Pleiotropic Drug Resistance
28	Os.11851.1.S1_at	LOC_Os03g11900.1	3.18 +	Transporter Family Protein
30	Os.4380.1.S1_at	LOC_Os12g02300.1	2.85 +	LTPL26 - Seed Storage Protein
31	Os.49111.1.S1_at	LOC_Os04g48930.2	2.23 +	Ferric-Chelate Reductase
32	Os.20541.1.S1_at	LOC_Os03g37840.1	2.18 +	Potassium Transporter
33	OsAffx.30475.5.S1_x_at	LOC_Os12g10570.1	2.17 +	ATP Synthase Subunit
34	Os.6037.1.S1_at	LOC_Os09g27580.2	2.16 +	Potassium Transporter
35	Os.50175.2.S1_at	LOC_Os04g51820.1	2.34 -	Na+ Transporter
36	Os.2617.2.S1_a_at	LOC_Os06g48810.1	2.10 -	Na+ Transporter
37	Os.15729.2.S1_at	LOC_Os04g49570.1	2.00 -	Glutamate Receptor

E.	**Chloroplast precursor genes**			
38	Os.16233.1.S1_a_at	LOC_Os04g09900	15.8 +	Chloroplast Precursor
39	Os.13008.1.S1_at	LOC_Os08g39850.1	5.76 +	Lipoxygenase
40	Os.27751.1.S1_at	LOC_Os02g36210.1	3.4 +	Ent-Kaurene Synthase
41	Os.405.1.S1_a_at	LOC_Os12g37260.1	3.39 +	Lipoxygenase 2.1
42	Os.9145.1.S1_at	LOC_Os05g09440.1	3.19 +	NADP-dependent Malic enzyme
43	Os.15171.1.S1_s_at	LOC_Os12g37260.1	2.92 +	Lipoxygenase 2.1
44	Os.12738.1.S1_a_at	LOC_Os01g48960.1	2.4 +	Glutamate Synthase,
45	Os.24822.1.A1_at	LOC_Os04g10060.1	2.38 +	Ent-Kaurene Synthase
46	Os.4416.1.S1_at	LOC_Os08g39840.1	2.36 +	Lipoxygenase
47	Os.50053.1.A1_at	LOC_Os07g05940.1	2.23 -	9-Cis-Epoxycarotenoid Dioxygenase 1
F.	**Genes for Hydrolases**			
48	Os.10166.1.S1_at	LOC_Os04g41680.1	11.1 +	Hydrolase and Chitinase Activity
49	Os.7991.1.S1_at	LOC_Os04g41620.1	6.34 +	Chitinase Activity
50	Os.22000.1.S1_at	LOC_Os06g51060.1	3.52 +	CHIT8 - Chitinase Family Protein Precursor
51	OsAffx.28731.1.S1_s_at	LOC_Os07g33780.1	3.42 +	Pleiotropic Drug Resistance
52	Os.1385.1.S1_at	LOC_Os01g71670.1	2.97 +	Glycosyl Hydrolases
53	Os.3415.1.S1_at	LOC_Os10g39680.1	2.95 +	CHIT14 - Chitinase
54	Os.38984.1.S1_s_at	LOC_Os01g23580.1	2.91 +	Inorganic H+ Pyrophosphatase
55	Os.51172.1.S1_x_at	LOC_Os06g51050.1	2.51 +	CHIT7 - Chitinase
56	Os.3415.1.S1_s_at	LOC_Os10g39680.1	2.41 +	CHIT14 - Chitinase
57	Os.2692.1.S1_x_at	LOC_Os06g51050.1	2.31 +	CHIT7 - Chitinase
58	Os.2416.1.S1_a_at	LOC_Os01g71340.1	2.29 +	Glycosyl Hydrolases
59	Os.17509.1.S1_at	LOC_Os02g50490.1	2.26 +	Endoglucanase
60	Os.38984.1.S1_at	LOC_Os01g23580.1	2.19 +	H+ Pyrophosphatase
G.	**Novel Genes**			
61	OsAffx.30475.1.S1_s_at	LOC_Os10g21270.1	3.05 +	Unknown
62	Os.26761.1.S1_s_at	LOC_Os02g24600	3.28 +	Unkown

4.3.1.4 Genes related to chlorophyll content

When the annotations of the differentially expressed genes were studied, it was found that a total of nine genes encoding for chloroplast precursors like lipoxygenases were up-regulated while only one gene which encodes for epoxycarotenoid dioxygenase (LOC_Os07g05940.1) was down-regulated (Fig. 4.3C).

4.3.1.5 Genes related to Gibberellic acid response

A set of eight genes responsible for gibberellic acid metabolism was identified to be differentially expressed with higher fold change in the mutant (Fig. 4.3D). This indicated that the synthesis and metabolism of GA was normal, rather higher in the mutant. The microarray data thus revealed that *OsAPC6* was not a GA responsive mutant. Two genes (LOC_Os03g57640, LOC_Os03g15270) encoding for GA receptor GID1 were also found to be up-regulated. The SLR1 Della protein which is a negative regulator of GA signaling was up-regulated by 1.07 fold in the mutant transcriptome. Many RING finger proteins LOC_Os02g35329.1 and LOC_Os01g52110.9 encoding RING-H2 finger protein and RING finger and CHY zinc finger domain-containing protein respectively were found to have changed expression in the mutant genome.

4.3.1.6 Validation of Microarray Data

We investigated the reliability of our microarray data using qRT-PCR. The data was normalized based on expression data of the housekeeping gene *E1Fα*. Two genes encoding *Cytochrome P450* and *ATP synthase* were randomly picked for validation purpose from the list of differentially expressed genes. The expression profiles of these selected genes were found to be 2.94 and 14.72 up-regulated (Fig. 4.2E) which were similar in both microarray and qRT-PCR the experiments.

4.3.2 *Ossl* mutant

4.3.2.1 Phenotypic analysis of Ossl mutant

A T-DNA insertional mutant of Basmati 370, *Ossl* showed 5-15% of albino seedlings when kept for germination. These albino seedlings died soon after germination while among non-albino 10-20% died slowly without tillering after transplantation (Fig. 4.4A and B). Due to lethality it is most likely being maintained as heterozygote which is further confirmed by hygromycin resistance and *hpt* PCR results (Fig. 4.4C and D). The T-DNA flanking sequence

obtained after TAIL-PCR was used for BLAST alignment which showed the position of insertion at chromosome 11 of rice. The flanking sequence was found to match the region between 15706842 to 15706985 of the rice genome in Gramene database. There were no ORFs or microRNA found in this region.

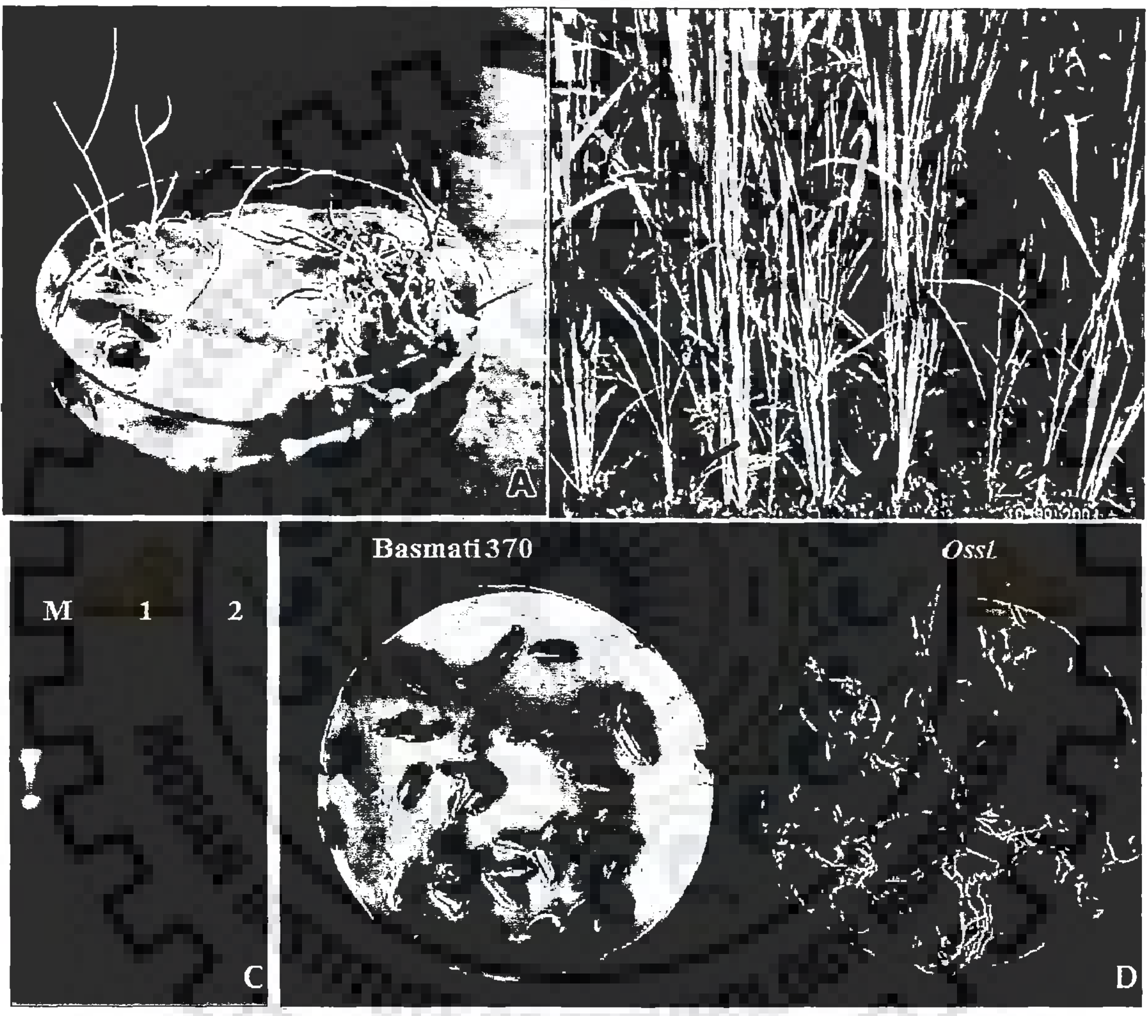

Figure 4.4 Phenotype of Seedling Lethal (Ossl) mutant A. Ossl albino seedlings shown in arrow B. Seedling lethal (shown in arrow) within a month of transplantation in field, C. hpt PCR result, lane 1 has Basmati 370, lane 2= Ossl mutant with T-DNA insertion, M= 100 bp ladder, D. Resistance of Ossl seeds against 80ppm hygromycin.

4.3.2.2 Genome wide expression analysis

The transcriptome profiles of a T-DNA insertional mutant *Ossl* and wild type Basmati 370 was studied using microarrays. The average hybridization and percentage of probe sets

detected in *Ossl* mutant was found to be 42.4% of the total 57,381 probe sets. For expression data analysis logarithmic (Log_{10}) expression values were normalized using PLIER algorithm. At two-fold change and probability $p \leq 0.05$ the differential expression pattern of genes in each case was clustered. A total of 86 genes were found to have differential expression out of which 52.6% genes were found to be up-regulated (Table 4.2). A scatter plot showing the differential expression genes in the mutant as compared to the wild type has been shown in Fig. 4.5A and B. Gene Ontology studies of these genes revealed that 53.4% belong to molecular function (MF), 76.7% were of biological process (BP) and 24.4% come under the category of cellular component (CC). A heat map was prepared for these differentially regulated set of genes by comparing their expression level in the wild type and mutant (Fig. 4.6B). Further, these differentially regulated genes were assigned into nine GO subcategories (Table 4.2). Among these genes list five genes involved in catalytic activity were up-regulated while one was down-regulated in mutant. Similarly, five of the genes involved in binding were found up-regulated while four were down-regulated. Interestingly, a high number of stress related genes were found to have differential expression, seven out of which were down regulated and five were up-regulated. Some other genes involved in transcription and transport were found to be differentially regulated (Fig. 4.7).

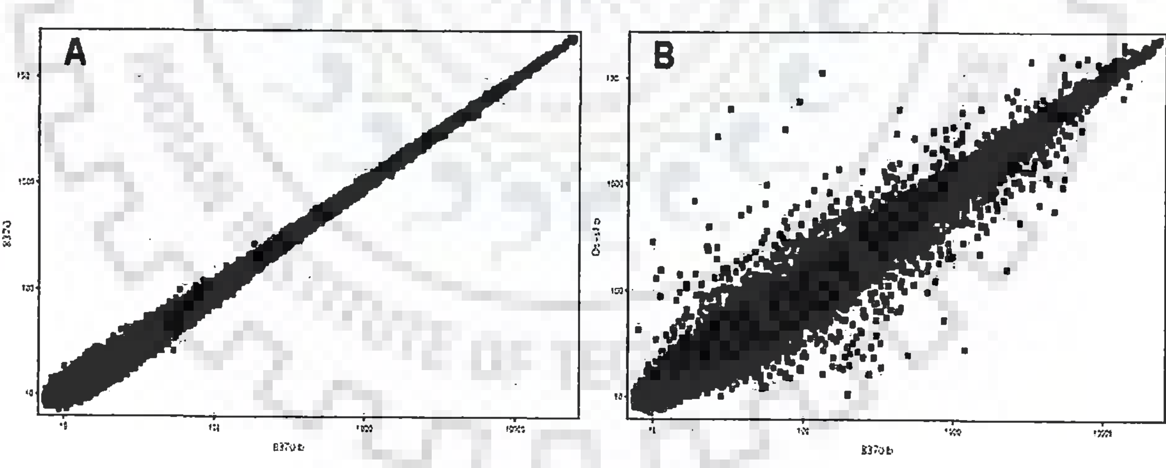

***Figure 4.5** Scatter plot obtained from Arraystar software A. shows technical replicates of wild type with no scattering of genes, B. shows scattering of differentially expressed genes between the wild type Basmati 370 & Ossl.*

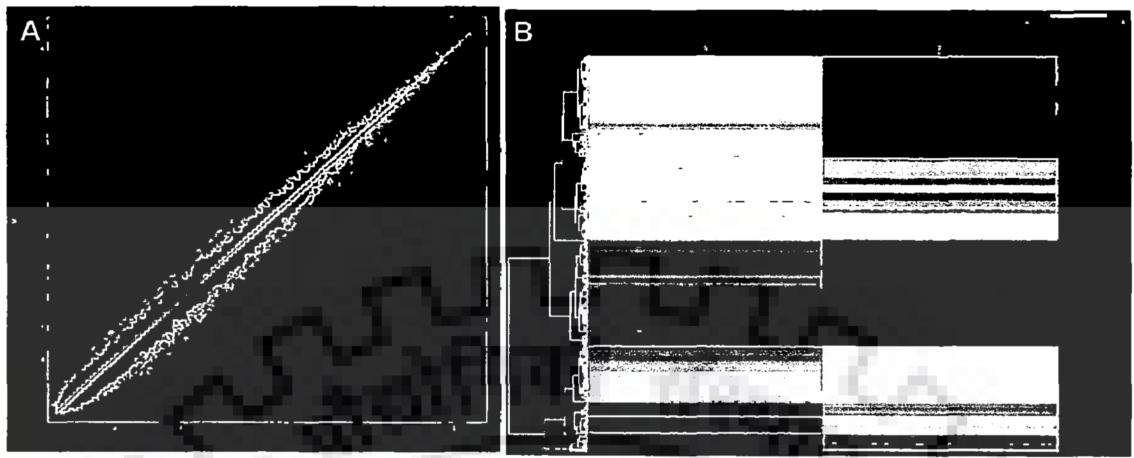

Figure 4.6 *Scatter plot and Heat Map for Ossl mutant showing the differentially expressed genes as compared to wild type Basmati 370.*

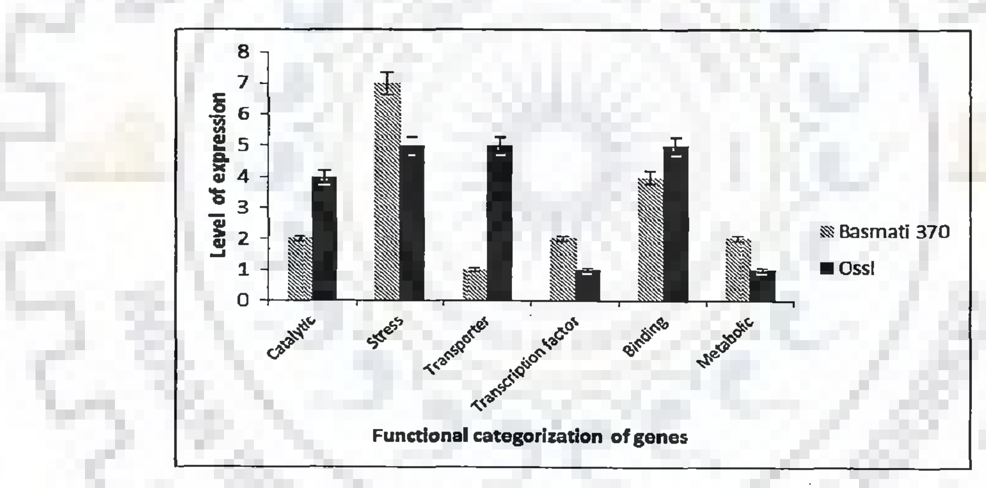

Figure 4.7 *Histogram of functionally categorized genes for Ossl mutant showing the differentially expressed genes as compared to wild type Basmati 370.*

4.3.2.3 Differentially regulated genes in Ossl

The annotations of the differentially expressed genes were studied and it was found that some genes related to the mutant phenotype were expressed differentially. A chloroplast precursor gene lipoxygenases was found to be down-regulated by more than 10-folds. Another gene encoding the phenylalanine ammonia-lyase (PAL) which has a role in anthocyanin

biosynthetic pathway is also down-regulated (3.2 folds). An oxidoreductase gene having a role in post embryonic development is 4.11 folds down-regulated. Again a large number of genes encoding zinc finger domain containing proteins were differentially expressed (Table 4.2).

Table 4.2: Differentially regulated genes in *Ossl* mutant

S. No	Probe set ID	TIGR ID	Fold Change	Gene Function
		Transcription factors		
1.	Os.4164.2.S1_a_at	LOC_Os03g03164.4	4.22 -	Transcription factor
2	Os.11450.1.S1_at	LOC_Os01g52110.9	2.70 +	RING finger domain
3	OsAffx.24166.1.S1_at	LOC_Os02g08440.3	3.48 -	zinc finger domain
		Stress related		
1	OsAffx.30475.5.S1_x_at	LOC_Os12g10570.1	7.33 +	ATP synthase subunit beta
2	Os.12738.1.S1_a_at	LOC_Os01g48960.1	4.50 +	glutamate synthase
3	Os.2210.1.S1_at	LOC_Os08g08970.1	4.26 -	Cupin domain containing protein
4	Os.25687.1.S1_at	LOC_Os02g41680.1	3.21 -	phenylalanine ammonia-lyase
5	Os.12633.1.S1_at	LOC_Os11g26790.1	3.94 -	Dehydrin
6	Os.51718.1.S1_at	LOC_Os11g26780.1	4.68 -	Dehydrin
7	Os.11510.1.S1_at	LOC_Os09g31430.2	3.75-	Os9bglu30 - beta-glucosidase
8	OsAffx.24166.1.S1_at	LOC_Os02g08440.3	3.48 -	zinc finger domain
9	Os.47761.1.S1_at	LOC_Os03g13300.1	3.25 +	glutamate decarboxylase
10	Os.9145.1.S1_at	LOC_Os05g09440.1	2.95 +	NADP-dependent malic enzyme

11	Os.22000.1.S1_at	LOC_Os06g51060.1	2.62 +	Chitinase family protein
12	Os.9563.1.S1_at	LOC_Os08g32160.1	4.11 -	2OG-FeII oxygenase domain
		Binding		
1	Os.4184.1.S1_at	LOC_Os02g15350.1	5.46 +	zinc finger domain
2	Os.1229.2.S1_at	LOC_Os01g13740	3.25 +	myb-like DNA-binding domain
3	Os.405.1.S1_a_at	LOC_Os12g37260.1,	18.35 –	lipoxygenase 2.1
4	Os.11450.1.S1_at	LOC_Os01g52110.9	2.70 +	RING finger domain
5	Os.12346.1.S1_at	LOC_Os01g62480.1	7.54 -	laccase precursor
6	Os.47761.1.S1_at	LOC_Os03g13300.1	3.25 +	glutamate decarboxylase
7	Os.6043.1.S1_at	LOC_Os04g23550.1	5.80 -	DNA binding
8	Os.4164.2.S1_a_at	LOC_Os03g03164.4	4.22 -	DNA binding
9	OsAffx.26050.1.S1_at	LOC_Os12g02320.1	2.52 +	LTPL12 - Protease inhibitor/seed protein precursor
		Catalytic		
1	Os.25687.1.S1_at	LOC_Os02g41680.1	3.21 -	phenylalanine ammonia-lyase
2	Os.7879.2.S1_at	LOC_Os04g56400.4	2.80 +	glutamine synthetase
3	Os.6671.1.S1_a_at	LOC_Os05g39930.1	2.88-	catalytic
4	Os.9145.1.S1_at	LOC_Os05g09440.1	2.95 +	NADP-dependent malic enzyme
5	Os.22000.1.S1_at	LOC_Os06g51060.1	2.62 +	Chitinase family protein
6	Os.7505.1.S1_at	LOC_Os05g33840	2.84 +	transketolase

		Metabolic		
1	Os.12452.1.S1_s_at	LOC_Os08g36910.2	3.89 -	alpha-amylase precursor
2	Os.11510.1.S1_at	LOC_Os09g31430.2	3.75 -	Os9bglu30 - beta-glucosidase
3	Os.47761.1.S1_at	LOC_Os03g13300.1	3.25 +	glutamate decarboxylase
		Biosynthesis		
1	Os.1478.1.S1_at	LOC_Os03g19436.1	3.43 -	Biosynthesis
		Oxidoreductase		
1	Os.9563.1.S1_at	LOC_Os08g32160.1	4.11 -	2OG-FeII oxygenase domain
		Electron Transporter		
1	Os.12738.1.S1_a_at	LOC_Os01g48960.1	4.50 +	glutamate synthase
2	Os.26761.1.S1_s_at	LOC_Os02g24600	2.45 +	Electron transporter
3	Os.46144.1.S1_at	LOC_Os10g38292.2	3.34 +	Electron transporter
4	OsAffx.32208.1.S1_x_at	LOC_Os10g38292.2	3.04 +	Electron transporter
		Transporter		
1	OsAffx.30475.5.S1_x_at	LOC_Os12g10570.1	7.33 +	ATP synthase subunit beta
2	OsAffx.26050.1.S1_at	LOC_Os12g02320.1	2.52 +	LTPL12 - Protease inhibitor protein precursor

4.3.2.4 Validation of microarray data

We investigated the reliability of our microarray data using qRT-PCR. The data was normalized based on expression data of the housekeeping gene *EIFα*. Two genes encoding Cytochrome P450, PAL and Cupin domain containing protein encoding gene were randomly picked for validation purpose from the list of differentially expressed genes. The expression profiles of these selected genes were found to be 4.92, 3.21 and 4.26 down-regulated (Fig. 4.8) which were similar in both microarray and qRT-PCR the experiments.

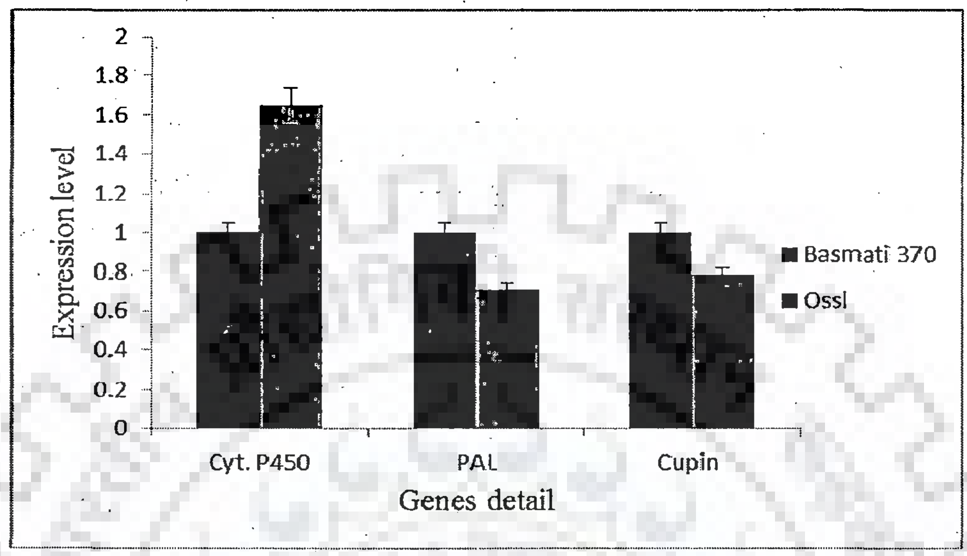

Figure 4.8 *qRT data to show the validation of the differential regulation of genes in Microarray data.*

4.4 Discussion

The *OsAPC6* mutant was dwarf with dark and broad leaves which are the typical symptoms of GA insensitivity. The dwarf mutants have been classified into GA-sensitive and GA-insensitive types, depending upon their responsiveness to exogenously applied GA (Kumar and Singh, 1984, Milach *et al.*, 1997). The stem elongation assay performed to evaluate the GA responsiveness of the mutant plants showed only 6.4% increase in height as compared to more than 38% increase in the wild type which indicated that the *OsAPC6* mutant was GA-insensitive. In the GA-insensitive type mutants the response to GA is affected although their GA level is normal or even higher than those found in the wild-type (Martin et a., 1996). The expression profile of *OsAPC6* mutant revealed that the genes encoding enzymes involved in GA biosynthesis were highly up-regulated. The bioactive GAs are synthesized from trans-geranylgeranyl diphosphate (GGDP), which is converted to a tetracyclic hydrocarbon ent-kaurene by CPS and ent-kaurene synthase (KS) enzymes (Hedden and Kamiya 1997, Hedden and Phillips 2000). In our study, one CPS2 (ent-copalyl diphosphate synthase 2), a terpene synthase and three ent-kaurene synthase- KS2, KS6 and KS10 were found to be up-regulated. Also two genes encoding *Cytochrome P450* which catalyzes the conversion of ent-kaurene to

GA_{12} were up-regulated in the mutant. These results indicate that the biosynthesis of GA in the mutant plant was normal, or even higher than the wild type.

The mutant had dark green leaves which indicated presence of more chlorophyll in the leaves. An increase in the chlorophyll content was further confirmed in the mutant as compared to the wild type. In the microarray study a large number of genes encoding the chloroplast precursor were found to be up-regulated. High chlorophyll content is generally associated with dwarfism and a large number of dwarf mutants like *gid1-1* (Ueguchi-Tanaka *et al.*, 2005), *gid2* (Sasaki *et al.*, 2003) of rice were reported to have higher chlorophyll content together with reduced height.

Some biologically significant genes were found to be differentially regulated in the mutant. The APC6 protein encoding gene (LOC_Os03g13370) was found to be down-regulated in the mutant. The expression of the gene was reduced in the mutant due to T-DNA insertion (Awasthi *et al.*, 2012). The phylogenetic analysis and multiple alignment results of APC6 protein of Basmati 370 and other organisms revealed a high conservation of this protein among organisms from prokaryotes to eukaryotes. These results also indicated the presence of TPR domains involved in protein-protein interaction, which are also reported to be involved in the signaling of plant hormones (Schapire *et al.*, 2006) like ethylene (Yoshida *et al.*, 2005), cytokinin (Greenboim-Wainberg *et al.*, 2005) and auxins (Gray *et al.*, 2003). The SPINDLY protein of *Arabidopsis* which is a negative regulator of GA signaling is a TPR protein (Greenboim-Wainberg *et al.*, 2005). APC6 protein is a subunit of APC/C which is a kind of E3 ubiquitin ligase enzyme (Moon *et al.*, 2004). APC/C regulates the mitotic progression and exit by controlling the stability of cell cycle regulatory proteins like cyclins (Irniger *et al.*, 1995) and securins (Pellman, 2001). APC/C was found to be involved in the targeted proteolysis of A- and B-type cyclins in clams and Xenopus (Hershko *et al.*, 1991). In *Arabidopsis* a mutation in *APC6* gene results in arrested cell division at two nucleate stage during embryo sac development. In this *nomega* mutant the embryo sac was unable to degrade the cyclin B protein (Kwee and Sundaresan, 2003). The role of APC/C in auxin signaling during root development has been reported in *HOBBIT* mutant of *Arabidopsis* (Blilou *et al.*, 2002). A role of APC10 for APC/C-directed proteolysis in auxin and ethylene regulation has been reported in *Arabidopsis* (Lindsay *et al.*, 2011). A link between the APC/C and vascular tissue differentiation in stems and cotyledons, processes associated with auxin regulation, has

also been documented, indicating a complex role for the APC/C in plant development (Marrocco *et al.*, 2009).

APC/C is a highly conserved multiprotein complex consisting of 11 subunits in plants while 13 subunits in other eukaryotes (Gieffers *et al.*, 2001; Capron *et al.*, 2003), out of which two subunits APC2 and APC11 have homology to CULLIN and RBX1 components of SCF, respectively (Tang *et al.*, 2001). This indicates that there is an evolutionary relationship between APC/C and SCF, which is another kind E3 ligase (Schwechheimer and Villalobos, 2004).

Recent studies reveals that the key components of GA signaling in both *Arabidopsis* and rice, which have been identified using mutation analysis, include the DELLA proteins, the GA receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1), and the F-box proteins SLEEPY1 (SLY1) and SNEEZY (SNZ) in *Arabidopsis* and GIBBERELLIN INSENSITIVE DWARF2 (GID2) in rice (Peng *et al.*, 1997; McGinnis *et al.*, 2003; Sasaki *et al.*, 2003; Ueguchi-Tanaka *et al.*, 2005). The sensing of GA is mediated by the GID1 protein, and the complex between GA and GID1 allows the capture of the nuclear growth-repressing DELLA proteins. The N-terminal region of SLR1 interacts with the GID1–GA complex, effectively stabilizing it. This tripartite structure functions as the 'ubiquitination chaperone', stimulating its recognition and proteolysis by E3 ligase SCF^{GID2} complex (Gao *et al.*, 2011). Thus, the DELLA proteins act to restrain plant growth, while GA promotes it by targeting them for destruction (Olszewski, 2006; Jiang and Fu, 2007; Davière *et al.*, 2008; Gao *et al.*, 2008; Hartweck and Shimada *et al.*, 2008).

In our microarray data, the genes encoding for GID1 and SLR1 were found to be up-regulated, indicating that the signaling is normal till this tripartite formation. In the present study we have speculated a pathway for GA signaling where APC/C plays a role in SLR1 degradation instead of SCF^{GID2} complex (Fig. 4.9). Since the APC6 is a part of holo-enzyme APC/C, which has a role in ubiquitin-mediated proteolytic degradation, mutation in APC6 probably somehow does not allow the degradation of SLR1 protein, thereby leading to GA-insensitive dwarf phenotype of *OsAPC6* mutant.

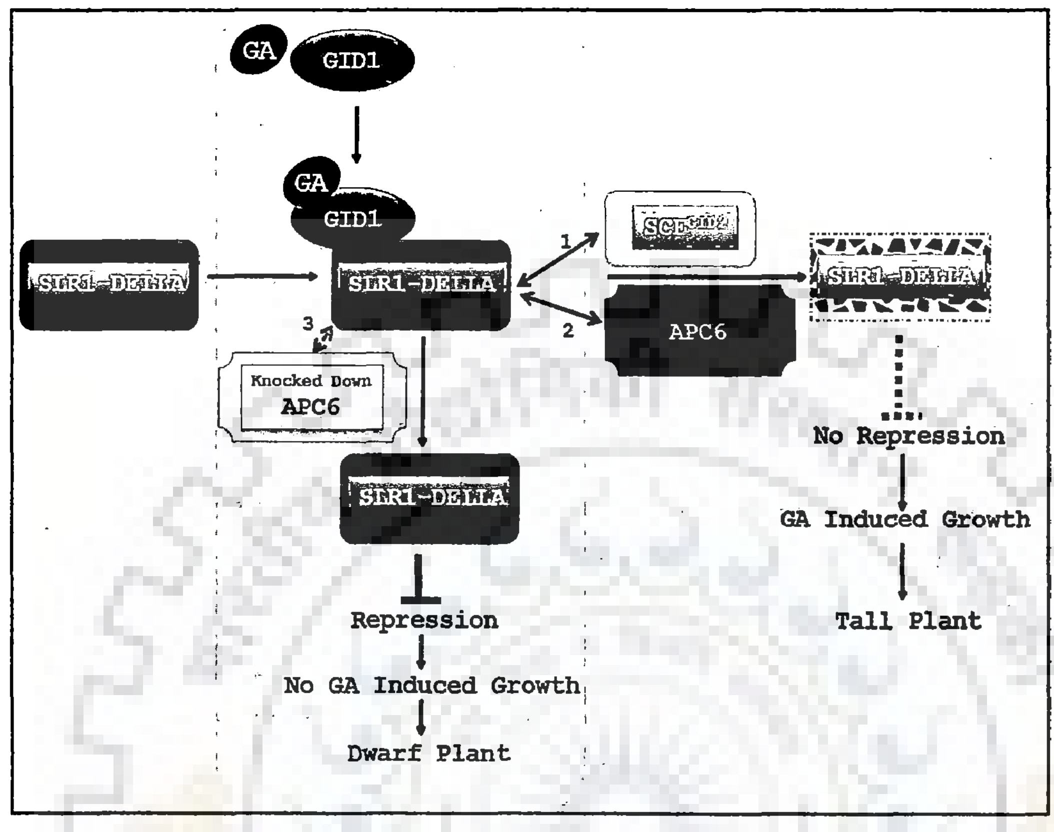

Figure 4.9 *Proposed GA signaling pathway for rice. The membrane bound GID1 receptor binds to the gibberellic acid molecule (GA) which again attaches to the SLR1 protein to form a tripartite complex. This complex is then degraded by 26S-proteasome using E3-ligase enzyme. Path 1 shows the reported degradation of SLR1 protein through SCFGID2 E3 ligase; Path 2 explains the proposed role of APC6 (a component of APC/C, another E3 ligase) in SLR1 degradation; and Path 3 represents the effect of mutation in APC6 gene on GA signaling.*

The expression analysis of the seedling lethal mutant revealed some genes related to the development of chlorophyll like the lipoxygenase gene which is a chloroplast precursor. It was found to be down-regulated by more than 10 folds. The albinism in the mutant could be attributed to this gene. Again PAL gene was found to be down-regulated. This gene has been reported to have a role in anthocyanin development. But the microarray results show a number of genes being differentially regulated which indicates that some important biological function is being disrupted due to this insertion.

Chapter V

IN SILICO ANALYSIS AND MODELING OF APC6 PROTEIN

5.1 Introduction

The anaphase-promoting complex/cyclosome (APC/C) is a multisubunit member of the RING finger family of ubiquitin ligases. The anaphase-promoting complex/cyclosome (APC/C) is an E3 ubiquitin ligase which specifically ubiquitylates cell cycle regulatory proteins containing destruction (D) and/or KEN box motifs, including Pds1/securin, mitotic cyclins, Cdc5/Polo like kinase, and Cdc20 (Harper *et al.*, 2002; Peters, 2002). It is composed of ~13 distinct subunits required for progression through meiosis, mitosis, and the G1 phase of the cell cycle. Studies on yeast and *Xenopus* APC/C identified two subcomplexes (Vodermaier, 2004; Thornton *et al.*, 2006): one binds the UBC (Ubiquitin conjugating enzyme) and is composed of the catalytic subunits APC11, APC2, and APC10, and the other subcomplex is composed of proteins with multiple tetratricopeptide (TPR) motifs that pack together to form a right-handed superhelix with a protein-binding groove.

TPR domains are present in a number of functionally unrelated proteins mediate a variety of different protein-protein interactions and were discovered in the first identified APC subunits (Lamb *et al.*, 1994; Irniger *et al.*, 1995; King *et al.*, 1995). Although TPR subunits of the APC are essential for viability in yeast (Yanagida *et al.*, 1999) and represent the largest group of structurally related proteins within the APC, their precise role has remained unknown. Tetratricopeptide repeat domains typically contains 34 amino acids [WLF]-X(2)-[LIM]-[GAS]-X(2)-[YLF]-X(8)-[ASE]-X(3)-[FYL]-X(2)-[ASL]-X(4)-[PKE] are the consensus sequence and are found in a variety of organisms including bacteria, cyanobacteria, yeast, fungi, plants, and humans in various subcellular locations including cytosol, nucleus, mitochondria and peroxisomes. They are involved in a variety of functions including protein-protein interactions, but common features in the interaction partners have not been defined. They are also involved in chaperone, cell-cycle, transciption, and protein transport complexes (Blatch and Lassle, 1999). Proteins do not normally contain an individual TPR motif, but consists of 3 to 16 tandem-repeats of TPRs that can be grouped or dispersed throughout the protein (Main *et al.*, 2003). Three-dimensional structure data have shown that a TPR motif contains two antiparallel α-helices such that tandem arrays of TPR motifs generate a right-handed helical structure with an amphipathic channel that might accommodate the complementary region of a target

protein (Blatch and Lassle, 1999). It has been proposed that TPR proteins preferably interact with WD-40 repeat proteins, but in many instances several TPR-proteins seem to aggregate to multi-protein complexes; examples of TPR-proteins include, Cdc16 (APC6), Cdc23 (APC8)and Cdc27 (APC3) components of the cyclosome/APC (Schapire *et al.*, 2006).

Proteins containing TPR domains are becoming a common theme in plant hormone signaling. There have been recent reports on TPR proteins involved in gibberellin, cytokinin and auxin responses as well as ethylene biosynthesis (Gray *et al.*, 2003; Greenboim-Wainberg *et al.*, 2005). In *Arabidopsis*, the ETO1 (ETHYLENE-OVERPRODUCER1) protein negatively regulates ethylene biosynthesis in seedlings through direct interaction of its TPR domains with a 1-aminocyclopropane-1-carboxylate synthase isoform (Blatch and Lassle, 1999; Wang *et al.*, 2004; Yoshida *et al.*, 2005). The spindly (*spy*) mutant was selected because of its capacity to germinate in the presence of an inhibitor of gibberellin (GA) biosynthesis (Jacobsen and Olszewski, 1993). The SPY protein contains TPR domains in its N terminus, whereas the C terminus sequence shows high homology to Ser/Thr O-linked N-acetylglucosamine (O-GlcNAc) transferases (OGTs) from animals (Jacobsen *et al.*, 1996; Roos and Hanover, 2000). The TPR domains of SPY physically interact with two transcription factors forming complexes that act as negative regulators of GA responses (Robertson, 2004). Mutations in the ETO3/STG1b enhanced the auxin-dependent phenotype of the auxin-receptor mutant *tir1-1* mutant. SGT1b was previously identified as a factor involved in plant disease resistance signaling (Takahashi *et al.*, 2003). The ETA3/SGT1b protein is required for SCF^{TIR1}-mediated degradation of Aux/IAA proteins, although the molecular mechanism has not been yet established (Gray *et al.*, 2003). Here, we have designed an *in silico* 3D-model of rice Cdc16/APC6 using yeast Cdc16/Cut 9 as a template.

5.2 Materials and methods

5.2.1 Sequence and phylogenetic analysis

The APC6 nucleotide and amino acid sequences for *Oryza sativa* was obtained from Gramene database. Sequence identity was verified by doing homology searches using the basic local alignment search tool (BLAST) algorithm (Altschul *et al.*, 1997).

Primary structure analysis was done using the ProtParam (http://www.expasy.ch/tools/protparam.html). The ClustalW multiple sequence alignment program was used to align the APC6 sequences (Larkin *et al.*, 2007). The ESPript server was used for generating secondary structure elements and to produce a representation of the sequence alignment (Gouet *et al.*, 1999). The phylogenetic tree was inferred using Clustal W2.

5.2.2 Comparative molecular modeling

Homology modeling for APC6 was performed in the following sequential steps: template selection from Protein Data Bank (PDB), sequence-template alignment, model building, model refinement and validation (Marti-Renom *et al.*, 2000). Template search for APC6 was done using NCBI BLAST search tool against PDB database. Blastp program was run with BLOSUM62 as a scoring matrix, word size of 3, gap penalty of 11 and gap extension penalty of 1. Crystal structure of *Saccharomyces pombe* (PDB ID: 2XPI) having 36% sequence identity with *Oryza sativa* APC6 was obtained as the best hit. *Saccharomyces pombe* crystal structure was used as template to generate a comparative 3D model of *Oryza sativa* APC6 by MODELLER 9v7 (Sali and Blundell, 1993).

ClustalW program was used for multiple sequence alignment of query sequence with template sequence (Larkin *et al.*, 2007). Some manual corrections were done in the alignment file for missing residues in the template sequence. This was then used to build alignment file in PIR/PAR format as an input for MODELLER. Based on sequence alignment analysis, it was assumed that ligand binding modes of *O. sativa* APC6 are similar to *S. pombe* Cut9. Using MODELLER, several preliminary models were generated which were ranked based on their DOPE scores. Five sets of models having lowest DOPE scores were selected and stereo-chemical quality of each was assessed by PROCHECK (Laskowski *et al.*, 1993). The model with the least number of residues in the disallowed region was further refined for relieving steric clashes and improper contacts. Energy minimization of the selected model was performed using Swiss-Pdb Viewer 4.01 (http://www.expasy.org/spdbv/). SPDBV implements GROMOS96 force field to compute energy and to execute energy minimization. PROCHECK was again used to evaluate the stereo-chemical quality of the model.

Loop refinement tool of MODELLER was used in an iterative fashion to refine the loop conformation of the model. Structural validation after each loop refinement step was done using ERRAT plot which gives a measure of the structural error at each residue in the protein. This process was repeated iteratively until most of the amino acid residues were below 95% cut-off value in ERRAT plot (Colovos and Yeates, 1993). The refined model was further validated by VERIFY-3D of SAVES server (http://nihserver.mbi.ucla.edu/SAVES/). ProSA 2003 was used to evaluate the generated 3D structure model of protein for potential errors (Wiederstein and Sippl, 2007).

5.2.3 Molecular dynamics simulations and APC6 three-dimensional structure analysis

Model assessment was done by molecular dynamics (MD) and simulation studies to determine the stability of the predicted 3D model of *Oryza sativa* APC6. GROMACS simulation suite version v. 4.0.7 was used for molecular dynamics work (Hess *et al.*, 2008). After solvating, the system was made electroneutral by adding the positive ions as counter ions and was energy minimized to remove high-energy interatomic contacts. Energy minimization was done using 2000 steps of the steepest descent method. Molecular dynamics simulations were performed in the isothermal isobaric ensemble (NPT). A constant pressure of 1 bar was applied independently in all the directions with Berendsen temperature coupling of 0.5 ps. Protein, solvent and ligands were coupled separately to the thermal bath at 300 K using a coupling constant of 0.1 ps. Finally, simulation was done which consisted of two phases: a short 100 ps canonical ensemble MD simulation allowing randomization of water molecules surrounding the protein molecule and a 1 ns isobaric-isothermal ensemble simulation. MD simulations were performed with GROMACS program installed in Red Hat Enterprise Linux 5 operation system (Red Hat Inc. Raleigh, NC) on a Dell Precision T5400 workstation. The protein stability during MD simulation time was assessed by calculating the root mean square deviation (RMSD) between the structures generated from the simulations and the starting structure. The generated model was visualized, inspected and analyzed using PyMOL (DeLano, 2002).

5.3 Results

5.3.1 Phylogenetic analysis

The APC6 amino acid sequence was obtained from Gramene Database (Fig. 5.1).

```
ATGCCTCTCTCAGCAGCTTCCATCAACCGTGCATCCTACCAAGTTCTCCTCCTCCTCGCC
GCCGCCGCCGTGTCCACCACGGGCGGCGACGGCAACACGGCGCCCGGCAATGCCACCGCC
ACGGCGACGACTGGCGGCGACGACACGGAGATGTACATCTGCTACCTCTGCACCGGGCGC
AACCCGATCCTGATCAGGAGGTGCCCCATCTACTGGGACTACTGCCACCTCAACTGCTTT
GACGACGCGCCCTCCACCGCCGCCGCCGCCGACGACGTCGCCGCCGTTCCCGTGGCCTCG
CCGGCGGCGCCTGCGCGACGGGTGGGCGGCGTCCCGCGCGAGACCCTCGAGGACGAGGAG
TGCTACGTCATGAAGCTGTACGAGAACGGCAGCTACGTCATCGTGACCACGCTGGGCTGC
TCCCAGACCGCCTCGTGCCTCCTCTCCTGCGGCGGCGGCGACCTCGCAGCCGACGGCGAA
GAAGCCCTGGCGGCGGCGCACCCGGCCGGCGCCGTCGGCGTCTCGCCGCCGTGGCGAATG
TGGGATACAAAATTCGGTTTCCCTCCAGCAGCGCCGCCCACCACCGCCGCCGCAGCGCAG
AAAAATCCCAAGCGGAGGAGAGAGGCGGAGGCGGAGGGGGAGGTGGCGGCGGAGATGAGG
GAGGAGGCGGTGGAGCGGCTGCGCGGGGTGGTGCGGGACAGCGTCGGGAAGCACCTGTAC
GCGTCCGCCATCTTCCTCGCCGACAAGGTGGCCGCGGCCACGGGGGACCCCGCCGACGTC
TACATGCTCGCGCAGGCGCTCTTCCTGGGCCGCCACTTCCGCCGCGCGCTCCACATCCTC
AACTCCTCCAAGCTCCTCCGCGACCTCCGCTTCCGCTTCCTCGCAGCCAAGTGCCTCGAG
GAGTTGAAAGAATGGCATCAGTGTTTGATCATACTTGGAGATGCAAAAATAGATGAGCAT
GGAAACGTTGTTGATCAGGATGATGGCAGTGACATTTACTTTGATAAGGATGCTGAAGAC
CATGAGATCAATATCAAAGCGGCAATATGTTTTTTACGTGGCAAGGCATACGAAGCACTG
GACAACTGTGACCTTGCTCGACAATGGTACAAAGCTGCAGTGAAGGCTGATCCTTTGTGC
TATGAGGCCCTTGAATGCCTTGTTGATAACTACATGTTGACATGCGAGGAAGAATCTGAG
CTATTGTCCTCTCTAAAATTTGGAAAAGAAGATGGGTGGCTCTCAGCATTCTACTCTTGT
TTGATAAGGAAGCATGAAAAAGAATATATAGTGGAAGCAAAGTTCAAGGAATTTGAACGA
GAATCTTGTAGTATTTCATCTTTGAGTTCAGGACTGACACTGAAAAATAATATTGACGTG
TTGGCTTGCAAAGCTGAATACTATCATCAGAGTGGAGAGTACCAAAAATGTTTCGAACTC
ACATCTGCGTTACTTGAAAGGGACCCTTTTCATTTGAAATGCACGTTAGTTCATTTGGCA
GCTGCAATGGAGCTTGGCCATTCAAATGACCTTTATATTTTGGCCTGCAATCTAGTGAAG
GACTATCCTCAGAAAGCTCTTTCCTGGTTTGCTGTTGGGTGTTATTACTACTGTATTAAG
AAGTATGATCAAGCACGCAGATACTTTGGCAAAGCTACAGGGTTAGATGGACATTTCCT
CCTGCTTGGATTGGTACTGGTATTGCTTATGCTGCACAGGAGGAGGGTGATCAAGCAATG
GCTGCATTTCGGACAGCAGCTCGGCTATTTCCTGGATGTCATCTGCCAACTTTATACATG
GGCATGCAATATTTGCGAATGCACAATTTCAAACTTGCAGAGCAGTTCTTCACGCAAGCA
AAATCTATCTGCCCATCTGATCCGCTTATATATAACGAGATGGGGGTTGTAGCTTATAAT
ATGAAAGAGTATCAAAAAGCAGTTCAGTGGTTTGAGCTAACACTGGAGCATACTTCATCC
TCCTTGAATGAAATGTGGGAACCAACATTGGTGAATCTTGGGCATGCATTGCGGAAACTC
AAGAAATATCAAAAGGCAATATCATATTATGAAAAGGCACTCACCTTTCAAACCAAAAGT
TTGAGCGCGTTTGCTGGTCTTGCTTATACTTACCACCTTATGGATAAATTCGAGGCTGCG
ATAACTTACTACCACAAGGCTTTATGGTTGAAACCAGACGATCAATTCTCCACAGACATG
CTAACGTTAGCCCTCGAGTCCAGCTGTCAAATCACTGCTCGGACAAGATAG
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Protein sequence

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MWDTKFGFPPAAPPTTAAAAQKNPKRRREAEAEGEVAAEMREEAVERLRGVVRDSVGKHL
YASAIFLADKVAAATGDPADVYMLAQALFLGRHFRRALHILNSSKLLRDLRFRFLAAKCL
EELKEWHQCLIILGDAKIDEHGNVVDQDDGSDIYFDKDAEDHEINIKAAICFLRGKAYEA
LDNCDLARQWYKAAVKADPLCYEALECLVDNYMLTCEEESELLSSLKFGKEDGWLSAFYS
CLIRKHEKEYIVEAKFKEFERESCSISSLSSGLTLKNNIDVLACKAEYYHQSGEYQKCFE
LTSALLERDPFHLKCTLVHLAAAMELGHSNDLYILACNLVKDYPQKALSWFAVGCYYYCI
KKYDQARRYFGKATGLDGTFPPAWIGTGIAYAAQEEGDQAMAAFRTAARLFPGCHLPTLY
MGMQYLRMHNFKLAEQFFTQAKSICPSDPLIYNEMGVVAYNMKEYQKAVQWFELTLEHTS
SSLNEMWEPTLVNLGHALRKLKKYQKAISYYEKALTFQTKSLSAFAGLAYTYHLMDKFEA
AITYYHKALWLKPDD QFSTDMLTLALESSCQITARTR
```

***Figure 5.1** CDS and amino acid sequence of Oryza APC6 protein (Source_Gramene Database)*

The amino acid sequence of *Os*APC6 protein (577 amino acids) was compared with already characterized APC6 proteins of other organisms using BlastP, *Os*APC6 had highest identity with that from genus *Zea mays* (99%), *Sorghum bicolor* (99%), *Hordeum vulgare* (94%), *Arabidopsis thaliana* (92%), *Arabidopsis lyrata* (92%), *Saccharomyces pombe* (90%) and a phylogenetic tree was prepared showing the evolutionary history of APC6 protein (Fig.5.2).

Figure 5.2 *Phylogenetic tree analysis of APC6 protein in Arabidopsis thaliana (B3DNN5), Arabidopsis lyrata (D7KVU7), Ricinus communis (B9SCC8), Populus trichocarpa (B9N961), Oryza sativa japonica (B9F6N8), Oryza sativa indica (B8AK21), Hordeum vulgare (F2ELV9), Zea mays (B4FWL0), Sorghum bicolor (C5WRM5), Picea sitchensis (D5ADE4), Homo sapiens (Q13042), Mus musculus (Q8R349), Sachhromyces cerevisiae (P09798), Sachhromyces pombe (P41889), Dictyostelium discoideum (Q1ZXE6) , Medicago truncaluta (D0VFU6).*

The amino acid sequences of these organisms were aligned together to look for the conserved motifs using ClustalW software (Fig. 5.3). The conserved TPR motifs in *Os*APC6 were predicted based on results of multiple alignments and a web logo was obtained (Fig. 5.4A). The sequences and position of these domains and a domain architecture showing comparative position of TPR domains in these proteins indicated that the rice APC6 was highly conserved among human, yeast, *Mus musculus* and *Arabidopsis* APC6 (Fig. 5.4B).

***Figure 5.3** Multiple sequence alignment of amino acid sequences of APC6 for Arabidopsis thaliana, Arabidopsis lyrata, Oryza sativa japonica, Oryza sativa indica, Homo sapiens, Saccharomyces cerevisiae and Saccharomyces pombe using Clustal W software.*

The conserved TPR motifs in *Oryza sativa* were predicted based on results of multiple alignments. Six TPR domains were found to be conserved among *Arabidopsis thaliana*, *Homo sapiens*, *Saccharomyces pombe* and *Oryza sativa* (Table 5.1). *Saccharomyces pombe* and *Oryza sativa* shared seven conserved domains. The sequences and position of these domains was also predicted based on sequence homology and which are highlighted in Fig. 5.5.

Table 5.1 TPR Domains in characterized APC6 protein

S. No.	*Saccharomyces cerevisiae*	*Saccharomyces pombe*	*Homo sapiens*	*Mus musculus*	*Arabidopsis thaliana*	*Oryza sativa (Basmati 370)*
1.	263-295	117-149	130-163	130-163	309-342	78-109
2.	296-329	150-184	299-333	299-333	344-376	110-142
3.	359-392	200-233	334-367	334-367	378-410	168-201
4.	497-530	339-372	368-401	369-401	411-444	312-346
5.	531-564	373-406	403-435	403-435	450-483	347-380
6.	565-598	407-440	445-478	445-478	484-517	381-414
7.	599-632	441-474	479-512	479-512		415-448
8.	633-666	475-508				449-482
9.	674-707	515-549				488-521
10.	708-741	550-583				522-555

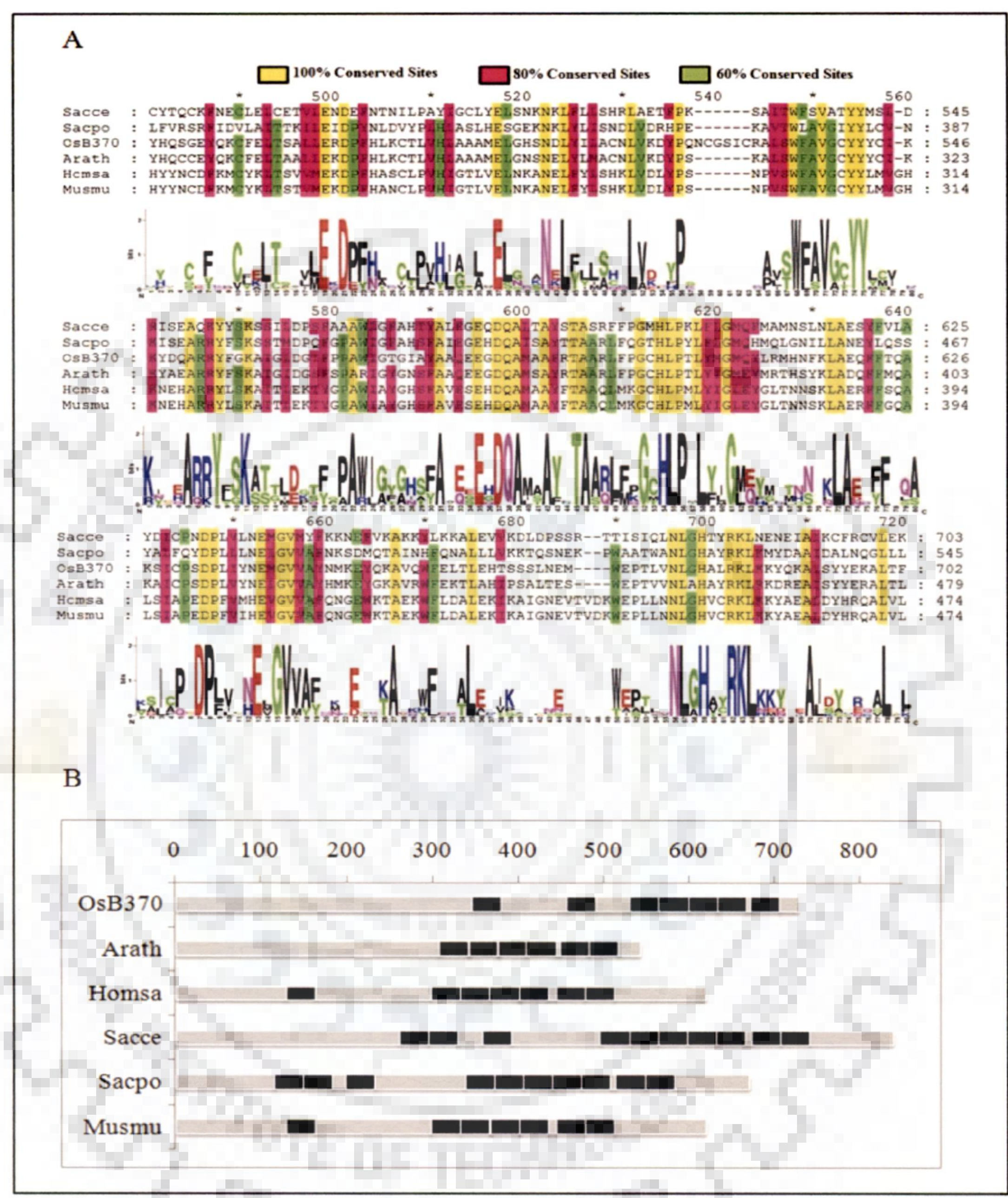
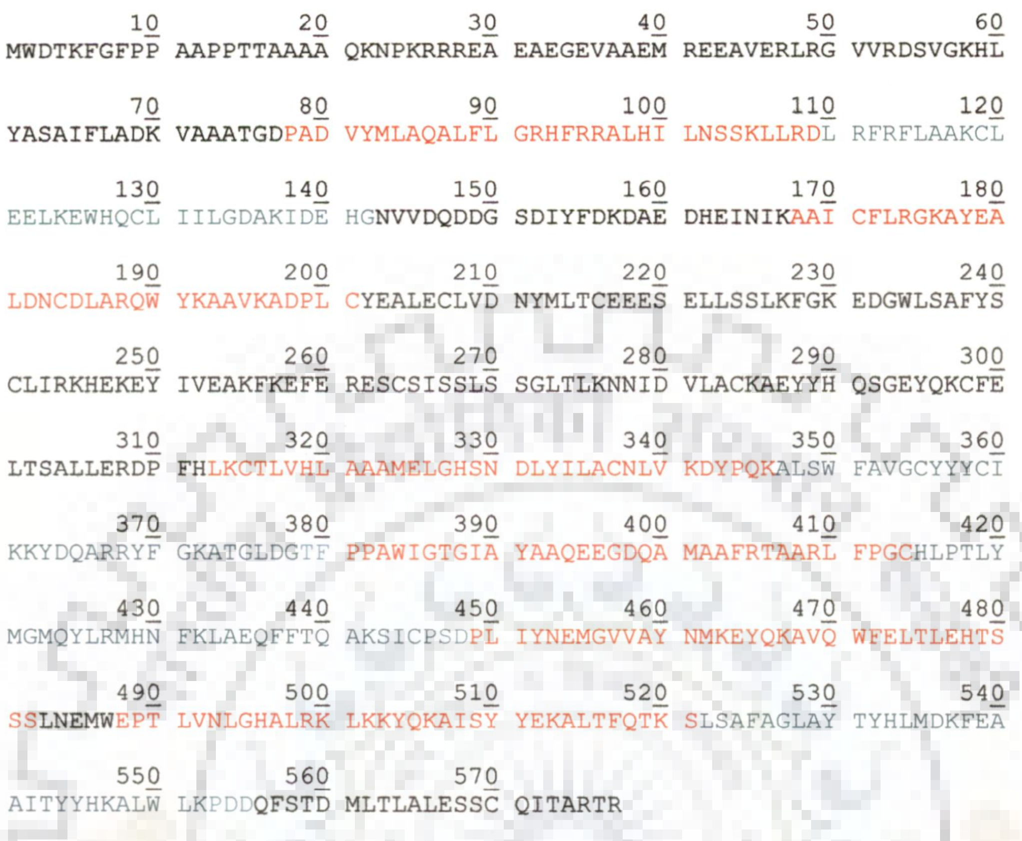


Figure 5.4: *In silico analysis of APC6 proteins of different organisms. A. Multiple alignment and web logo of APC6 protein in Saccharomyces cerevisiae (Sacce), Saccharomyces pombe (Sacpo), Orysa sativa Basmati 370 (OsAPC6), Arabidopsis thaliana (Arath), Homo sapiens (Homsa) and Mus musculus (Musmu) B. Domain architecture showing the distribution of the TPR motifs of APC6 protein in these organisms. The black coloured rectangular box represents the TPR motifs.*



```
        10         20         30         40         50         60
MWDTKFGFPP AAPPTTAAAA QKNPKRRREA EAEGEVAAEM REEAVERLRG VVRDSVGKHL

        70         80         90        100        110        120
YASAIFLADK VAAATGDPAD VYMLAQALFL GRHFRRALHI LNSSKLLRDL RFRFLAAKCL

       130        140        150        160        170        180
EELKEWHQCL IILGDAKIDE HGNVVDQDDG SDIYFDKDAE DHEINIKAAI CFLRGKAYEA

       190        200        210        220        230        240
LDNCDLARQW YKAAVKADPL CYEALECLVD NYMLTCEEES ELLSSLKFGK EDGWLSAFYS

       250        260        270        280        290        300
CLIRKHEKEY IVEAKFKEFE RESCSISSLS SGLTLKNNID VLACKAEYYH QSGEYQKCFE

       310        320        330        340        350        360
LTSALLERDP FHLKCTLVHL AAAMELGHSN DLYILACNLV KDYPQKALSW FAVGCYYYCI

       370        380        390        400        410        420
KKYDQARRYF GKATGLDGTF PPAWIGTGIA YAAQEEGDQA MAAFRTAARL FPGCHLPTLY

       430        440        450        460        470        480
MGMQYLRMHN FKLAEQFFTQ AKSICPSDPL IYNEMGVVAY NMKEYQKAVQ WFELTLEHTS

       490        500        510        520        530        540
SSLNEMWEPT LVNLGHALRK LKKYQKAISY YEKALTFQTK SLSAFAGLAY TYHLMDKFEA

       550        560        570
AITYYHKALW LKPDDQFSTD MLTLALESSC QITARTR
```

***Figure 5. 5** TPR Domains of Oryza sativa APC6 (shown in red and blue colour) as predicted from alignment of Sachhromyces pombe Cut9 protein and Oryza sativa APC6 sequences.*

In order to predict the 3D model of *Os*APC6 protein, *Sachhromyces pombe* was chosen as template since it was the most identical characterized protein. 534 out of 597 amino acid residues of *Sp*APC6 are similar to *Os*APC6 amino acids with 190 identites and 293 positives (Fig. 5.6).

pdb|2XPI|A Chain A, Crystal Structure Of ApcC HETERO-Tetramer Cut9-Hcn1

Score = 311 bits (797), Expect = 6e-96, Method: Compositional matrix adjust. Identities = 190/534 (36%), Positives = 293/534 (55%), Gaps = 35/534 (7%)

```
Query  48    LRGVVRDSVGKHLYASAIFLADKVAAATGDPADVYMLAQALFLGRHFRRALHILNSSKLL  107
             LR    D++ +  Y  A F+ +KV   TG+P D + LAQ       + RA  +L    L
Sbjct  87    LRLWRHDALMQQQYKCAAFVGEKVLDITGNPNDAFWLAQVYCCTGDYARAKCLLTKEDLY  146

Query  108   -RDLRFRFLAAKCLEELKEWHQCLIILGDA----KIDEHGNVVDQDDGSDIYFDKDAEDH  162
              R    R+LAA CL +L +W   L +LG+     K +++ N +   DG
Sbjct  147   NRSSACRYLAAFCLVKLYDWQGALNLLGETNPFRKDEKNANKLLMQDGG-----------  195

Query  163   EINIKAAICFLRGKAYEALDNCDLARQWYKAAVKADPLCYEALECLVDNYMLTCEEESEL  222
              I ++A++C+LRG+ Y  L N D A++ YK A+  D  CYEA + LV N++LT +EE +L
Sbjct  196   -IKLEASMCYLRGQVYTNLSNFDRAKECYKEALMVDAKCYEAFDQLVSNHLLTADEEWDL  254

Query  223   LSSLKFG---KEDG-WLSAFYSCLIRKHEKEYIVEAKFKEFERESCSISSLSSGLTLKNN  278
             +  L +    KED  +L + Y   + K   E        E  R    +SS++    L+ +
Sbjct  255   VLKLNYSTYSKEDAAFLRSLYMLKLNKTSHE-------DELRRAEDYLSSING---LEKS  304

Query  279   IDVLACKAEYYHQSGEYQKCFELTSALLERDPFHLKCTLVHLAAAMELGHSNDLYILACN  338
              D+L CKA+        +     +T+ +LE DP++L    +HLA+  E G  N LY+++ +
Sbjct  305   SDLLLCKADTLFVRSRFIDVLAITTKILEIDPYNLDVYPLHLASLHESGEKNKLYLISND  364

Query  339   LVKDYPQKALSWFAVGCYYYCIKKYDQARRYFGKATGLDGTFPPAWIGTGIAYAAQEEGD  398
             LV  +P+KA++W AVG YY C+ K  +ARRYF K++ +D  F PAWIG   ++A + E D
Sbjct  365   LVDRHPEKAVTWLAVGIYYLCVNKISEARRYFSKSSTMDPQFGPAWIGFAHSFAIEGEHD  424

Query  399   QAMAAFRTAARLFPGCHLPTLYMGMQYLRMHNFKLAEQFFTQAKSICPSDPLIYNEMGVV  458
             QA++A+ TAARLF G HLP L++GMQ++++ N  LA ++   + ++   DPL+ NE+GVV
Sbjct  425   QAISAYTTAARLFQGTHLPYLFLGMQHMQLGNILLANEYLQSSYALFQYDPLLLNELGVV  484

Query  459   AYNMKEYQKAVQWFE--LTLEHTSSSLNEMWEPTLVNLGHALRKLKKYQKAISYYEKALT  516
             A+N  + Q A+  F+  L L   + S  + W  T  NLGHA RKLK Y  AI    + L
Sbjct  485   AFNKSDMQTAINHFQNALLLVKKTQSNEKPWAATWANLGHAYRKLKMYDAAIDALNQGLL  544

Query  517   FQTKSLSAFAGLAYTYHLMDKFEA-AITYYHKALWLKPDDQFSTDMLTLALESS  569
               T   +    +A  Y L  K    AIT+ H++L + P++  ++D+L  ALE +
Sbjct  545   LSTNDANVHTAIALVY-LHKKIPGLAITHLHESLAISPNEIMASDLLKRALEEN  597
```

Figure 5. 6 *Alignment result of Oryza sativa APC6 sequence and Saccharomyces pombe Cut9 sequence using BlastP.*

5.3.2: APC6 three-dimensional structure analysis

The sequence homology between *Os*APC6 and the template was 36 % identity and 55 % similarity. *Os*APC6 ligand-supported homology model was constructed based on the crystallographic 3D structure of *Sp*APC6 (PDB ID: 2XPI) (Fig. 5.7).

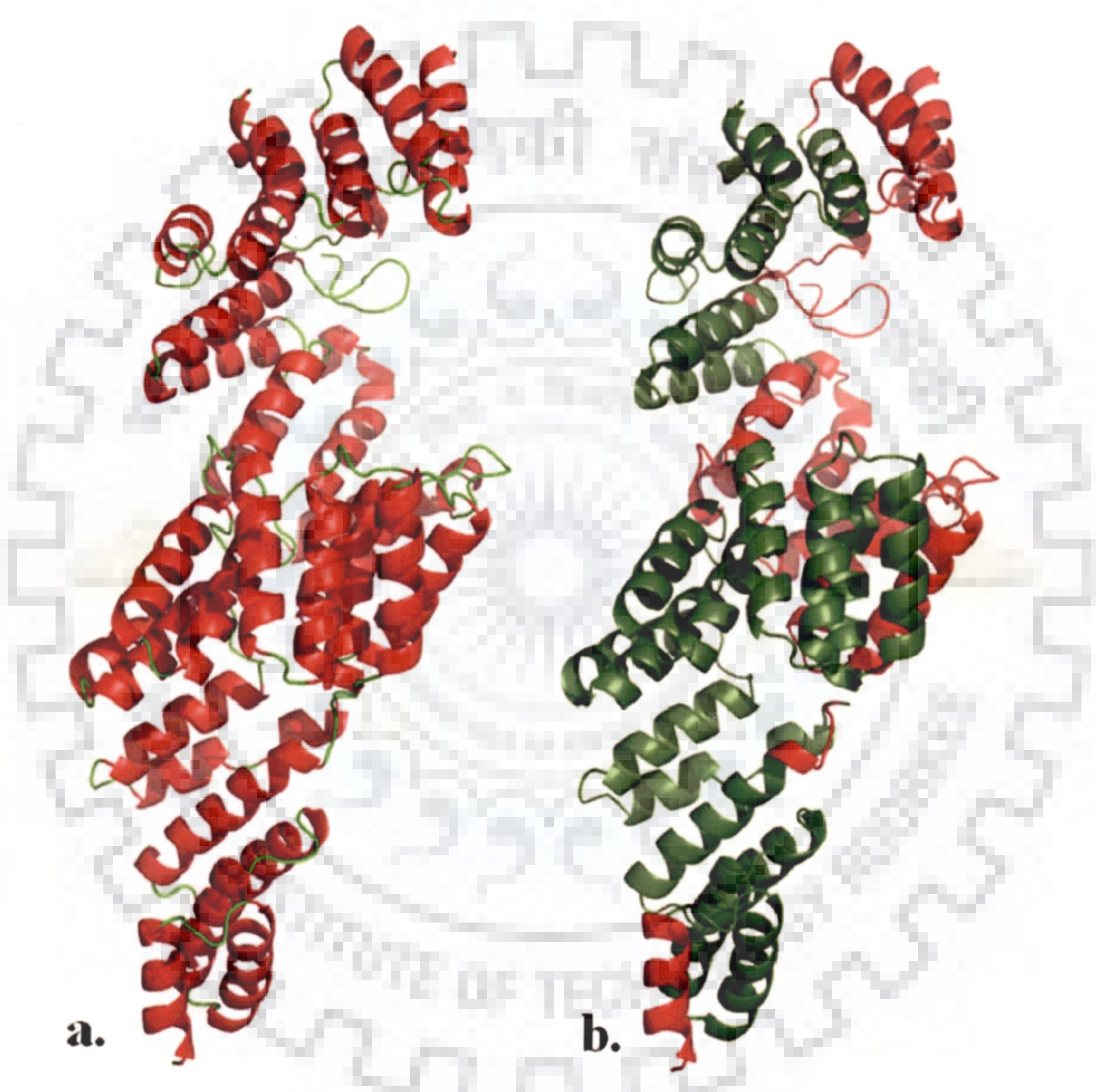


***Figure 5. 7 a**. Cartoon representation of molecular model of OsAPC6, helices are shown in red coloured spiral cylindrical ribbons, **b**. OsAPC6 3D molecular model, green coloured spiral cylindrical ribbons represents TPR domains. Figure prepared using PyMOL.*

The generated *Os*APC6 model was superimposed on the *Sp*APC6 model for comparative molecular modeling (Fig. 5.8). The generated model was subjected to refinement, loop modeling and energy minimization. PROCHECK, Verify-3D and ERRAT plot were used for determining the stereo-chemical parameters of the energy minimized model of *Os*APC6. Ramachandran plot of the 3D model generated by PROCHECK shows 90.5% residues are present in the core region, 9.0% in allowed region, 0.3% in generously allowed region and 0.3% in disallowed region which includes only one residue. This residue in disallowed region could be ignored as it is not present near the active site nor is involved in ligand binding.

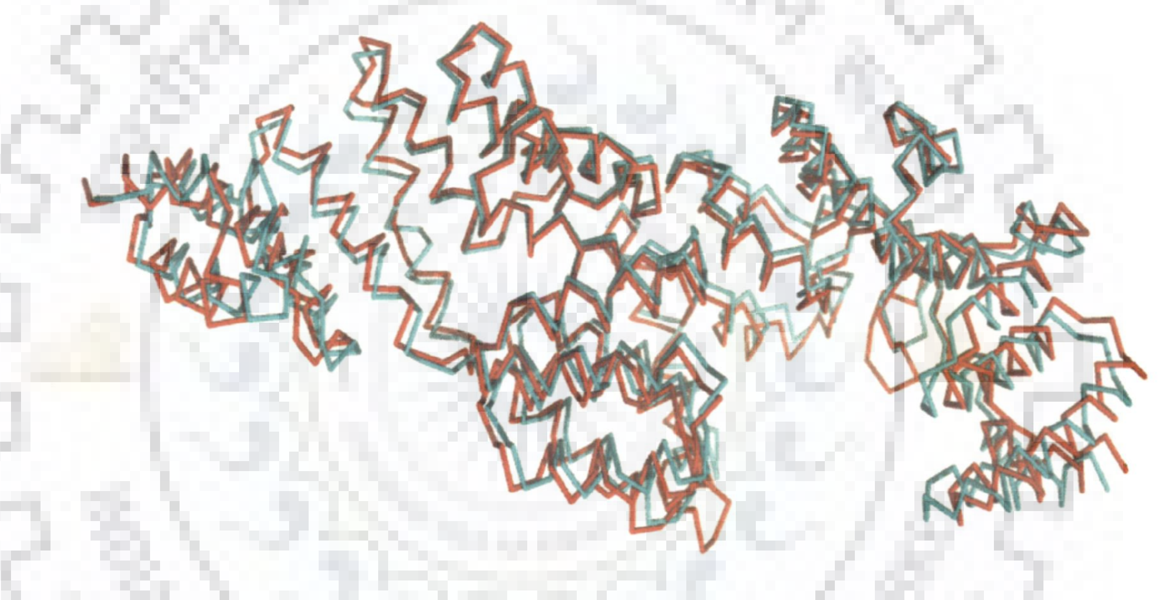


Figure 5. 8 *Superimposition of Oryza sativa APC6 (red color) with Saccharomyces pombe APC6 (Blue color) developed in PyMOL.*

Molecular dynamics simulations were carried out using the predicted 3D structure of *Os*APC6 protein to determine the stability of the model in equilibration with solvent molecules, i.e., in the physiological state. The obtained MD trajectories during the simulation run of 1 ns were monitored and found to be stable. An overall RMSD of 0.23 nm was obtained which indicates that the 3D modeled structure of *Os*APC6 is good and has a stable configuration.

5.4 Discussion

The anaphase-promoting complex/cyclosome (APC/C) is an unusually complex multi-subunit E3 ubiquitin ligase endowed with elaborate regulatory, catalytic and specificity properties. By mediating the ubiquitylation of a diverse array of mitotic regulatory proteins, the APC/C controls the cell cycle processes responsible for chromatid segregation at the metaphase to anaphase transition, the completion of mitosis, and the establishment and maintenance of G1 (Peters, 2006; Thornton *et al.*, 2006). Structural studies of the APC/C have focused on crystallographic analysis of isolated APC/C subunits and small sub-complexes whereas single particle electron microscopy (EM) has defined the molecular envelope of the APC/C and its complexes with co-activators and the mitotic checkpoint complex. Although approximate locations of the termini of most APC/C subunits have been reported, no systematic assignment of the EM molecular envelope to individual APC/C subunits has been attempted, limiting our understanding of APC/C molecular mechanisms. The APC/C is a highly conserved complex consisting of thirteen subunits in yeast (Passmore and Barford, 2004) while eleven subunits have been identified in plants (Gieffers *et al.*, 2001; Capron *et al.*, 2003a). The APC/C is essential for the ubiquitin-dependent degradation of cell cycle regulatory proteins. The complex multisubunit structure of APC/C facilitates its intimate involvement in the formation of substrate-ubiquitin conjugates, and thus determines substrate specificity of the whole process. The function of the anaphase promoting complex/cyclosome (APC/C) components is not known in detail.

Functional genomics approaches are exploited to develop APC/C mutants so that the function of the respective subunits can be determined (Carroll and Morgan, 2002; Kwee and Sundaresan, 2003; Kuppusamy *et al.*, 2009). The mutation in rice *APC6* gene was observed in a T-DNA insertional mutant (Dhaliwal *et al.*, 2001; Kumar *et al.*, 2010). The APC6 was found to be involved in female gametophyte development and gibberellic acid insensitivity.

The Clustal W results showed that the anaphase promoting complex is highly conserved. It is present in almost all organisms ranging from prokaryotes like fission yeast to eukaryotes including plants, animals and human. Several studies to determine the protein structure of APC/C complex are carried out, one which

explains the structural basis of subunit assembly in APC/C complex (Schreiber *et al.*, 2011). The three-dimensional structure of the human APC/C complex has been solved at a resolution of 24 Å (Gieffers *et al.*, 2001). But research on the APC/C has been restricted to the use of native systems. Because most APC/C subunits are essential, genetic manipulations are intrinsically difficult, and the low natural abundance of APC/C has limited structural studies further. Recombinant production of large protein complexes is a significant challenge (Schreiber *et al.*, 2011). So *in silico* modeling could come up to be a good source for studying the protein structures and interactions. The protein structure of APC6 was generated using comparative modeling. An *in silico* 3D-structure of APC6 was modeled using *Saccharomyces pombe* APC6 (PDB ID: 2XPI) as template. The TPR domains were identified in *Oryza sativa* protein sequence based on comparative phylogenetic analysis and multiple alignment results. Seven TPR domains were predicted in rice which were spanning the C-end terminal of the protein. The APC6 protein bears tetratricopeptide repeat (TPR) motifs as functional domains. The TPR motif was originally identified in yeast as a protein-protein interaction module in cell cycle proteins. It has since been found in organisms ranging from bacteria to humans. The TPR motif is a degenerate sequence of ~34 amino acids loosely based around the consensus residues -W-LG-Y-A-F-A-P-. The sequence occurs in tandem arrays and is present in over 800 different proteins. TPR motif-containing proteins act as scaffolds for the assembly of different multiprotein complexes including the anaphase promoting, the peroxisomal import receptor and the NADPH oxidase complexes. TPR motifs are present in functionally divergent proteins and thought to mediate protein-protein interactions and the assembly of multiprotein complexes. TPR-containing proteins are involved in a diverse spectrum of cellular functions with the majority of them participating in cell cycle control, transcription and splicing events, protein transport especially protein import, regulatory phosphate turnover, and protein folding (Blatch and Lassle, 1999).

Chapter VI

PREPARATION AND TRANSFORMATION OF RNAi CONSTRUCT FOR APC6 GENE

6.1 Introduction

Genetic engineering is defined as the modification of an organism, involving isolation and manipulation from one organism and its reintroduction of DNA into cells of another organism usually with the purpose to express a protein (Schouten *et al.*, 2006; Sahoo *et al.*, 2007). In plants, genetic transformation is a critical technology to study the functions of genes (i.e. complementation, overexpression, gene silencing) and also an important tool in crop improvement. Gene silencing is a novel gene regulatory mechanism that limits the transcript level by either suppressing transcription (TGS) or by activating a sequence- specific RNA degradation process (RNA interference, RNAi) (Agrawal, 2003). RNAi can specifically silence individual genes, creating knockout phenotypes in transformants that can produce the required hairpin RNAs.

In plant functional genomic studies, gene cloning into binary vectors for plant transformation is a routine procedure. Traditionally, gene cloning has relied on restriction enzyme digestion and ligation. In recent years, however, Gateway® cloning technology (Invitrogen Co.) has developed a fast and reliable alternative cloning methodology which uses a phage recombination strategy (Xu and Li, 2008). The protocol takes advantage of unique characteristics of the replication origins of plasmids used and eliminates the necessity for restriction enzyme digestion in plasmid selections.

6.2 Materials and methods

6.2.1 Plant material

The RNAi construct was prepared using Gateway Cloning method using the Invitrogen kit. The entry and destination vectors were pENTR/D-TOPO and pANDA vector respectively and the plant material chosen for transformation was an *indica* variety IR-64.

6.2.2 RNA isolation and cDNA synthesis

Total RNA was isolated from the two week old seedlings of Basmati 370 using Genei plant RNA isolation kit. In order to check the resolution and quality of RNA denaturing formaldehyde gel was prepared using MOPS, formamide and formaldehyde. All the solutions were prepared using DEPC treated water. 4 μL of RNA with 1.5 μL RNA loading dye and 23.5 μL of the formamide master mix was loaded on the 1% MOPS gel. Gel was visualized and photographed.

The mRNA was reverse transcribed using superscript™ II reverse transcriptase (RT) enzyme. 20 µL reaction was set using 1 µg of total RNA, oligo $(dT)_{12-18}$ (500 µg/mL), 1 µL dNTP mix 10 mM each. The mixture was heated to 65°C for 5 min and quick chilled on ice and after brief centrifuge. To the reaction mixture 4 µL of 5X first strand buffer; 2 µL of 0.1 M DDT; RNase OUT (40 U/µL) and 1 µL (200 U) of Super Script II RT were added. The entire 20 µL reaction was mixed thoroughly and incubated at 42°C for 1 h, followed by inactivation at 95°C for 2 min. 2 µL of this cDNA was used for the RT-PCR.

6.2.3 Target gene amplification and its recovery for cloning

The candidate gene used for RNAi construct preparation was *APC6*. Primers were designed based on its mRNA sequence using primer 3 software. Four extra nucleotides CACC- were added to the 5' end of the forward primer. The sequence of the primers used was Forward 5'-**CACC**TCTCTCAGCAGCTTCCATCA-3' and Reverse 5'- ACGCGCTCAAACTTTTGGT-3'. These primers were designed from the first exonic region of the *APC6* gene (Fig. 6.1). PCR was performed in PTC-200 Mastercycler (Eppendorf) using a 20 µL reaction containing 2 µL of cDNA, 0.1 mM of each of the dNTPs, 0.25 µM of forward and reverse primers, 1U of high fidelity Taq DNA polymerase and 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$) and the condition using 94°C for 2 min, followed by 40 cycles denaturation at 94°C for 15 sec, annealing at 54°C for 30 sec and extension at 72°C for 1min with final extension at 72°C for 5min.

The PCR product was subjected to agarose gel electrophoresis. The desired band was excised from the agarose gel with a clean and sharp scalpel. Three volumes of gel solubilizing buffer L3 (provide in the PureLink™ Quick gel extraction kit from Invitrogen) was added to 1 volume of the gel and incubated for 10 min at 50°C. It was then transferred to the wash tube containing the extraction column and centrifuged at maximum speed (12000 rpm) for 1 min. The pellet was washed with 500 µL of wash buffer (W1). The washed pellet containing extraction column was placed into a recovery tube and eluted with 30µl of elution buffer (E5) followed by centrifugation at maximum speed for 1 min. The purified PCR product was stored at -20°C for long term use.

ATGCCTCTCTCAGCAGCTTCCATCAACCGTGCATCCTACCAAGTTCTCCTCCTCCTCGCCGCCGCCGCCGTGTCCACCACGGGCGGCGACG
GCAACACGGCGCCCGGCAATGCCACCGCCACGGCGACGACTGGCGGCGACGACACGGAGATGTACATCTGCTACCTCTGCACCGGGCGC
AACCCGATCCTGATCAGGAGGTGCCCCATCTACTGGGACTACTGCCACCTCAACTGCTTTGACGACGCGCCCTCCACCGCCGCCGCCGCCG
ACGACGTCGCCGCCGTTCCCGTGGCCTCGCCGGCGGCGCCTGCGCGACGGGTGGGCGGCGTCCCGCGCGAGACCCTCGAGGACGAGGA
GTGCTACGTCATGAAGCTGTACGAGAACGGCAGCTACGTCATCGTGACCACGCTGGGCTGCTCCCAGACCGCCTCGTGCCTCCTCTCCTGC
GGCGGCGGCGACCTCGCAGCCGACGGCGAAGAAGCCCTGGCGGCGGCGCACCCGGCCGGCGCCGTCGGCGTCTCGCCGCCGTGGCGAA
TGTGGGATACAAAATTCGGTTTCCCTCCAGCAGCGCCGCCCACCACCGCCGCCGC AGCGCAGAAAAATCCCAAGCGGAGGAGAGAGGCGG
AGGCGGAGGGGGAGGTGGCGGCGGAGATGAGGGAGGAGGCGGTGGAGCGGCTGCGCGGGGTGGTGCGGGACAGCGTCGGGAAGCACCT
GTACGCGTCCGCCATCTTCCTCGCCGACAAGGTGGCCGCGGCCACGGGGGACCCCGCCGACGTCTACATGCTCGCGCAGGCGCTCTTCCT
GGGCCGCCACTTCCGCCGCGCGCTCCACATCCTCAACTCCTCCAAGCTCCTCCGCGACCTCCGCTTCCGCTTCCTCGCAGCCAAGTGCCTC
GAGGAGTTGAAAGAATGGCATCAGTGTTTGATCATACTTGGAGATGCAAAAATAGAGCATGGAAACGTTGTTGATCAGGATGATGGCAGTGAC
ATTTACTTTGATAAGGATGCTGAAGACCATGAGATCAATATCAAAGCGGCAATATGTTTTTTACGTGGCAAGGCATACGAAGCACTGGACAACT
GTGACCTTGCTCGACAATGGTACAAAGCTGCAGTGAAGGCTGATCCTTTGTGCTATGAGGCCCTTGAATGCCTTGTTGATAACTACATGTTGA
CATGCGAGGAAGAATCTGAGCTATTGTCCTCTCTAAAATTTGGAAAAGAAGATGGGTGGCTCTCAGCATTCTACTCTTGTTTGATAAGGAAGCA
TGAAAAAGAATATATAGTGGAAGCAAAGTTCAAGGAATTTGAACGAGAATCTTGTAGTATTTCATCTTTGAGTTCAGGACTGACACTGAAAAATA
ATATTGACGTGTTGGCTTGCAAAGCTGAATACTATCATCAGAGTGGAGAGTACCAAAAATGTTTCGAACTCACATCTGCGTTACTTGAAAGGGA
CCCTTTTCATTTGAAATGCACGTTAGTTCATTTGGCAGCTGCAATGGAGCTTGGCCATTCAAATGACCTTTATATTTTGGCCTGCAATCTAGTGA
AGGACTATCCTCAGAACTGTGGTTCCATCTGTAGAGCTCTTTCCTGGTTTGCTGTTGGGTGTTATTACTACTGTATTAAGAAGTATGATCAAGCA
CGCAGATACTTTGGCAAAGCTACAGGGTTAGATGGGACATTTCCTCCTGCTTGGATTGGTACTGGTATTGCTTATGCTGCACAGGAGGAGGGT
GATCAAGCAATGGCTGCATTTCGGACAGCAGCTCGGCTATTTCCTGGATGTCATCTGCCAACTTTATACATGGGCATGCAATATTTGCGAATGC
ACAATTTCAAACTTGCAGAGCAGTTCTTCACGCAAGCAAAATCTATCTGCCCATCTGATCCGCTTATATATAACGAGATGGGGGTTGTAGCTTA
TAATATGAAAGAGTATCAAAAAGCAGTTCAGTGGTTTGAGCTAACACTGGAGCATACTTCATCCTCCTTGAATGAAATGTGGGAACCAACATTG
TGAATCTTGGGCATGCATTGCGGAAACTCAAGAAATATCAAAAGGCAATATCATATTATGAAAAGGCACTCACCTTTCAAACCAAAAGTTTGAG
CGCGTTTGCTGGTCTTGCTTATACTTACCACCTTATGTTTGACACTT CCACAGTTACTTTC**TGA**

Figure 6.1 *APC6 CDS sequence showing red colored region where gene specific primers were designed.*

6.2.4 Cloning of target gene in Entry clone vector and its transformation in E. coli

4µl of freshly purified PCR product of the target gene was mixed with 1 µL of pENTR™ TOPO® vector (Fig. 6.2), 1 µL of salt solution (provided in the kit), 1 µL of sterile water and incubated for 5 min at room temperature. 2 µL of TOPO® cloning reaction mixture was used for transformation in competent *E. coli* (DH5α) cells, by heat shock at 42°C for 90 sec, followed by immediate transfer to ice for 1 min. Cells were suspended with LB medium and kept for shaking at 37°C, 200 rpm maximum for 2 h for proper growth. Cells were concentrated and spread to LB plate containing 50 µg/mL kanamycin, and incubated at 37°C for overnight.

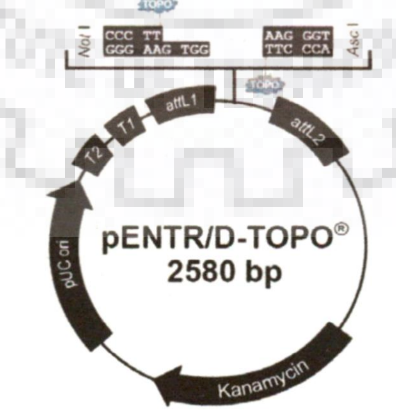


Figure 6.2 *Entry vector map obtained from Invitrogen.*

6.2.5 LR recombination reaction and transformation to E. coli

The Gateway® LR Clonase™ enzyme mix kit (Invitrogen) was used to perform the LR recombination reaction using pANDA destination vector (Fig. 6.3). A 10µl of reaction mixture was prepared with 2 µL of Entry clone (150ng), 1 µL of destination vector (pANDA – 150 ng/µL), 5 µL of TE buffer and 2 µL of LR clonase. The mixture was vortexed briefly followed by a short spin and incubation at 25°C for 1 h. To stop the reaction 1 µL of Proteinase K (2 µg/µL) was added and incubated at 37°C for 10 min. 5 µL of the LR reaction mix was used for transformation in competent *E. coli* (DH5α) cells, and was spread on LB plate containing 50 µg/mL kanamycin and incubated overnight at 37°C.

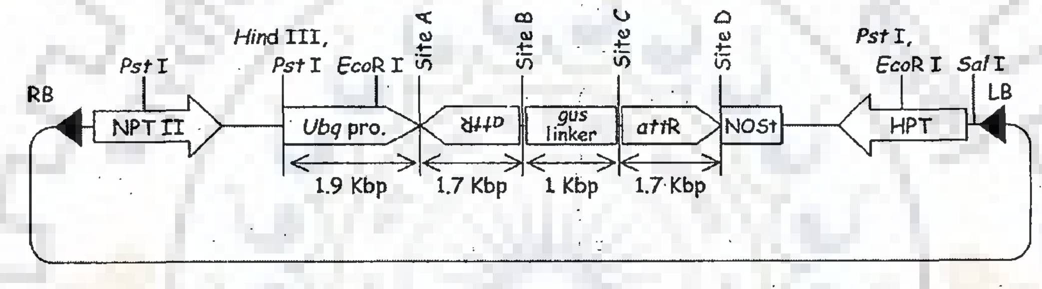


Figure 6.3 *Destination vector map for LR cloning.*

6.2.6 Plasmid DNA isolation and confirmation of LR reaction

To confirm the LR reaction, colonies from the LB plate were harvested and plasmid isolation was done by using miniprep kit from Invitrogen. 1-5 mL of an overnight culture (*E. coli* in LB medium) was pellet and 250 µL resuspension buffer (R3) with RNase A was added to it. Then 250 µL lysis buffer (L7) was added and mixed gently by inverting the capped tube 5 times. Now the tube was incubated for 5 minutes at room temperature and 350 µL precipitation buffer (N4) was added to it. The mixture was then centrifuged at ~12,000 x g for 10 min at room temperature and the supernatant was loaded onto a spin column. This was again subjected to centrifugation at ~12,000 x g for 1min, followed by a washing with 500 µL Wash Buffer (W10). The column was then centrifuged at ~12,000 x g for 1 min. The flow-through was discarded and the column was placed back into the wash tube. 75 µL of preheated TE Buffer (TE) was added to the center of the column and incubated for 1 min at room temperature. After centrifugation at ~12,000 x g for 2 min the purified plasmid DNA was obtained.

The plasmid isolated after LR reaction was subjected to agarose gel electrophoresis along with empty plasmid. The LR transformed plasmid DNA was subjected to PCR with gene specific primer and GUS primer. PCR conditions were same as used for target gene amplification.

6.2.7 Transformation of recombinant LR clone in Agrobacterium

For the transformation electro-competent *Agrobacterium* cells were used. To prepare electro-competent cells, *Agrobacterium tumefaciens* strain LBA4404 was streaked on LB plate containing 50 μg/mL kanamycin and 10 μg/mL rifampicin and incubated for 3 days at 28°C. Colonies were harvested in LB liquid medium containing same antibiotic selection and kept at incubation for overnight at 28°C, 200 rpm. 50 mL cell culture was transferred to fresh 500 mL LB media and allowed to grow at 28°C, 200 rpm until OD_{600} = 1-1.5. Cells were then transferred to cold centrifuge tubes (250 mL) on ice for 20 min followed by centrifugation at 4°C, 4000 rpm for 15 min. The pellet was resuspended very gently in 250 mL of sterile cold water and centrifuged like before. In this way cells were washed four more times. After the washing steps cells were resuspended with 25 mL of cold sterile 10% glycerol and combined suspended cells from each tube into one cold 50 mL polypropylene falcon centrifuge tube. Cells were again centrifuged at 4°C, 3500 rpm for 10 min and finally the pellet was resuspended completely but very gently in 2 mL of cold 10% glycerol.

1 μg of plasmid DNA was added to 0.1 mL of electro-competent cells and mixed gently but thoroughly and then freezed in liquid N_2 for 1 min. Immediately the cells were thawed at 37°C for 5 min. Then YEP liquid media (150 μL) was added and incubated at 28°C for 2 h. Cells were then transferred to YEP plate containing 50 μg/mL kanamycin and 10 μg/mL rifampicin and incubated for 3 days at 28°C.

6.2.8 Verification of positive clones

A colony PCR was performed by directly using the colonies from YEP plate with the same PCR conditions and gene specific and GUS primers used for confirmation of LR reaction, mentioned above.

6.2.9 In planta transformation

Rice seeds (IR64) were soaked in distilled water for overnight and were germinated in petriplates at 30°C. Three-day old seedlings were taken for *Agrobacterium* infection.

Agrobacterium tumefaciens strain LBA4404 containing the RNAi construct in pANDA vector was used for transformation. *Agrobacterium* was grown overnight at 28°C in 1 mL LB medium (pH 7.0) containing 50 μg/mL kanamycin and 10 μg/mL rifampicin. The inoculum from this was used to prepare 3 mL LB culture containing same concentration of kanamycin and rifampicin. This bacterial culture was later resuspended in 100 mL of Winan's AB medium (Winans *et al.*, 1988) (pH 5.2) and grown for 18 h. For *vir* gene induction treatment, wounded tobacco leaf extract was added to the *Agrobacterium* suspension in Winan's AB medium 5 h before extraction. The seedlings with plumule just emerging were pricked at the apical meristem of the axis and at the intercotyledonary region with a sterile sewing needle and co-cultivated by immersion in the suspension of *Agrobacterium* for 1 h. Following infection co-cultivation, the seedlings were washed briefly with sterile water and later placed on autoclaved soilrite (vermiculite equivalent), moistened with water and allowed to grow in the growth room. The growth room was maintained at 26-28°C under 14 h photoperiod with fluorescent light of intensity 35 mmol m^{-2} s^{-1}. After two weeks plantlets were transferred to pots containing sand-soil mix and grown in the greenhouse. Upon flowering and ripening seeds from the T_0 plants were harvested.

6.2.10 Screening of transformed plants

The seeds obtained from T_0 plants were grown on autoclaved soilrite (vermiculite equivalent) with G418 antibiotic added to it.

6.2.11 Callus induction for biolistic transformation

Mature seeds of rice cultivar PR118 were dehulled and surface sterilized for 1 min with 70% ethyl alcohol then with 35% sodium hypochlorite for 20 min. After rinsing three times with sterile distilled water, seeds were plated on MS media. Twenty seeds were placed on each plate. For three weeks, the plates were kept in the dark at 25° C until calli appeared. These calli were subcultured in the same medium for another two weeks.

6.2.12 Particle gun-mediated genetic transformation of rice and regeneration

The embryogenic calli was taken and cut into small pieces of 2-3 mm size. 20-25 such pieces were placed in the centre of a petri-plate with osmoticum media (MS media with mannitol and 2.5 ppm 2, 4-D) for 4 h in the incubation room.

For bombardment, tungsten particles (0.6 μm) were coated with the plasmid DNA carrying

the selectable marker and RNAi construct. The calli was subjected to bombardment using Bio-Rad gun (PDS-1000/He system). The same plate was bombarded for the second time after 4-6 h and incubated overnight. The bombarded calli was transferred to recovery media (same media as used for growing embryogenic calli) for two days to revive the growth of calli. Now the calli was moved to selection media containing the G418 antibiotic (50 mg/L). The selection was carried out for two cycles of two week each. Some of the calli were analyzed for transformation by histochemical GUS (β-glucuronidase) assay using X-gluc. The remaining G418 resistant calli were transferred to regeneration media (MS with 4 ppm BAP and 0.5 ppm NAA). As the shoots start appearing in the regeneration media, they were transferred to the rooting media and incubated for 10-15 days to get good rooting. The plantlets were then taken, washed thoroughly in the running tap water and placed in moist cotton for hardening in water filled tray for two weeks. Finally the individual plantlets were transferred to soil in transgenic greenhouse.

6.2.13 Confirmation of selectable marker in RNAi construct

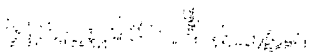
Leaf tissues from greenhouse grown plants were taken for molecular characterization. The RNAi construct carried *npt*II gene, so PCR was carried out using *npt*II specific primer. The PCR conditions were 94°C for 5 min, followed by 35 cycles denaturation at 94°C for 45 sec, annealing at 62°C for 1 min and extension at 72°C for 1 min with final extension at 72°C for 10 min.

6.3 Results and Discussion

6.3.1 Cloning of target gene in Entry clone

The target gene *APC6* was amplified using gene specific primers and the amplified product obtained was of 177 bp. The gene was then cloned into the pENTR/ D-TOPO entry vector through the BP reaction, which produces an Entry clone.

For this purpose Gateway-compatible primer with a 4-bp (CACC) sequence added to the forward primer was designed. This allows the PCR product to be cloned directionally into the TOPO vector to generate an Entry clone, which carries two recombination sites (*attL1* & *attL2*) for LR Clonase reaction. Since no restriction enzyme was involved during the entire cloning process, the TOPO technology allows easy production of entry clones. After cloning the entry vector plasmid was transformed into chemically competent *E.coli* cells. For selection



kanamycin was used and the colonies grown against the kanamycin antibiotic were considered to be positive with entry clones.

6.3.2 Cloning of target gene into destination vector and its confirmation

The resulting Entry clone with target gene (*OsAPC6*) and the recombination sites (*attL1* & *attL2*) was subcloned into pANDA destination vector by the LR recombination reaction between the entry clone and the destination vector. In this reaction, the PCR-derived fragments are inserted into two regions flanked by two recombination sites (*attB1* and *attB2*) in opposite directions, and the *gus* linker sequence is flanked by the two inverted repeats (Fig. 6.4). The cloned destination vector was transformed into *E. coli* and kanamycin resistant colonies were selected for plasmid isolation.

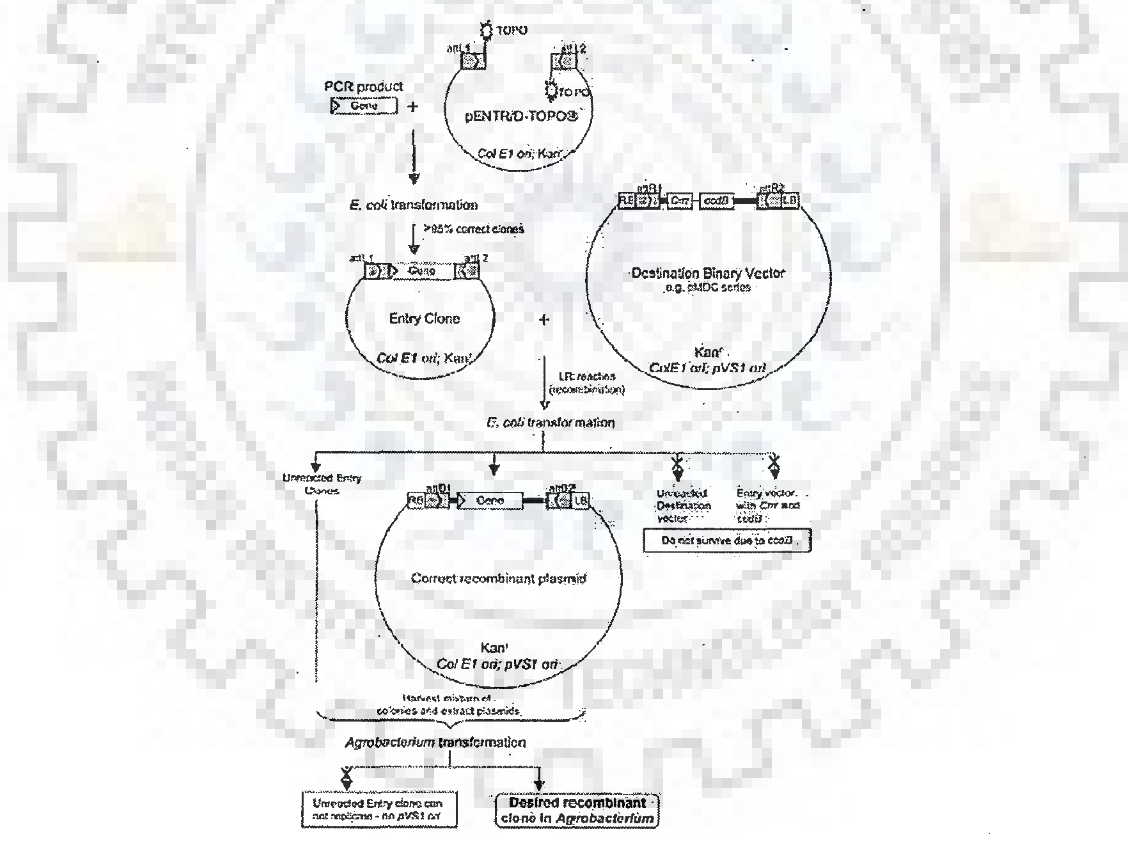


Figure 6.4 *Schematic from PCR gene cloning to Agrobacterium expression clones using the Gateway® cloning technology. Col E1 ori: origin of replication for E. coli. pVS1 ori: origin of replication for Agrobacteria. Kan^r: kanamycin resistance gene. Cm^r: chloramphenicol resistance gene. RB: T-DNA right border. LB: T-DNA left border. attL, attR, attB: recombination sites. ccdB: E. coli ccdB gene.*

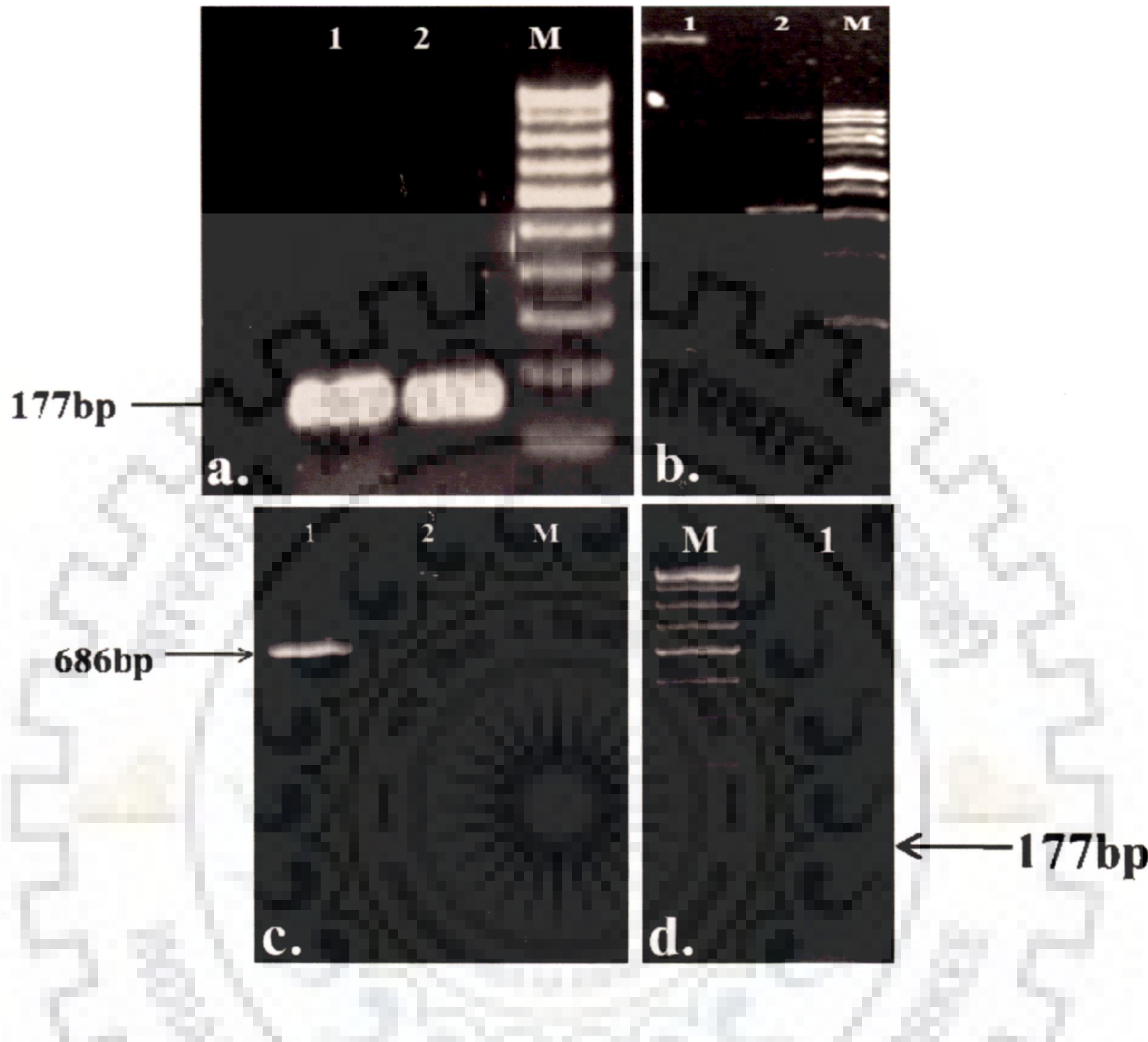


Figure 6.5 *Confirmation of cloning using PCR* ***a.*** *Confirmation of the LR cloning in destination vector, lane 1= PCR amplification of target gene in cDNA, lane 2= PCR amplification of target gene in pANDA destination vector, M= 100 bp ladder,* ***b.*** *pANDA plasmid DNA lane 1= before LR reaction, lane 2= after LR reaction, M= 1kbp ladder,* ***c.*** *Confirmation of LR cloning using GUS primers, lane 1= using pANDA plasmid before LR reaction, lane 2= using pANDA plasmid after LR cloning of target gene, M= 100 bp ladder,* ***d.*** *Colony PCR result using gene specific primer on Agrobacterium transformed colony, lane 1= bacterial DNA as template, M= 100 bp ladder.*

The *APC6* gene specific primers were used to check the presence of target gene in the construct. The cDNA was used as control to amplify the target gene. The PCR results (Fig. 6.5a) showed the presence of target gene sequence in the plasmid of construct after LR cloning. The gel run of untransformed pANDA plasmid (before LR reaction) and the transformed plasmid (after LR reaction) showed a difference in size. The untransformed plasmid was bigger in size as compared to the transformed one (Fig. 6.5b). This could be attributed to the replacement of 177 bp of gene sequence in the two side of GUS linker inplace if the attR sequence with 1.7 kbp size.

The GUS gene specific primers were further used to confirm the presence of GUS linker in between the sense and anti-sense target gene orientation. The PCR results showed the presence of GUS gene and the position of the amplified product was found to be same on gel for both the untransformed pANDA vector and the transformed construct (Fig. 6.5c).

6.3.3 Confirmation of RNAi construct in Agrobacterium

After LR cloning, the RNAi construct in pANDA vector was transformed in electro-competent cells of *Agrobacterium tumefaciens* strain LBA4404. The positive colonies resistant to kanamycin and rifampicin were picked for further confirmation. The plasmid DNA of transformed pANDA vector was taken and gene specific primers were used to look for the presence of the gene in the RNAi construct in *Agrobacterium.* The PCR result showed the presence of APC6 gene sequence of 177 bp (Fig. 6.5d). The resistance shown against kanamycin also confirmed the presence of *nptII* gene.

6.3.4 Results of in planta transformation

The IR64 variety of *indica* rice was taken for the purpose of in-planta transformation. Seedlings grown for two days were taken for transformation (Fig. 6.6a). Around 80 seedlings were pricked in a single batch of transformation and the plants were transferred to soilrite for further growth (Fig 6.6b & c). The experiment was carried out in three batches and around 15 plants were survived from each batch resulting in survival of 46 healthy plants (Fig. 6.6d).

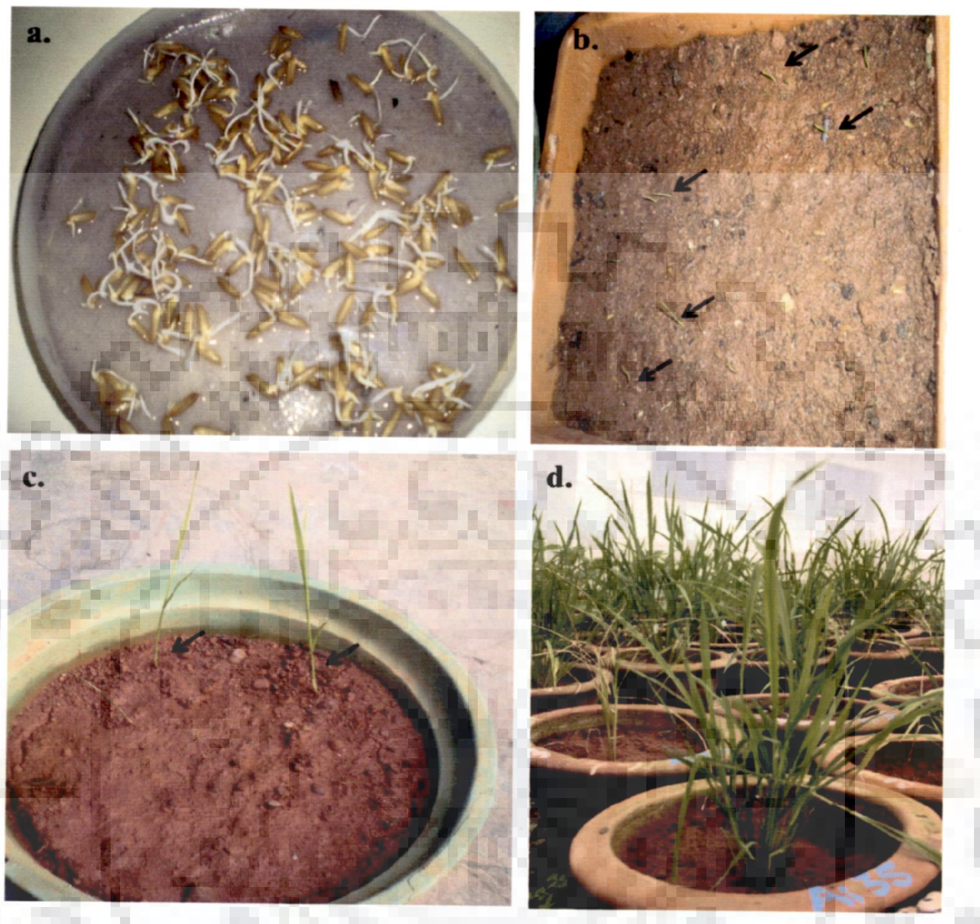


Figure 6.6 *In planta transformation in rice seedlings* ***a.*** *Two days old seedlings of IR64 rice ready for transformation,* ***b.*** *One-week old plantlets after transformation,* ***c.*** *Two-week old plantlets after transformation,* ***d.*** *transformed plants grown in green house condition.*

6.3.5 Regeneration and particle bombardment of rice calli

The PR118 rice variety of *indica* rice was selected for the purpose of calli regeneration and transformation using particle bombardment. The calli suitable for transformation was grown on MS media with 2.5ppm 2,4-D (Fig. 6.7a). The scutellar calli from rice embryo was regenerated. Only embryogenic calli (white to light yellowish in color, compact and friable) were selected and non-embryogenic calli (mucilaginous and smooth) were discarded (Fig.

6.7b). Three weeks old scutellum derived calli were transferred on to the osmoticum media for particle bombardment (Fig. 6.7c).

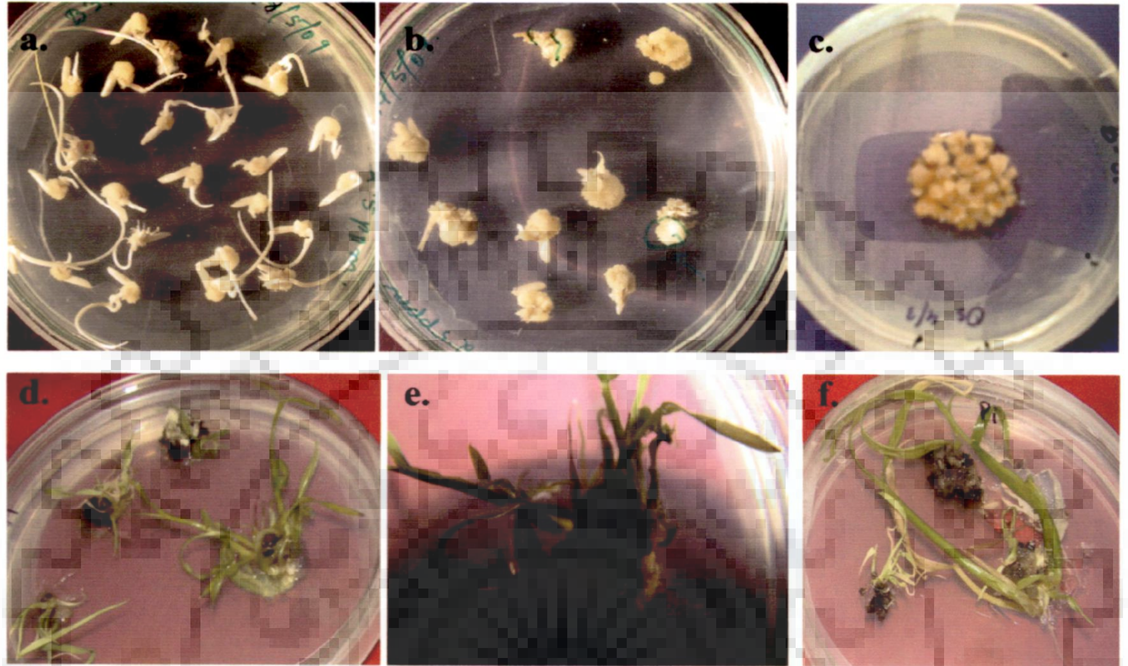


Figure 6.7 *Calli development and regeneration of PR-118 rice seeds* ***a.*** *rice seeds kept for callus generation in MS-media,* ***b.*** *three week old scutellar calli of rice,* ***c.*** *rice calli placed in osmoticum media for particle bombardment,* ***d, e & f.*** *regeneration of shoots from the transformed calli*

The plasmid DNA with the RNAi construct was coated on the tungsten beads which were placed on macrocarriers of the biolistic gene gun for bombardment (Fig. 6.8a & b). The rice scutellar calli was subjected to bombardment. Some of the bombarded calli was taken for GUS assay. The normal calli which was not subjected to bombardment appeared white but the transformed calli was blue in colour after the GUS assay (Fig. 6.8c & d). The calli was further subjected to selection using G418 antibiotic. The further regeneration was carried out and around seven plantlets were found to survive the selection (Fig. 6.7d, e & f). The plantlets were transferred to the green house for further growth.

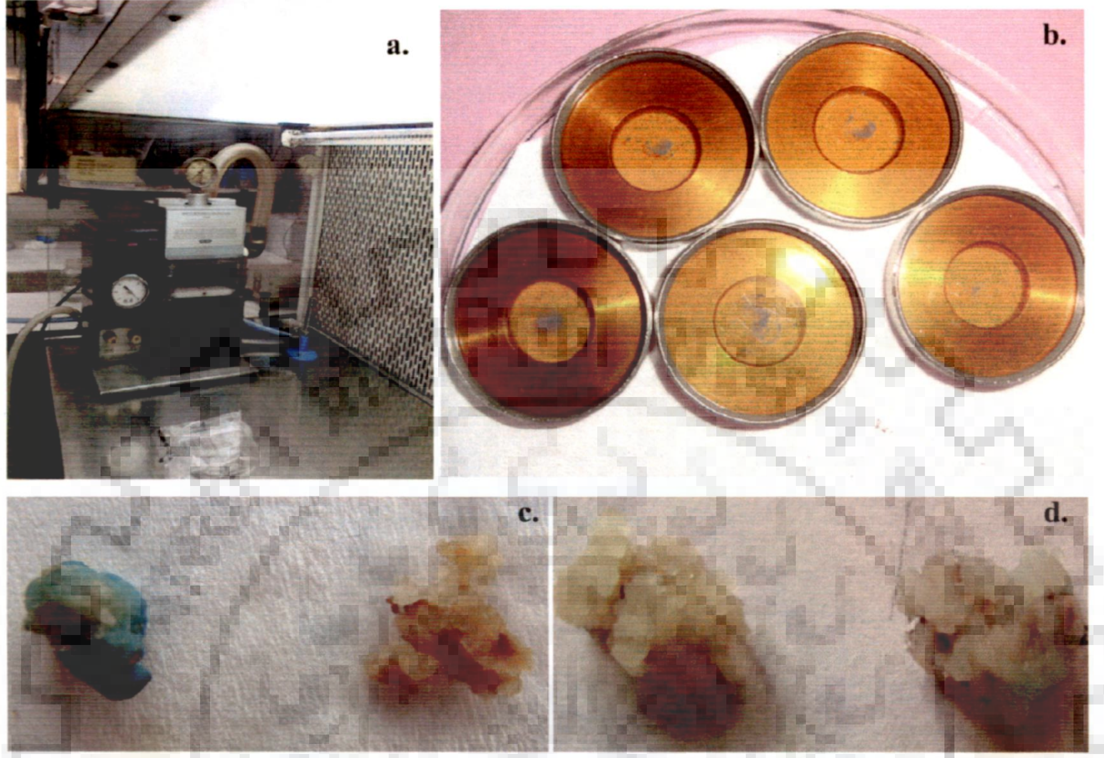


Figure 6.8 *Biolistic transformation* ***a.*** *Particle bombardment gun,* ***b.*** *Tungsten beads coated with plasmid DNA placed on macro-carriers for bombardment,* ***c.*** *GUS assay of the calli soon after transformation, blue coloured callus is transformed while the yellow coloured is untransformed one,* ***d.*** *GUS positive callus after one week, the blue colouration is reduced as there is division in the cells, left one is untransformed callus.*

Chapter VII

CONCLUSION AND FUTURE SCOPE

The present work was carried out on two T-DNA insertional mutants *OsAPC6* and *Ossl* of Basmati rice. Among these, *OsAPC6* was GA insensitive, with reduced height and seed set and high chlorophyll content. The seedling lethal mutant *Ossl* showed 5-15% of albino seedlings during germination. These albino seedlings died soon after germination while among non-albino 10-20% died slowly after transplantation without tillering. Further characterization of these mutants and validation of candidate gene was carried out using microarray, confocal laser scanning microscopy and RNAi.

In the *OsAPC6* mutant, pollen viability and cytological analysis was done and it was found that the male gametophyte development was normal. The female gametophyte development and the embryo sac of rice mutant were studied under the confocal laser scanning microscopy. The CLSM results revealed that the endosperm development was abnormal in the mutant which was attributed to the delay or arrest in cell division during the second or third mitotic division during megagametogenesis. The mutant was found to be homozygous lethal and it was being maintained as heterozygote. The RT-PCR analysis for *APC6* gene was carried out and it was found that the expression of APC6 gene in the mutant was reduced as compared to the wild type.

The transcriptomic profiling of the *OsAPC6* mutant was carried out using the microarray. Around ten genes related to chloroplast precursors were found to be up regulated and the chlorophyll estimation results confirm the presence of high chlorophyll in the mutant leaves. The expression profiling data also showed that many genes related to GA metabolism were up-regulated indicating that the synthesis of GA in the mutant could be normal. The stem elongation assay indicated that mutant is GA insensitive and hence has abnormal GA signaling. Based on this we have speculated a pathway showing role of APC6 in GA signaling. The sensing of GA is reported to be mediated by the GID1 receptor protein, and the complex between GA and GID1 allows the capture of the growth-repressing DELLA protein SLR1 which forms a GA-GID1-SLR1 complex. The SLR1 protein in this tripartite which acts as a repressor is then targeted by SCF^{GID2} ubiquitin complex for proteolytic degradation. In our signaling pathway we have speculated the role of APC6 in GA signaling and its interaction with SLR1 protein for proteolytic degradation. This role of APC6 could be confirmed further by performing western blot using SLR1 antibody and yeast two hybrid assay.

The transcriptomic profiling of the *Ossl* mutant showed down regulation of some chloroplast precursors including lipoxygenase and the dehydrin encoding gene. The hygromycin results and lethality in the mutant seedlings indicated that the mutant could be maintained as heterozygote.

The APC6 protein is reported to be a subunit of anaphase promoting complex which is one of the E3 ligase enzyme. The *in silico* studies revealed that this protein is highly conserved and it has TPR domains which are responsible for protein-protein interaction. Further *in vivo* studies of this protein and its domains may help to unravel the complex interactions of this protein in biological pathways.

The functional validation of *APC6* gene was taken up using RNA interference. The RNAi construct was prepared using the binary vector pANDA and TOPO cloning. The two days old seedlings were infected using *Agrobacterium* carrying transformed plasmid. The transgenics have been prepared till now. In the future further molecular analysis of the transgenics is proposed to be done.

REFERENCES

Achard P, Gusti A, Cheminant S, Alioua M, Dhondt S, Coppens F, Beemster GTS and Genschik P (2009) Gibberellin signaling controls cell proliferation rate in *Arabidopsis*. *Current biology* **19**:1188-1193.

Achard P, Liao L, Jiang C, Desnos T, Bartlett J, Fu X and Harberd NP (2007) DELLAs contribute to plant photomorphogenesis. *Plant Physiology* **143**:1163-1172.

Agrawal N, Dasaradhi PVN, Mohmmed A, Malhotra P, Bhatnagar RK and Mukherjee SK (2003) RNA interference: biology, mechanism, and applications. *Microbiology and Molecular Biology Reviews* **67**:657-685.

Alba R, Fei Z, Payton P, Liu Y, Moore SL, Debbie P, Cohn J, D'Ascenzo M, Gordon JS, Rose JKC, Martin G, Tanksley SD, Bouzayen M, Jahn MM and Giovannoni J (2004) ESTs, cDNA microarrays, and gene expression profiling: tools for dissecting plant physiology and development. *The Plant Journal* **39**:697-714.

Albone KS, Gaskin P, MacMillan J, Phinney BO and Willis CL (1990) Biosynthetic origin of gibberellins A3 and A7 in cell-free preparations from seeds of *Marah macrocarpus* and *Malus domestica*. *Plant Physiology* **94**:132-142.

Aldemita RR and Hodges TK (1996) *Agrobacterium tumefaciens*-mediated transformation of japonica and indica rice varieties. *Planta* **199**:612-617.

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W and Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**:3389-3402.

Alvarez JP, Pekker I, Goldshmidt A, Blum E, Amsellem Z and Eshed Y (2006) Endogenous and synthetic microRNAs stimulate simultaneous, efficient and localized regulation of multiple targets in diverse species. *The Plant Cell* **18**:1134-1151.

Amador V, Monte E, Garcia-Martinez J-L and Prat S (2001) Gibberellins signal nuclear import of PHOR1, a Photoperiod-Responsive protein with homology to *Drosophila* armadillo. *Cell* **106**:343-354.

Amos WB and White JG (2003) How the Confocal Laser Scanning Microscope entered Biological Research. *Biology of the Cell* **95**:335-342.

Ariizumi T, Lawrence PK and Steber CM (2011) The role of two F-Box proteins, SLEEPY1 and SNEEZY, in *Arabidopsis* gibberellin signaling. *Plant Physiology* **155**:765-775.

Arnaud N, Girin T, Sorefan K, Fuentes S, Wood TA, Lawrenson T, Sablowski R and Ostergaard L (2010) Gibberellins control fruit patterning in *Arabidopsis thaliana*. *Genes and Development* **24**:2127-2132.

Ashikari M, Wu J, Yano M, Sasaki T and Yoshimura A (1999) Rice gibberellin-insensitive dwarf mutant gene Dwarf 1 encodes the alpha-subunit of GTP-binding protein. *Proceeding of the National Academy of Sciences, USA* **96**:10284-10289.

Ashraf M, Karim F and Rasul E (2002) Interactive effects of gibberellic acid (GA) and salt stress on growth, ion accumulation and photosynthetic capacity of two spring wheat (*Triticum aestivum L.*) cultivars differing in salt tolerance. *Plant Growth Regulation* **36**:49-59.

Awasthi A, Paul P, Kumar S, Verma SK, Prasad R and Dhaliwal HS (2012) Abnormal endosperm development causes female sterility in rice insertional mutant *OsAPC6*. *Plant Science* **183**:167-174.

Azpiroz-Leehan and Feldman (1997) T-DNA insertion mutagenesis in *Arabidopsis*: going back and forth. *Trends in Genetics* **13**:152-156.

Bai X, Peirson BN, Dong F, Xue C and Makaroff CA (1999) Isolation and characterization of *SYN1*, a *RAD21-like* gene essential for meiosis in *Arabidopsis*. *The Plant Cell* **11**:417-430.

Barrell PJ and Grossniklaus U (2005) Confocal microscopy of whole ovules for analysis of reproductive development: the *elongate1* mutant affects meiosis II. *The Plant Journal* **43**:309-320.

Baxevanis AD and Ouellette BFF (2001) Bioinformatics: A practical guide to the analysis of genes and proteins, *John Wiley and Sons*, New York.

Becich MJ (2000) The Role of the pathologist as tissue refiner and data miner: the impact of functional genomics on the modern pathology laboratory and the critical roles of pathology informatics and bioinformatics. *Molecular Diagnosis* **5**:287-299.

Bemer M, Wolters-Arts M, Grossniklaus U and Angenent GC (2008) The MADS domain protein DIANA acts together with AGAMOUS-LIKE80 to

specify the central cell in *Arabidopsis* ovules. *The Plant Cell* **20**:2088-2101.

Benková E and Hejátko J (2009) Hormone interactions at the root apical meristem. *Plant Molecular Biology* **69**:383-396.

Bevan M, Barnes WM and Chilton MD (1983) Structure and transcription of the nopaline synthase gene region of T-DNA. *Nucleic Acids Research* **11**:369–385.

Bhatt AM, Lister C, Page T, Fransz P, Findlay K, Jones GH, Dickinson HG and Dean C (1999) The *DIF1* gene of *Arabidopsis* is required for meiotic chromosome segregation and belongs to the REC8/RAD21 cohesin gene family. *The Plant Journal* **19**:463-472.

Blatch GL and Lassle M (1999) The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. *Bioessays* **21**:932-939.

Blazquez MA, Soowal LN, Lee I and Weigel D (1997) LEAFY expression and flower initiation in *Arabidopsis*. *Development* **124**:3835-3844.

Blazquez MA and Weigel D (2000) Integration of floral inductive signals in *Arabidopsis*. *Nature* **404**:889-892.

Blilou I, Frugier F, Folmer S, Serralbo O, Willemsen V, Wolkenfelt H, Eloy NbB, Ferreira PCG, Weisbeek P and Scheres B (2002) The *Arabidopsis HOBBIT* gene encodes a *CDC27* homolog that links the plant cell cycle to progression of cell differentiation. *Genes and Development* **16**:2566-2575.

Bouchez D and Hofte H (1998) Functional Genomics in Plants. *Plant Physiology* **118**:725-732.

Brady SM, Long TA and Benfey PN (2006) Unraveling the Dynamic Transcriptome. *The Plant Cell* **18**:2101-2111.

Breitler JC, Labeyrie A, Meynard D, Legavre T and Guiderdoni E (2002) Efficient microprojectile bombardment-mediated transformation of rice using gene cassettes. *Theoretical and Applied Genetics* **104**:709-719.

Breyne P and Zabeau M (2001) Genome-wide expression analysis of plant cell cycle modulated genes. *Current Opinion in Plant Biology* **4**:136-142.

Brown PO and Botstein D (1999) Exploring the new world of the genome with DNA microarrays. *Nature Genetics* **21**:33-37.

Burton JL and Solomon MJ (2000) Hsl1p, a Swe1p inhibitor, is degraded via the anaphase-promoting complex. *Molecular and Cellular Biology* **20**:4614-4625.

Buttery RG, Ling LC, Juliano BO and Turnbaugh JG (1983) Cooked rice aroma and 2-acetyl-1-pyrroline. *Journal of Agricultural and Food Chemistry* **31**:823-826.

Cao J, Duan X, McEiroy D and Wu R (1992) Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. *Plant Cell Reports* **11**:586-591.

Capron A, Okresz L and Genschik P (2003a) First glance at the plant APC/C, a highly conserved ubiquitin-protein ligase. *Trends in Plant Science* **8**:83-89.

Capron A, Serralbo O, Fulop K, Frugier F, Parmentier Y, Dong A, Lecureuil A, Guerche P, Kondorosi E, Scheres B and Genschik P (2003b) The *Arabidopsis* anaphase-promoting complex or cyclosome: molecular and genetic characterization of the *APC2* subunit. *The Plant Cell* **15**:2370-2382.

Carroll CW and Morgan DO (2002) The Doc1 subunit is a processivity factor for the anaphase-promoting complex. *Nature Cell Biology* **4**:880-887.

Cass DD, Peteya DJ and Robertson BL (1985) Megagametophyte development in *Hordeum vulgare* early megagametogenesis and the nature of cell wall formation. *Canadian Journal of Botany* **63**:2164-2171.

Causse MA, Fulton TM, Cho YG, Ahn SN, Chunwongse J, Wu K, Xiao J, Yu Z, Ronald PC, Harrington SE, Second G, McCouch SR and Tanksley SD (1994) Saturated Molecular Map of the Rice Genome Based on an Interspecific Backcross Population. *Genetics* **138**:1251-1274.

Cebolla A, Maria Vinardell J, Kiss E, Olah B, Roudier F, Kondorosi A and Kondorosi E (1999) The mitotic inhibitor *ccs52* is required for endoreduplication and ploidy-dependent cell enlargement in plants. *EMBO Journal* **18**:4476-4484.

Chandler PM, Marion-Poll A, Ellis M and Gubler F (2002) Mutants at the Slender1 locus of barley cv. Himalaya molecular and physiological characterization. *Plant Physiology* **129**:181-190.

Chang HH and Chan MT (1991) *Agrobacterium tumefaciens*-mediated transformation of soybean (*Glycine max* (L.) Merr.) is promoted by the inclusion of potato suspension culture, *Botanical Bulletin of Academia Sinica* **32**:171-178.

Cheadle C, Fan J, Cho-Chung YS, Werner T, Ray J, Do L, Gorospe M and Becker KG (2005) Stability regulation of mRNA and the control of gene expression. *Annals of the New York Academy of Sciences* **1058**:196-204.

Chen L, Zhang S, Beachy RN and Fauquet CM (1998) A protocol for consistent, large-scale production of fertile transgenic rice plants. *Plant Cell Reports* **18**:25-31.

Chen Y, Cheng G and Mahato R (2008) RNAi for treating hepatitis B viral infection. *Pharmaceutical Research* **25**:72-86.

Chien JC and Sussex IM (1996) Differential regulation of trichome formation on the adaxial and abaxial leaf surfaces by gibberellins and photoperiod in *Arabidopsis thaliana* (L.) Heynh. *Plant Physiology* **111**:1321-1328.

Chin HG, Choe MS, Lee S-H, Park SH, Park SH, Koo JC, Kim NY, Lee JJ, Oh BG, Yi GH, Kim SC, Choi HC, Cho MJ and Han Cd (1999) Molecular analysis of rice plants harboring an Ac/Ds transposable element-mediated gene trapping system. *The Plant Journal* **19**:615-623.

Cho HT and Kende H (1997) Expression of *expansin* genes is correlated with growth in deep water rice. *The Plant Cell* **9**:1661-1671.

Christensen CA, King EJ, Jordan JR and Drews GN (1997) Megagametogenesis in *Arabidopsis* wild type and the *Gf* mutant. *Sexual Plant Reproduction* **10**:49-64.

Christensen CA, Subramanian S and Drews GN (1998) Identification of gametophytic mutations affecting female gametophyte development in *Arabidopsis*. *Developmental Biology* **202**:136-151.

Christou P, Ford TL and Kofron M (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important *indica* and *japonica* varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Nature Biotechnology* **9**:957-962.

Christou P (1993) Particle gun mediated transformation. *Current Opinion in Biotechnology* **4**:135-141.

Chuang CF and Meyerowitz EM (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana. Proceedings of the National Academy of Sciences, USA* **97**:4985-4990.

CohenFix O, Peters JM, Kirschner MW and Koshland D (1996) Anaphase initiation in *Saccharomyces cerevisiae* is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes and Development* **10**:3081-3093.

Colovos C and Yeates TO (1993) Verification of protein structures: Patterns of nonbonded atomic interactions. *Protein Science* **2**:1511-1519.

Conley EJ, Nduati V, Gonzalez-Hernandez JL, Mesfin A, Trudeau-Spanjers M, Chao S, Lazo GR, Hummel DD, Anderson OD, Qi LL, Gill BS, Echalier B, Linkiewicz AM, Dubcovský J, Akhunov ED, Dvorak J, Peng JH, Lapitan NLV, Pathan MS, Nguyen HT, Ma XF, Miftahudin, Gustafson JP, Greene RA, Sorrells ME, Hossain KG, Kalavacharla V, Kianian SF, Sidhu D, Dilbirligi M, Gill KS, Choi DW, Fenton RD, Close TJ, McGuire PE, Qualset CO and Anderson JA (2004) A 2600-locus chromosome bin map of wheat homoeologous group 2 reveals interstitial gene-rich islands and colinearity with rice. *Genetics* **168**:625-637.

Coux O, Tanaka K and Goldberg AL (1996) Structure and functions of the 20S and 26S proteasomes. *Annual Review of Biochemistry* **65**:801-847.

Cove DJ and Knight CD (1993) The moss *Physcomitrella patens*, a model system with potential for the study of plant reproduction. *The Plant Cell* **4**:1483-1488.

Dai Xi-mei, Qun-ce H, Qiu-xia L and Guang-yong Q (2009) Observation on the double fertilization and early stage development of embryo in autotetraploid polyembryonic rice. *Rice Science* **16**:124–130.

Das AK, Cohen PTW and Barford D (1998) The structure of the tetratricopeptide repeats of protein phosphatase 5: implications for TPR-mediated protein-protein interactions. *EMBO Journal* **17**:1192-1199.

Datanet (2011) www.indiastat.com.

Davière JM, de Lucas M and Prat S (2008) Transcriptional factor interaction: a central step in DELLA function. *Current Opinion in Genetics and Development* **18**:295-303.

Deak P, Pal M, Nagy O, Menesi D and Udvardy A (2007) Structurally related TPR subunits contribute differently to the function of the anaphase-promoting complex in Drosophila melanogaster. *Journal of Cell Science* **120**:3238-3248.

DeLano WL (2002) The PyMOL Molecular Graphics System. DeLano Scientific, San Carlos, CA, USA. http://www.pymol.org.

Dellaporta SL and Calderon-Urrea A (1993) Sex determination in flowering plants. *The Plant Cell* **5**:1241-1251.

Deng Y, Bennink JR, Kang HC, Haugland RP and Yewdell JW (1995) Fluorescent conjugates of Brefeldin, a selectively stain the endoplasmic reticulum and golgi complex of living cells. *Journal of Histochemistry and Cytochemistry* **43**:907-915.

Deshaies RJ (1999) SCF and Cullin/RING H_2-based ubiquitin ligases. *Annual Review of Cell and Developmental Biology* **15**:435-467.

Dhaliwal HS, Das A, Singh A and Gupta VK (2001) Isolation of insertional mutants in indica rice using Ds transposable element of maize. *Rice Genetics Newsletter* **18**:98-99.

Di Serio F, Schöb H, Iglesias A, Tarina C, Bouldoires E and Meins F (2001) Sense- and antisense-mediated gene silencing in tobacco is inhibited by the same viral suppressors and is associated with accumulation of small RNAs. *Proceedings of the National Academy of Sciences, USA* **98**:6506-6510.

Diaz-Munoz M, de la Rosa Santander P, Juarez-Espinosa A, Arellano R and Morales-Tlalpan V (2008) Granulosa cells express three inositol 1,4,5-trisphosphate receptor isoforms: cytoplasmic and nuclear Ca^{2+} mobilization. *Reproductive Biology and Endocrinology* **6**:60.

Diehn M and Relman DA (2001) Comparing functional genomic datasets: lessons from DNA microarray analyses of host and pathogen interactions. *Current Opinion in Microbiology* **4**:95-101.

Dill A and Sun TP (2001) Synergistic derepression of gibberellin signaling by removing RGA and GAI function in *Arabidopsis thaliana. Genetics* **159**:777-785.

Dill A, Thomas SG, Hu J, Steber CM and Sun TP (2004) The *Arabidopsis* F-Box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation. *The Plant Cell* **16**:1392-1405.

Doherty FJ, Dawson S and Mayer RJ (2002) The ubiquitin proteasome pathway of intracellular proteolysis. *Essays in Biochemistry* **38**:51-63.

Drews GN, Lee D and Christensen CA (1998) Genetic analysis of female gametophyte development and function. *The Plant Cell* **10**:5-18.

Duan X, Li X, Xue Q, Abo-Ei-Saad M, Xu D and Wu R (1996) Transgenic rice plants harboring an introduced potato proteinase inhibitor II gene are insect resistant. *Nature Biotechnology* **14**:494-498.

Duggan DJ, Bittner M, Chen Y, Meltzer P and Trent JM (1999) Expression profiling using cDNA microarrays. *Nature Genetics* **21**:10-14.

Ellis MH, Rebetzke GJ, Azanza F, Richards RA and Spielmeyer W (2005) Molecular mapping of gibberellin-responsive dwarfing genes in bread wheat. *Theoretical and Applied Genetics* **111**:423-430.

Eloy NB, Coppens F, Beemster GT Hemerly AS, Ferreira PCG (2006) The *Arabidopsis* anaphase promoting complex (APC): Regulation through subunit availability in plant tissues. *Cell Cycle* **5**:1957-1965.

Evans T, Rosenthal ET, Youngblom J, Distel D and Hunt T (1983) Cyclin: A protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* **33**:389-396.

Fang G, Yu H and Kirschner MW (1998) The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. *Genes and Development* **12**:1871-1883.

FAO (2011) http://www.fao.org/economic/est/publications/rice-publications/rice-market-monitor-rmm/en/.

Fedoroff NV, Furtek DB and Nelson OE (1984) Cloning of the bronze locus in maize by a simple and generalizable procedure using the transposable controlling element Activator (Ac). *Proceedings of the National Academy of Sciences, USA* **81**:3825-3829.

Filleur S, Dorbe M-F, Cerezo M, Orsel M, Granier F, Gojon A and Daniel-Vedele F (2001) An *Arabidopsis* T-DNA mutant affected in *Nrt2* genes is impaired in nitrate uptake. *FEBS Letters* **489**:220-224.

Finer JJ and MCmullen MD (1990) Transformation of cotton (*Gossypium hirsuram* L.) via particle bombardment. *Plant Cell Reproduction* **8**:586-589.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE and Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**:806-811.

Fleck B and Harberd NP (2002) Evidence that the *Arabidopsis* nuclear gibberellin signaling protein GAI is not destabilized by gibberellin. *The Plant Journal* **32**:935-947.

Fowler S and Thomashow MF (2002) *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *The Plant Cell* **14**:1675-1690.

Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS, Galluppi GR, Goldberg SB, Hoffmann NL and Woo SC (1983) Expression of bacterial genes in plant cells. *Proceedings of the National Academy of Sciences, USA* **80**:4803-4807.

Freemont PS (2000) Ubiquitination: RING for destruction? *Current Biology* **10**:R84–R87.

Fridborg I, Kuusk S, Moritz T and Sundberg E (1999) The *Arabidopsis* dwarf mutant *shi* exhibits reduced gibberellin responses conferred by overexpression of a new putative zinc finger protein. *The Plant Cell* **11**:1019-1031.

Fridborg I, Kuusk S, Robertson M and Sundberg E (2001) The *Arabidopsis* protein SHI represses gibberellin responses in *Arabidopsis* and Barley. *Plant Physiology* **127**:937-948.

Fu X, Fu N, Guo S, Yan Z, Xu Y, Hu H, Menzel C, Chen W, Li Y, Zeng R and Khaitovich P (2009) Estimating accuracy of RNA-Seq and microarrays with proteomics. *BMC Genomics* **10**:161.

Fu X and Harberd NP (2003) Auxin promotes *Arabidopsis* root growth by modulating gibberellin response. *Nature* **421**:740-743.

Fu X, Richards DE, Ait-ali T, Hynes LW, Ougham H, Peng J and Harberd NP (2002) Gibberellin-Mediated Proteasome-Dependent Degradation of the Barley DELLA Protein SLN1 Repressor. *The Plant Cell* **14**:3191-3200.

Fu X, Richards DE, Fleck B, Xie D, Burton N and Harberd NP (2004) The *Arabidopsis* Mutant sleepy1gar2-1 Protein Promotes Plant Growth by Increasing the Affinity of the SCFSLY1 E3 Ubiquitin Ligase for DELLA Protein Substrates. *The Plant Cell* **16**:1406-1418.

Fujisawa Y, Kato T, Ohki S, Ishikawa A, Kitano H, Sasaki T, Asahi T and Iwasaki Y (1999) Suppression of the heterotrimeric G protein causes abnormal morphology, including dwarfism, in rice. *Proceedings of the National Academy of Sciences, USA* **96**:7575-7580.

Funabiki H and Murray AW (2000) The Xenopus chromokinesin Xkid is essential for metaphase chromosome alignment and must be degraded to allow anaphase chromosome movement. *Cell* **102**:411-424.

Fusaro AF, Matthew L, Smith NA, Curtin SJ, Dedic-Hagan J, Ellacott GA, Watson JM, Wang MB, Brosnan C, Carroll BJ and Waterhouse PM (2006) RNA interference-inducing hairpin RNAs in plants act through the viral defence pathway. *EMBO Reports* 7:1168-1175.

Gale MD and Devos KM (1998) Comparative genetics in the grasses. *Proceedings of the National Academy of Sciences, USA* **95**:1971-1974.

Gale MD and Youssefian S (1985.) Dwarfing genes in wheat. In Progress in Plant Breeding,. *Russell G E (ed), Butterworths, London,*: 1-35.

Gao XH, Huang XZ, Xiao SL and Fu XD (2008) Evolutionarily conserved DELLA-mediated gibberellin signaling in plants. *Journal of Integrative Plant Biology* **50**:825-834.

Gao XH, Xiao SL, Yao QF, J. WY and Fu D (2011) An updated GA signaling 'Relief of Repression' regulatory mode. *Molecular Plant,* doi: 10.1093/mp/ssr046.

Gieffers C, Dube P, Harris JR, Stark H and Peters JM (2001) Three-dimensional structure of the anaphase-promoting complex. *Molecular Cell* **7**:907-913.

Gieffers C, Schleiffer A and Peters JM (2000) Cullins and cell cycle control. *Protoplasma* **211**:20-28.

Gilchrist E and Haughn G (2010) Reverse genetics techniques: engineering loss and gain of gene function in plants. *Briefings in Functional Genomics* **9**:103-110.

Gill BS, Appels R, Botha-Oberholster A-M, Buell CR, Bennetzen JL, Chalhoub B, Chumley F, Dvorak J, Iwanaga M, Keller B, Li W, McCombie WR, Ogihara Y, Quetier F and Sasaki T (2004) A workshop report on wheat genome sequencing: international genome research on wheat consortium. *Genetics* **168**:1087-1096.

Gill RT, Katsoulakis E, Schmitt W, Taroncher-Oldenburg G, Misra J and Stephanopoulos G (2002) Genome-wide dynamic transcriptional profiling of the light-to-dark transition in synechocystis sp. strain PCC 6803. *The Journal of Bacteriology* **184**:3671-3681.

Gmachl M, Gieffers C, Podtelejnikov AV, Mann M and Peters JM (2000) The RING-H2 finger protein APC11 and the E2 enzyme UBC4 are sufficient to ubiquitinate substrates of the anaphase-promoting complex. *Proceedings of the National Academy of Sciences, USA* **97**:8973-8978.

Gocal GFW, Sheldon CC, Gubler F, Moritz T, Bagnall DJ, MacMillan CP, Li SF, Parish RW, Dennis ES, Weigel D and King RW (2001) GAMYB-like genes, flowering, and gibberellin signaling in Arabidopsis. *Plant Physiology* **127**:1682-1693.

Goff SA (1999) Rice as a model for cereal genomics. *Current Opinion in Plant Biology* **2**:86-89.

Goff SA, Ricke D, Lan T-H, Presting G, Wang R, Dunn M, Glazebrook J, Sessions A, Oeller P, Varma H, Hadley D, Hutchison D, Martin C, Katagiri F, Lange BM, Moughamer T, Xia Y, Budworth P, Zhong J, Miguel T, Paszkowski U, Zhang S, Colbert M, Sun W-l, Chen L, Cooper B, Park S, Wood TC, Mao L, Quail P, Wing R, Dean R, Yu Y, Zharkikh A, Shen R, Sahasrabudhe S, Thomas A, Cannings R, Gutin A, Pruss D, Reid J, Tavtigian S, Mitchell J, Eldredge G, Scholl T, Miller RM, Bhatnagar S, Adey N, Rubano T, Tusneem N, Robinson R, Feldhaus

J, Macalma T, Oliphant A and Briggs S (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). *Science* **296**:92-100.

Gordon-Kamm WJ, Spencer TM, Mangano. ML, Adams TR and Daines RJ (1990) Transformation of maize cells and regeneration of fertile transgenic plants. *Plant Cell* **2**:603-618.

Gouet P, Courcelle E, Stuart DI and Metoz F (1999) ESPript: analysis of multiple sequence alignments in PostScript. *Bioinformatics* **15**:305-308.

Gould J, Devey M, Hasegawa M, Ulian EC, Peterson G and Smith RH (1991) Transformation of *Zea mays* L. using *Agrobacterium tumefaciens* and the shoot apex. *Plant Physiology* **95**.

Gray D and Finer J (1993) Development and operation of five particle guns for introduction of DNA into plant cells. *Plant Cell, Tissue and Organ Culture* **33**:219-219.

Gray WM, Muskett PR, Chuang H-w and Parker JE (2003) *Arabidopsis* SGT1b is required for SCFTIR1-mediated auxin response. *The Plant Cell* **15**:1310-1319.

Greenboim-Wainberg Y, Maymon I, Borochov R, Alvarez J, Olszewski N, Ori N, Eshed Y and Weiss D (2005) Cross talk between gibberellin and cytokinin: the *Arabidopsis* GA response inhibitor SPINDLY plays a positive role in cytokinin signaling. *The Plant Cell* **17**:92-102.

Griffiths J, Murase K, Rieu I, Zentella R, Zhang ZL, Powers SJ, Gong F, Phillips AL, Hedden P, Sun TP and Thomas SG (2006) Genetic characterization and functional analysis of the GID1 gibberellin receptors in *Arabidopsis*. *The Plant Cell* **18**:3399-3414.

Groll M and Huber R (2003) Substrate access and processing by the 20S proteasome core particle. *The International Journal of Biochemistry and Cell Biology* **35**:606-616.

Gubler F, Chandler PM, White RG, Llewellyn DJ and Jacobsen JV (2002) Gibberellin signaling in barley aleurone cells. Control of SLN1 and GAMYB expression. *Plant Physiology* **129**:191-200.

Hacia JG (1999) Resequencing and mutational analysis using oligonucleotide microarrays. *Nature Genetics* **21**:42-47.

Hamada, S, Ishiyama K, Salkulsingharoj C, Choi S-B, Wu Y, Hwang C, Singh S, Kawai N, Messing J and Okita TW (2003) Multiple RNA transport pathways to the cortical region in developing rice endosperm. *The Plant Cell* **15**:2265-2272.

Hames RS, Wattam SL, Yamano H, Bacchieri R and Fry AM (2001) APC/C-mediated destruction of the centrosomal kinase Nek2A occurs in early mitosis and depends upon a cyclin A-type D-box. *EMBO Journal* **20**:7117-7127.

Han MJ, Jung KH, Yi G, Lee DY and An G (2006) Rice immature pollen 1 (RIP1) is a regulator of late pollen development. *Plant and Cell Physiology* **47**:1457-1472.

Harper JW (2002) A phosphorylation-driven ubiquitination switch for cell-cycle control. *Trends in Cell Biology* **12**:104-107.

Harper JW, Burton JL and Solomon MJ (2002) The anaphase-promoting complex: it's not just for mitosis any more. *Genes and Development* **16**:2179-2206.

Hartweck LM and Olszewski NE (2006) Rice GIBBERELLIN INSENSITIVE DWARF1 is a gibberellin receptor that illuminates and raises questions about ga signaling. *The Plant Cell* **18**:278-282.

Harushima Y, Yano M, Shomura A, Sato M, Shimano T, Kuboki Y, Yamamoto T, Lin SY, Antonio BA, Parco A, Kajiya H, Huang N, Yamamoto K, Nagamura Y, Kurata N, Khush GS and Sasaki T (1998) A high-density rice genetic linkage map with 2275 markers using a single F2 population. *Genetics* **148**:479-494.

Hayashi H, Czaja I, Lubenow H, Schell J and Walden R (1992) Activation of a plant gene by T-DNA tagging: auxin-independent growth in vitro. *Science* **258**:1350-1353.

Hedden P, Kamiya Y (1997) Gibberellin biosynthesis: enzymes, genes and their regulation. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**:431–460.

Hedden P and Phillips AL (2000) Gibberellin metabolism: new insights revealed by the genes. *Trends in Plant Science* **5**:523-530.

Hejátko J, Pernisová M, Eneva T, Palme K and Brzobohatý B (2003) The putative sensor histidine kinase CKI1 is involved in female gametophyte development in *Arabidopsis*. *Molecular Genetics and Genomics* **269**:443-453.

Hellmann H and Estelle M (2002) Plant development: regulation by protein degradation. *Science* **297**:793-797.

Hershko A and Ciechanover A (1998) The Ubiquitin System. *Annual Review of Biochemistry* **67**:425-479.

Herskowitz I (1987) Functional inactivation of genes by dominant negative mutations. *Nature* **329**:219-222.

Hess B, Kutzner C, van der Spoel D and Lindahl E (2008) GROMACS 4: Algorithms for highly efficient, load-balanced, and scalable molecular simulation. *Journal of Chemical Theory and Computation* **4**:435-447.

Hibbs AR (2004) *Confocal Microscopy for Biologists*. New York: Kluwer Academic/Plenum Publishers.

Hiei Y, Ohta S, Komari T and Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *The Plant Journal* **6**:271-282.

Hieter P and Boguski M (1997) Functional Genomics: It's all how you read it. *Science* **278**:601-602.

Hirano K, Asano K, Tsuji H, Kawamura M, Mori H, Kitano H, Ueguchi-Tanaka M and Matsuoka M (2010) Characterization of the molecular mechanism underlying gibberellin perception complex formation in rice. *The Plant Cell* **22**:2680-2696.

Howard E and Citovsky V (1990) The emerging structure of the *Agrobacterium* T-DNA transfer complex. *Bioessays* **12**:103-108.

Hou X, Lee LYC, Xia K, Yan Y and Yu H (2010) DELLAs modulate jasmonate signaling via competitive binding to JAZs. *Developmental Cell* **19**:884-894.

Hsing YI, Chern CG, Fan MJ, Lu PC, Chen KT, Lo SF, Sun PK, Ho SL, Lee KW, Wang YC, Huang WL, Ko SS, Chen S, Chen JL, Chung CI, Lin YC, Hour AL, Wang YW, Chang YC, Tsai MW, Lin YS, Chen YC, Yen HM, Li CP, Wey CK, Tseng CS, Lai MH, Huang SC, Chen LJ and Yu

SM (2007) A rice gene activation/knockout mutant resource for high throughput functional genomics. *Plant Molecular Biology* **63**:351-364.

Hu C, Zeng Y, Guo H, Lu Y, Chen Z, Shahid MQ and Liu X (2010) Megasporogenesis and megagametogenesis in autotetraploid *indica/japonica* rice hybrid. *Rice Science* **17**:296-302.

Hui EKW, Wang PC and Lo SJ (1998) Strategies for cloning unknown cellular flanking DNA sequences from foreign integrants. *Cellular and Molecular Life Sciences* **54**:1403-1411.

Hussain A and Peng J (2003) DELLA proteins and GA signalling in *Arabidopsis*. *Journal of Plant Growth Regulation* **22**:134-140.

Hwang LH, Lau LF, Smith DL, Mistrot CA, Hardwick KG, Hwang ES, Amon A and Murray AW (1998) Budding Yeast Cdc20: A target of the spindle checkpoint. *Science* **279**:1041-1044.

Ikeda A, Ueguchi-Tanaka M, Sonoda Y, Kitano H, Koshioka M, Futsuhara Y, Matsuoka M and Yamaguchi J (2001) slender rice, a constitutive gibberellin response mutant, is caused by a null mutation of the SLR1 gene, an ortholog of the height-regulating gene GAI/RGA/RHT/D8. *The Plant Cell* **13**:999-1010.

IRGSP (2005) The map-based sequence of the rice genome. *Nature* **436**:793-800.

Irniger S, Piatti S, Michaelis C and Nasmyth K (1995) Genes involved in sister-chromatid separation are needed for B-type cyclin proteolysis in budding yeast. *Cell* **81**:269-278.

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T and Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nature Biotechnology* **14**:745-750.

Isshiki M, Yamamoto Y, Satoh H and Shimamoto K (2001) Nonsense-mediated decay of mutant waxy mRNA in rice. *Plant Physiology* **125**:1388-1395.

Itoh H, Ueguchi-Tanaka M, Sato Y, Ashikari M and Matsuoka M (2002) The gibberellin signaling pathway is regulated by the appearance and disappearance of SLENDER RICE1 in nuclei. *The Plant Cell* **14**:57-70.

Itoh H, Ueguchi-Tanaka M, Sentoku N, Kitano H, Matsuoka M and Kobayashi M (2001) Cloning and functional analysis of two gibberellin 3 beta-

hydroxylase genes that are differently expressed during the growth of rice. *Proceedings of the National Academy of Sciences, USA* **98**:8909-8914.

Itoh H, Ueguchi Tanaka M, Matsuoka M and Kwang WJ (2008) Molecular biology of gibberellins signaling in higher plants, *International Review of Cell and Molecular Biology*: 191-221.

Ivandic V, Malyshev S, Korzun V, Graner A and Borner A (1999) Comparative mapping of a gibberellic acid-insensitive dwarfing gene (Dwf2) on chromosome 4HS in barley. *Theoretical and Applied Genetics* **98**:728-731.

Izawa (1996) Becoming a model plant: the importance of rice to plant science. *Trends in Plant Science* **1**:95-99.

Izhaki A, Swain SM, Tseng TS, Borochov A, Olszewski NE and Weiss D (2001) The role of SPY and its TPR domain in the regulation of gibberellin action throughout the life cycle of *Petunia hybrida* plants. *The Plant Journal* **28**:181-190.

Jacobsen SE, Binkowski KA and Olszewski NE (1996a) SPINDLY, a tetratricopeptide repeat protein involved in gibberellin signal transduction *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* **93**:9292-9296.

Jacobsen SE and Olszewski NE (1993) Mutations at the SPINDLY locus of *Arabidopsis* alter gibberellin signal transduction. *The Plant Cell* 5:887-896.

Jeganathan KB, Baker DJ and Deursen JMv (2006) Securin associates with apccdh1 in prometaphase but its destruction is delayed by Rae1 and Nup98 until the metaphase/anaphase transition. *Cell Cycle* **5**:366-370.

Jeganathan KB, Malureanu L and van Deursen JM (2005) The Rae1-Nup98 complex prevents aneuploidy by inhibiting securin degradation. *Nature* **438**:1036-1039.

Jensen S, Gassama M-P and Heidmann T (1999) Taming of transposable elements by homology-dependent gene silencing. *Nature Genetics* **21**:209-212.

Jiang C and Fu X (2007) GA action: turning on the DELLA repressing signaling. *Current Opinion in Plant Biology* **10**:461-465.

Jiao Y, Jia P, Wang X, Su N, Yu S, Zhang D, Ma L, Feng Q, Jin Z, Li L, Xue Y, Cheng Z, Zhao H, Han B and Deng XW (2005) A tiling microarray expression analysis of rice chromosome 4 suggests a chromosome-level regulation of transcription. *The Plant Cell* **17**:1641-1657.

Juang YL, Huang J, Peters JM, McLaughlin ME, Tai CY and Pellman D (1997) APC-mediated proteolysis of Ase 1 and the morphogenesis of the mitotic spindle. *Science* **275**:1311-1314.

Jung KH, An G and Ronald PC (2008) Towards a better bowl of rice: assigning function to tens of thousands of rice genes. *Nature Reviews Genetics* **9**:91-101.

Kahn E, Baarine M, Pelloux S, Riedinger JM, Frouin F, Tourneur Y and Lizard G (2010) Iron nanoparticles increase 7-ketocholesterolinduced cell death, inflammation, and oxidation on murine cardiac HL1-NB cells. *International Journal of Nanomedicine* **5**:185-195.

Kalavacharla V, Hossain K, Gu Y, Riera-Lizarazu O, Vales MI, Bhamidimarri S, Gonzalez-Hernandez JL, Maan SS and Kianian SF (2006) High-resolution radiation hybrid map of wheat chromosome 1D. *Genetics* **173**:1089-1099.

Kennerdell JR and Carthew RW (2000) Heritable gene silencing in *Drosophila* using double-stranded RNA. *Nature Biotechnology* **18**:896-898.

Khan J, Saal LH, Bittner ML, Chen Y, Trent JM and Meltzer PS (1999) Expression profiling in cancer using cDNA microarrays. *Electrophoresis* **20**:223-229.

Khan N (1996) Effect of gibberellic acid on carbonic anhydrase, photosynthesis, growth and yield of mustard. *Biologia Plantarum* **38**:145-147.

Khan NA, Iqbal N, Nazar R, Khan MIR and Masood A (2011) Role of gibberellins in regulation of source-sink relations under optimal and limiting environmental conditions. *Current Science* **100**:998-1007.

Khan NA, Mir R, Khan M, Javid S and Samiullah (2002) Effects of gibberellic acid spray on nitrogen yield efficiency of mustard grown with different nitrogen levels. *Plant Growth Regulation* **38**:243-247.

Khush GS (1997) Origin, dispersal, cultivation and variation of rice. *Plant Molecular Biology* **35**:25-34.

Khvorova A, Reynolds A and Jayasena SD (2003) Functional siRNAs and miRNAs exhibit strand bias. *Cell* **115**:209-216.

Kikuchi S, Satoh K, Nagata T, Kawagashira N, Doi K, Kishimoto N, Yazaki J, Ishikawa M, Yamada H, Ooka H, Hotta I, Kojima K, Namiki T, Ohneda E, Yahagi W, Suzuki K, Li CJ, Ohtsuki K, Shishiki T, Otomo Y, Murakami K, Iida Y, Sugano S, Fujimura T, Suzuki Y, Tsunoda Y, Kurosaki T, Kodama T, Masuda H, Kobayashi M, Xie Q, Lu M, Narikawa R, Sugiyama A, Mizuno K, Yokomizo S, Niikura J, Ikeda R, Ishibiki J, Kawamata M, Yoshimura A, Miura J, Kusumegi T, Oka M, Ryu R, Ueda M, Matsubara K, Riken, Kawai J, Carninci P, Adachi J, Aizawa K, Arakawa T, Fukuda S, Hara A, Hashidume W, Hayatsu N, Imotani K, Ishii Y, Itoh M, Kagawa I, Kondo S, Konno H, Miyazaki A, Osato N, Ota Y, Saito R, Sasaki D, Sato K, Shibata K, Shinagawa A, Shiraki T, Yoshino M and Hayashizaki Y (2003) Collection, mapping, and annotation of over 28,000 cDNA clones from *japonica* rice. *Science* **301**:376-379.

King RW, Deshaies RJ, Peters J-M and Kirschner MW (1996) How proteolysis drives the cell cycle. *Science* **274**:1652-1659.

King RW, Peters JM, Tugendreich S, Rolfe M, Hieter P and Kirschner MW (1995) A 20s complex containing Cdc27 and Cdc16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin-B. *Cell* **81**:279-288.

Klein TM, Wolf ED, Wu R and Sanford JC (1987) High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature* **327**:70-73.

Kojima M, Shioiri H, Nogawa M, Nozue M, Matsumoto D, Wada A, Saiki Y, Kiguchi K (2004) *In planta* transformation of kenaf plants (*Hibiscus cannabinus* var. aokawa no.3) by *Agrobacterium tumefaciens*, *Journal of Biosciences and Bioengineering* **98**: 136-139.

Kojima M, Arai Y, Iwase N, Shiratori K, Shioiri H, Nozue M (2000) Development of a simple and efficient method for transformation of buckwheat plants (*Fugopyrum esculentum*) using *Agrobacterium tumefaciens*, *Biosciences, Biotechnology and Biochemistry* **64**: 845-847.

Koorneef M, Elgersma A, Hanhart CJ, van Loenen-Martinet EP, van Rijn L and Zeevaart JAD (1985) A gibberellin insensitive mutant of *Arabidopsis thaliana*. *Physiologia Plantarum* **65**:33-39.

Koornneeff M, Dellaert LWM and van der Veen JH (1982) EMS and relation-induced mutation frequencies at individual loci in *Arabidopsis thaliana* (L.) Heynh. *Mutation Research* **93**:109-123.

Koziel MG, Beland GL, Bowman C, Carozzi NB, Crenshaw R, Crossland L, Dawson J, Desai N, Hill M, Kadwell S, Launis K, Lewis K, Maddox D, McPherson K, Meghji MR, Merlin E, Rhodes R, Warren GW, Wright M and Evola SV (1993) Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. *Nature Biotechnology* **11**:194-200.

Kramer ER, Scheuringer N, Podtelejnikov AV, Mann M and Peters JM (2000) Mitotic regulation of the APC activator proteins CDC20 and CDH1. *Molecular Biology of the Cell* 11:1555-1569.

Krishnan A, Guiderdoni E, An G, Hsing YC, Han C-d, Lee MC, Yu S-M, Upadhyaya N, Ramachandran S, Zhang Q, Sundaresan V, Hirochika H, Leung H and Pereira A (2009) Mutant resources in rice for functional genomics of the grasses. *Plant Physiology* **149**:165-170.

Krysan PJ, Young JC and Sussman MR (1999) T-DNA as an insertional mutagen in *Arabidopsis*. *The Plant Cell* **11**:2283-2290.

Kumar M, Basha OP, Puri A, Rajpurohit D, Randhawa GS, Sharma TR and Dhaliwal HS (2010) A candidate gene *OsAPC6* of anaphase-promoting complex of rice identified through T-DNA insertion. *Functional and Integrative Genomics* **10**:349-358.

Kumar I and Singh TH. 1984. A rapid method for identifying different dwarfing genes in rice. *Rice Genetics Newsletter* **1**: 135.

Kunze R, Stochaj U, Laufs J and Starlinger P (1987) Transcription of transposable element Activator (Ac) of *Zea mays* L. *EMBO Journal* **6**:1555-1563.

Kuppusamy KT, Ivashuta S, Bucciarelli B, Vance CP, Gantt JS and VandenBosch KA (2009) Knockdown of *CELL DIVISION CYCLE16* reveals an inverse relationship between lateral root and nodule numbers and a link to auxin in *Medicago truncatula*. *Plant Physiology* **151**:1155-1166.

Kurata N, Nagamura Y, Yamamoto K, Harushima Y, Sue N, Wu J, Antonio BA, Shomura A, Shimizu T, Lin SY, Inoue T, Fukuda A, Shimano T, Kuboki Y, Toyama T, Miyamoto Y, Kirihara T, Hayasaka K, Miyao A, Monna L, Zhong HS, Tamura Y, Wang ZX, Momma T, Umehara

Y, Yano M, Sasaki T and Minobe Y (1994) A 300 kilobase interval genetic map of rice including 883 expressed sequences. *Nature Genetics* **8**:365-372.

Kusaba M, Miyahara K, Iida S, Fukuoka H, Takano T, Sassa H, Nishimura M and Nishio T (2003) Low glutelin content 1: a dominant mutation that suppresses the glutelin multigene family via RNA silencing in rice. *The Plant Cell* **15**:1455-1467.

Kwee HS and Sundaresan V (2003) The NOMEGA gene required for female gametophyte development encodes the putative APC6/CDC16 component of the Anaphase Promoting Complex in *Arabidopsis*. *The Plant Journal* **36**:853-866.

Lamb JR, Tugendreich S and Hieter P (1995) Tetratrico peptide repeat interactions - to TPR or not to TPR. *Trends in Biochemical Sciences* **20**:257-259.

Lamb JR, W A Michaud, R S Sikorski and Hieter PA (1994) Cdc16p, Cdc23p and Cdc27p form a complex essential for mitosis. *EMBO Journal* **13**:4321–4328.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ and Higgins DG (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**:2947-2948.

Laskowski RA, MacArthur MW, Moss DS and Thornton JM (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *Journal of Applied Crystallography* **26**:283-291.

Lee DH and Goldberg AL (1998) Proteasome inhibitors: valuable new tools for cell biologists. *Trends in Cell Biology* **8**:397-403.

Lee S, Kim J, Han J-J, Han M-J and An G (2004) Functional analyses of the flowering time gene *OsMADS50*, the putative SUPPRESSOR OF OVEREXPRESSION OF CO1/AGAMOUS-LIKE 20 (SOC1/AGL20) ortholog in rice. *The Plant Journal* **38**:754-764.

Lersten (2004) *Flowering Plant Embryology*: Blackwell Publishing Ltd, (Iowa, USA).

Li L, Qu R, Kochko A, Fauquet C and Beachy RN (1993) An improved rice transformation system using the biolistic method. *Plant Cell Reports* **12**:250-255.

Li FN and Johnston M (1997) Grr1 of *Saccharomyces cerevisiae* is connected to the ubiquitin proteolysis machinery through Skp1: coupling glucose sensing to gene expression and the cell cycle. *EMBO Journal* **16**:5629-5638.

Li N, Yuan L, Liu N, Shi D, Li X, Tang Z, Liu J, Sundaresan V and Yang W-C (2009) SLOW WALKER2, a NOC1/MAK21 Homologue, is essential for coordinated cell cycle progression during female gametophyte development in *Arabidopsis*. *Plant Physiology* **151**:1486-1497.

Li W, Zhang P, Fellers JP, Friebe B and Gill BS (2004) Sequence composition, organization, and evolution of the core *Triticeae* genome. *The Plant Journal* **40**:500-511.

Lima Md, Eloy N, Pegoraro C, Sagit R, Rojas C, Bretz T, Vargas L, Elofsson A, de Oliveira A, Hemerly A and Ferreira P (2010) Genomic evolution and complexity of the anaphase-promoting complex (APC) in land plants. *BMC Plant Biology* **10**:254.

Littlepage LE and Ruderman JV (2002) Identification of a new APC/C recognition domain, the A box, which is required for the Cdh1-dependent destruction of the kinase Aurora-A during mitotic exit. *Genes and Development* **16**:2274-2285.

Liu J and Qu LJ (2008) Meiotic and mitotic cell cycle mutants involved in gametophyte development in *Arabidopsis*. *Molecular Plant* **1**:564-574.

Liu M, Shi DQ, Yuan L, Liu J and Yang WC (2010) SLOW WALKER3, encoding a putative DEAD-box RNA Helicase, is essential for female gametogenesis in *Arabidopsis*. *Journal of Integrative Plant Biology* **52**:817-828.

Liu YG, Mitsukawa N, Oosumi T and Whittier RF (1995) Efficient isolation and mapping of Arabidopsis thaliana T-DNA insert junctions by thermal asymmetric interlaced PCR. *The Plant Journal* **8**:457-463.

Liu YG and Whittier RF (1995) Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. *Genomics* **25**:674-681.

Livingstone DM and Birch RG (1995) Plant regeneration and microprojectile-mediated gene transfer in embryonic leaflets of peanut (*Arachis hypogaea* L.). *Australian Journal of Plant Physiology* **22**:585-591.

Lucas M, Daviere JM, Rodriguez-Falcon M, Pontin M, Iglesias-Pedraz JM, Lorrain S, Fankhauser C, Blazquez MA, Titarenko E and Prat S (2008) A molecular framework for light and gibberellin control of cell elongation. *Nature* **451**:480-484.

Machida C, Onouchi H, Koizumi J, Hamada S, Semiarti E, Torikai S and Machida Y (1997) Characterization of the transposition pattern of the Ac element in *Arabidopsis thaliana* using endonuclease I-*Sce*I. *Proceedings of the National Academy of Sciences, USA* **94**:8675-8680.

Mackenzie DR, Anderson PM and Wernham CC (1966) A mobile air blast inoculator for plot experiments with maize dwarf mosaic virus. *Plant Disease Reporter* **50**:363-367.

Maheshwari P (1950) *An introduction to the embryology of angiosperms*. New York: McGraw-Hill.

Main ERG, Xiong Y, Cocco MJ, D'Andrea L and Regan L (2003) Design of stable alpha-helical arrays from an idealized TPR Motif. *Structure* **11**:497-508.

Mansfield SG, Briarty LG and Erni S (1990) Early embryogenesis in *Arabidopsis thaliana.* The mature embryo sac. *Canadian Journal of Botany* **69**:447-460.

Marcelo BS (1997) Identification and cloning of differentially expressed genes. *Current Opinion in Biotechnology* **8**:542-546.

Marrocco K, Thomann A, Parmentier Y, Genschik P and Criqui MC (2009) The APC/C E3 ligase remains active in most post-mitotic *Arabidopsis* cells and is required for proper vasculature development and organization. *Development* **136**:1475-1485.

Marteijn JAF, Jansen JH and van der Reijden BA (2006) Ubiquitylation in normal and malignant hematopoiesis: novel therapeutic targets. *Leukemia* **20**:1511-1518.

Marti-Renom MA, Stuart AC, Fiser A, Sanchez R, Melo F and Sali A (2000) Comparative protein structure modeling of genes and genomes. *Annual Review of Biophysics and Biomolecular Structure* **29**:291-325.

Martin DN, Proebsting WM, Parks TD, Dougherty WG, Lange T, Lewis M J, Gaskin P, Hedden P. 1996. Feedback regulation of gibberellin biosynthesis and gene expression in *Pisum sativum* L. *Planta.* **200**:159–166

Martienssen RA (1998) Functional genomics: Probing plant gene function and expression with transposons. *Proceedings of the National Academy of Sciences, USA* **95**:2021-2026.

Martinez J, Patkaniowska A, Urlaub H, Lührmann R and Tuschl T (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* **110**:563-574.

Maskos U and Southern EM (1993) A novel method for the parallel analysis of multiple mutations in multiple samples. *Nucleic Acids Research* **21**:2269-2270.

Mathur J, Szabados L, Schaefer S, Grunenberg B, Lossow A, Jonas-Straube E, Schell J, Koncz C and Koncz-Kálmán Z (1998) Gene identification with sequenced T-DNA tags generated by transformation of *Arabidopsis* cell suspension. *The Plant Journal* **13**:707-716.

McCabe DE, Swain WF, Martinell BJ and Christou P (1988) Stable transformation of soybean (*Glycine max*) by particle acceleration. *Nat Biotech* **6**:923-926.

McClintock B (1948) Mutable loci in maize. *Carnegie Institution Washington Yearbook* **47**:155-159.

McGarry TJ and Kirschner MW (1998) Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell* **93**:1043-1053.

McGinnis KM, Thomas SG, Soule JD, Strader LC, Zale JM, Sun TP and Steber CM (2003) The *Arabidopsis* SLEEPY1 gene encodes a putative F-Box subunit of an SCF E3 ubiquitin ligase. *The Plant Cell* **15**:1120-1130.

Medlin J (2001) Array of hope for gene technology. *Environmental Health Perspectives* **109**:34-37.

Mehta PA, Sivaprakash K, Parani M, Venkataraman G and Parida AK (2005) Generation and analysis of expressed sequence tags from the salt-tolerant mangrove species *Avicennia marina* (Forsk) Vierh. *Theoretical and Applied Genetics* **110**:416-424.

von Mering C, Krause R, Snel B, Cornell M, Oliver SG, Fields S, Bork, P (2002) Comparative assessment of large-scale data sets of protein–protein interactions, *Nature* **417**:399-403.

Miki D and Shimamoto K (2004) Simple RNAi vectors for stable and transient suppression of gene function in rice. *Plant and Cell Physiology* **45**:490-495.

Milach SCK, Rines HW, Phillips RL. 1997. Molecular genetic mapping of dwarfing genes in oat. *Theoretical and Applied Genetics* **95**: 783-790.

Minsky M (1988) Memoir on inventing the confocal scanning microscope. *Scanning* **10**:128-138.

Monaco JJ and Nandi D (1995) The genetics of proteasomes and antigen processing. *Annual Review of Genetics* **29**:729-754.

Moon J, Parry G and Estelle M (2004) The ubiquitin-proteasome pathway and plant development. *The Plant Cell* **16**:3181-3195.

Moore G, Devos KM, Wang Z and Gale MD (1995) Cereal genome evolution: grasses, line up and form a circle. *Current Biology* **5**:737-739.

Moore JM, Vielle Calzada JP, Gagliano W and Grossniklaus U (1997) Genetic characterization of *hadad*, a mutant disrupting female gametogenesis in *Arabidopsis thaliana. Cold Spring Harbor Symposia on Quantitative Biology* **62**:35-47.

Morgan DO (1999) Regulation of the APC and the exit from mitosis. *Nature Cell Biology* **1**:E47-E53.

Mu X, Jin B and Teng N (2010) Studies on the early development of zygotic and synergid embryo and endosperm in polyembryonic rice *ApIII. Flora - Morphology, Distribution, Functional Ecology of Plants* **205**:404-410.

Mueller LA, Zhang P and Rhee SY (2003) AraCyc: a biochemical pathway database for *Arabidopsis. Plant Physiology* **132**:453-460.

Multani DS, Briggs SP, Chamberlin MA, Blakeslee JJ, Murphy AS and Johal GS (2003) Loss of an MDR transporter in compact stalks of maize *br2* and sorghum *dw3* mutants. *Science* **302**:81-84.

Murray AW (2004) Recycling the cell cycle: cyclins revisited. *Cell* **116**:221-234.

Murray AW and Kirschner MW (1989) Cyclin synthesis drives the early embryonic-cell cycle. *Nature* **339**:275-280.

Myers JW, Jones JT, Meyer T and Ferrell JE (2003) Recombinant Dicer efficiently converts large dsRNAs into siRNAs suitable for gene silencing. *Nature Biotechnology* **21**:324-328.

Nakagahra M, Okuno K and Vaughan D (1997) Rice genetic resources: history, conservation, investigative characterization and use in Japan. *Plant Molecular Biology* **35**:69-77.

Nakayama KI and Nakayama K (2006) Ubiquitin ligases: cell-cycle control and cancer. *Nature Reviews Cancer* **6**:369-381.

Nannini M, Pantaleo MA, Maleddu A, Astolfi A, Formica S and Biasco G (2009) Gene expression profiling in colorectal cancer using microarray technologies: results and perspectives. *Cancer Treatment Reviews* **35**:201-209.

Napoli C, Lemieux C and Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes in trans. *The Plant Cell* **2**:279-289.

Oard JH, Paige DF, Simmonds JA and Gradziel TM (1990) Transient gene expression in maize, rice and wheat cells using an air gun apparatus. *Plant Physiology* **92**:334-339.

Ogas J, Cheng JC, Sung ZR and Somerville C (1997) Cellular differentiation regulated by gibberellin in the *Arabidopsis thaliana pickle* mutant. *Science* **277**:91–94.

Ogas J, Kaufmann S, Henderson J and Somerville C (1999) PICKLE is a CHD3 chromatin-remodeling factor that regulates the transition from embryonic to vegetative development in *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* **96**:13839-13844.

Ogawa M, Kusano T, Katsumi M and Sano H (2000) Rice gibberellin-insensitive gene homolog, *OsGAI*, encodes a nuclear-localized protein capable of gene activation at transcriptional level. *Gene* **245**:21-29.

Ogita S, Uefuji H, Morimoto M and Sano H (2004) Application of RNAi to confirm theobromine as the major intermediate for caffeine biosynthesis in coffee plants with potential for construction of decaffeinated varieties. *Plant Molecular Biology* **54**:931-941.

Osterlund T (2001) Structure-function relationships of hormone-sensitive lipase. *European Journal of Biochemistry* **268**:1899-1907.

Ouyang S, Zhu W, Hamilton J, Lin H, Campbell M, Childs K, Thibaud-Nissen F, Malek RL, Lee Y, Zheng L, Orvis J, Haas B, Wortman J and Buell CR (2007) The TIGR rice genome annotation resource: improvements and new features. *Nucleic Acids Research* **35**:D883-D887.

Paddock SW (1999) *Confocal Microscopy: Methods and Protocols*. Totowa, (ed.) New Jersey: Humana Press.

Pagnussat GC, Yu H-J, Ngo QA, Rajani S, Mayalagu S, Johnson CS, Capron A, Xie LF, Ye D and Sundaresan V (2005) Genetic and molecular identification of genes required for female gametophyte development and function in *Arabidopsis*. *Development* **132**:603-614.

Pal M, Nagy O, Menesi D, Udvardy A and Deak P (2007) Structurally related TPR subunits contribute differently to the function of the anaphase-promoting complex in *Drosophila melanogaster*. *Journal of Cell Science* **120**:3238-3248.

Pamer E and Cresswell P (1998) Mechanisms of MHC class I restricted antigen processing. *Annual Review of Immunology* **16**:323-358.

Parinov S and Sundaresan V (2000) Functional genomics in *Arabidopsis*: large-scale insertional mutagenesis complements the genome sequencing project. *Current Opinion in Biotechnology* **11**:157-161.

Passmore LA and Barford D (2004) Getting into position: the catalytic mechanisms of protein ubiquitylation. *Biochemical Journal* **379**:513-525.

Pedersen JF, Vogel KP and Funnell DL (2005) Impact of reduced lignin on plant fitness. *Crop Science* **45**:812-819.

Pellman D and Christman MF (2001) Separase anxiety: dissolving the sister bond and more. *Nature Cell Biology* **3**:E207-E209.

Peng H, Begg GE, Schultz DC, Friedman JR, Jensen DE, Speicher DW and Rauscher Iii FJ (2000) Reconstitution of the KRAB-KAP-1 repressor complex: a model system for defining the molecular anatomy of RING-B box-coiled-coil domain-mediated protein-protein interactions. *Journal of Molecular Biology* **295**:1139-1162.

Peng J, Carol P, Richards DE, King KE, Cowling RJ, Murphy GP and Harberd NP (1997) The *Arabidopsis GAI* gene defines a signaling pathway that negatively regulates gibberellin responses. *Genes and Development* **11**:3194-3205.

Peng J and Harberd NP (1997) Gibberellin deficiency and response mutations suppress the stem elongation phenotype of phytochrome-deficient mutants of *Arabidopsis*. *Plant Physiology* **113**:1051-1058.

Peng J, Richards DE, Hartley NM, Murphy GP, Devos KM, Flintham JE, Beales J, Fish LJ, Worland AJ, Pelica F, Sudhakar D, Christou P, Snape JW, Gale MD and Harberd NP (1999) 'Green revolution' genes encode mutant gibberellin response modulators. *Nature* **400**:256-261.

Perazza D, Vachon G and Herzog M (1998) Gibberellins promote trichome formation by up-regulating GLABROUS1 in *Arabidopsis*. *Plant Physiology* **117**:375-383.

Peters JM (2002) The anaphase-promoting complex: proteolysis in mitosis and beyond. *Molecular Cell* **9**:931-943.

Peters JM (2006) The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nature Reviews Molecular Cell Biology* 7:644-656.

Petersen BO, Wagener C, Marinoni F, Kramer ER, Melixetian M, Denchi EL, Gieffers C, Matteucci C, Peters JM and Helin K (2000) Cell cycle- and cell growth-regulated proteolysis of mammalian CDC6 is dependent on APC-CDH1. *Genes and Development* **14**:2330-2343.

Pfleger CM and Kirschner MW (2000) The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. *Genes and Development* **14**:655-665.

Phogat S, Burma PK and Pental D (2000) A four-element based transposon system for allele specific tagging in plants -Theoretical considerations. *Journal of Biosciences* **25** 57-63.

Pickart CM (2001) Mechanisms underlying ubiquitination. *Annual Review of Biochemistry* **70**:503-533.

Ping LX, Nogawa M, Nozue M, Makita M, Takeda M, Bao L, Kojima M (2003). *In planta* transformation of mulberry trees (*Morus alba* L.) by *Agrobactetium tumefaciens*, *Journal of Insect Biotechnology Sericology* **72**: 177-184.

Pohlman RF, Fedoroff NV and Messing J (1984) The nucleotide sequence of the maize controlling element Activator. *Cell* **37**:635-643.

Portereiko MF, Lloyd A, Steffen JG, Punwani JA, Otsuga D and Drews GN (2006) AGL80 is required for central cell and endosperm development in *Arabidopsis*. *The Plant Cell* **18**:1862-1872.

Prasad BD, Goel S, Krishna P (2010) *In Silico* identification of carboxylate clamp type tetratricopeptide repeat proteins in *Arabidopsis* and rice as putative co-chaperones of Hsp90/Hsp70. *PLoS ONE* **5**:1-18.

Puri A, Basha OP, Kumar M, Rajpurohit D, Randhawa GS, Kianian S, Rishi A and Dhaliwal HS (2010) The polyembryony gene (*OsPE*) in rice. *Functional and Integrative Genomics* **10**:359-366.

Pysh LD, Wysocka-Diller JW, Camilleri C, Bouchez D and Benfey PN (1999) The GRAS gene family in *Arabidopsis*: sequence characterization and basic expression analysis of the SCARECROW-LIKE genes. *The Plant Journal* **18**:111-119.

Raghavan V (2003) Some reflections on double fertilization, from its discovery to the present. *New Phytologist* **159**:565-583.

Ramachandran S and Sundaresan V (2001) Transposons as tools for functional genomics. *Plant Physiology and Biochemistry* **39**:243-252.

Ratcliff FG, MacFarlane SA and Baulcombe DC (1999) Gene silencing without DNA: RNA-mediated cross-protection between viruses. *The Plant Cell* **11**:1207-1216.

Reiser L and Fischer RL (1993) The ovule and the embryo sac. *The Plant Cell* **5**:1291-1301.

Robertson M (2004) Two transcription factors are negative regulators of gibberellin response in the HvSPY-signaling pathway in barley aleurone. *Plant Physiology* **136**:2747-2761.

Robertson M, Swain SM, Chandler PM and Olszewski NE (1998) Identification of a negative regulator of gibberellin action, HvSPY, in barley. *The Plant Cell* **10**:995-1007.

Rohini, VK, Sankara RK (2000) Transformation of peanut (*Arachis hypogeae L.*): a non-tissue culture based approach for generating transgenic plants. *Plant Science* **150**:41-49.

Rohini, VK, Sankara RK (2000) Embryo transformation, a practical approach for realizing transgenic plants of safflower (*Carthamus tinctorius* L.). *Annals of Botany* **86**:1043-1049.

Rohini, VK and Sankara RK (2001) Transformation of peanut (*Arachis hypogaea L.*) with tobacco Chitinase gene: variable response of transformants to leaf spot disease. *Plant Science* **160** (5):883-892.

Rojas C, Eloy N, de Freitas Lima M, Rodrigues R, Franco L, Himanen K, Beemster G, Hemerly A and Ferreira P (2009) Overexpression of the *Arabidopsis* anaphase promoting complex subunit *CDC27a* increases growth rate and organ size. *Plant Molecular Biology* **71**:307-318.

Roodbarkelari F, Bramsiepe J, Weinl C, Marquardt S, Béla N, Jakoby MJ, Lechner E, Genschik P and Schnittger A (2010) CULLIN 4-RING FINGER-LIGASE plays a key role in the control of endoreplication cycles in *Arabidopsis* trichomes. *Proceedings of the National Academy of Sciences, USA* **107**:15275–15280.

Roos MD and Hanover JA (2000) Structure of O-Linked GlcNAc transferase: mediator of glycan-dependent signaling. *Biochemical and Biophysical Research Communications* **271**:275-280.

Russell S, Meadows LA and Russell RR (2009) *Microarray Technology in Practice*: Academic Press, Elsevier, I Ed.

Russell SD (1993) The egg cell: development and role in fertilization and early embryogenesis. *The Plant Cell* **5**:1349-1359.

Sahoo L, Mishra S, Purkayastha J, Solleti SK and Sugla T (2007) Genetic engineering of rice: prospects for abiotic stress tolerance. *Journal of Applied Bioscience and Biotechnology* **3**: 1-28

Sakamoto T, Miura K, Itoh H, Tatsumi T, Ueguchi-Tanaka M, Ishiyama K, Kobayashi M, Agrawal GK, Takeda S, Abe K, Miyao A, Hirochika H, Kitano H, Ashikari M and Matsuoka M (2004) An overview of gibberellin metabolism enzyme genes and their related mutants in rice. *Plant Physiology* **134**:1642-1653.

Saldaña C, Díaz-Muñoz M, Antaramián A, González-Gallardo A, García-Solí P and Morales-Tlalpan V (2009) MCF-7 breast carcinoma cells express ryanodine receptor type 1: functional characterization and subcellular localization. *Molecular and Cellular Biochemistry* **323**:39-47.

Sali A and Blundell TL (1993) Comparative protein modeling by satisfaction of spatial restraints. *Journal of Molecular Biology* **234**:779-815.

Sanford JC, Klein TM, Wolf ED and Allen N (1987) Delivery of substances into cells and tissues using a particle bombardment process. *Particulate Science and Technology* **5**:27-37.

Sankara RK and Rohini VK (1999) *Agrobacterium*-mediated transformation of sunflower (Helianthus annuus L.): a simple protocol. *Annals of Botany* **83**: 347-354.

Sarkar N, Kim YK and Grover A (2009) Rice sHsp genes: genomic organization and expression profiling under stress and development. *BMC Genomics* **10**:393.

Sasaki A, Itoh H, Gomi K, Ueguchi-Tanaka M, Ishiyama K, Kobayashi M, Jeong DH, An G, Kitano H, Ashikari M and Matsuoka M (2003) Accumulation of phosphorylated repressor for gibberellin signaling in an F-box mutant. *Science* **299**:1896-1898.

Sasaki T, Matsumoto T, Antonio BA and Nagamura Y (2005) From mapping to sequencing, post-sequencing and beyond. *Plant and Cell Physiology*.

Sautter C (1993) Development of a microtargeting device for particle bombardment of plant meristems. *Plant Cell, Tissue and Organ Culture* **33**:251-257.

Schapire AL, Valpuesta V and Botella MA (2006) TPR proteins in plant hormone signaling. *Plant Signaling and Behavior* **1**:229-230.

Schena M (1996) Genome analysis with gene expression microarrays. *Bioessays* **18**:427-431.

Schena M, Heller RA, Theriault TP, Konrad K, Lachenmeier E and Davis RW (1998) Microarrays: biotechnology's discovery platform for functional genomics. *Trends in Biotechnology* **16**:301-306.

Schena M, Shalon D, Davis RW and Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270**:467-470.

Schlappi M and Hohn B (1992) Competence of immature maize embryos for *Agrobacterium*-mediated gene transfer. *The Plant Cell* **4**:7-16.

Schwarz DS, Hutvágner G, Haley B and Zamore PD (2002) Evidence that siRNAs function as guides, not primers, in the *Drosophila* and human RNAi pathways. *Molecular Cell* **10**:537-548.

Seki M, Komeda Y, Iida A, Yamada Y and Morikawa H (1991) Transient expression of beta - glucuronidase in *Arabidopsis thaliana* leaves and roots and *Brassica napus* stems using a pneumatic particle gun. *Plant Molecular Biology* 17:259-263.

Service RF (1998) Microchip arrays put DNA on the spot. *Science* **282**:396-399.

Shimada A, Ueguchi-Tanaka M, Nakatsu T, Nakajima M, Naoe Y, Ohmiya H, Kato H and Matsuoka M (2008) Structural basis for gibberellin recognition by its receptor GID1. *Nature* **456**:520-523.

Shimamoto K and Kyozuka J (2002) rice as a model for comparative genomics of plants. *Annual Review of Plant Biology* **53**:399-419.

Shirayama M, Zachariae W, Ciosk R and Nasmyth K (1998) The polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae*. *EMBO Journal* **17**:1336-1349.

Silverstone AL, Ciampaglio CN and Sun TP (1998) The *Arabidopsis* RGA gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. *The Plant Cell* **10**:155-170.

Silverstone AL, Jung HS, Dill A, Kawaide H, Kamiya Y and Sun TP (2001) Repressing a repressor: Gibberellin-induced rapid reduction of the RGA protein in *Arabidopsis*. *The Plant Cell* **13**:1555-1565.

Silverstone AL, Mak PYA, Casamitjana Martínez E and Sun TP (1997) The new RGA locus encodes a negative regulator of gibberellin response in *Arabidopsis thaliana*. *Genetics* **146**:1087-1099.

Singh ND, Sahoo L, Saini, R, Sarin NB and Jaiwal PK (2004) *In vitro* regeneration and recovery of primary transformants from shoot apices of pigeonpea using *Agrobacterium tumefaciens*. *Physiology and Molecular Biology of Plants*. **10**, 65-74.

Singh D, Sirohi A, Sharma V, Kumar S, Gaur V Chaudhary A and Chaudhary RN (2006) RAPD based identification of farmers collection of Kalanamak and traditional varieties of basmati rice. *Indian Journal of Crop Science* **1**:102-105.

Singh N, Dalal V, Batra K, Singh B, Chitra G, Singh A, Ghazi I, Yadav M, Pandit A, Dixit R, Singh P, Singh H, Koundal K, Gaikwad K, Mohapatra T and Sharma TR (2007) Single-copy genes define a conserved order between rice and wheat for understanding differences caused by duplication, deletion, and transposition of genes. *Functional and Integrative Genomics* 7:17-35.

Skowyra D, Craig KL, Tyers M, Elledge SJ and Harper JW (1997) F-Box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell* **91**: 209-219.

Smith EF and Townsend CO (1907) A plant tumour of bacterial origin. *Science* **25**:671-673.

Smith D, Yanai Y, Liu YG, Ishiguro S, Okada K, Shibata D, Whittier RF and Fedoroff NV (1996) Characterization and mapping of Ds-GUS T-DNA lines for targeted insertional mutagenesis. *The Plant Journal* **10**: 721-732.

Smith NA, Singh SP, Wang M-B, Stoutjesdijk PA, Green AG and Waterhouse PM (2000) Gene expression: Total silencing by intron-spliced hairpin RNAs. *Nature* **407**:319-320.

Soini H and Musser JM (2001) Molecular Diagnosis of Mycobacteria. *Clinical Chemistry* **47**:809-814.

Solanke AU and Sharma AK (2008) Signal transduction during cold stress in plants. *Physiology and Molecular Biology of Plants*. **14**: 69-79.

Somasundaram K, Mungamuri SK and Wajapeyee N (2002) DNA microarray technology and its applications in cancer biology. *Applied Genomics and Proteomics* **1**:1-10.

Springer PS (2000) Gene traps: tools for plant development and genomics. *The Plant Cell* **12**:1007-1020.

Springer PS, Holding DR, Groover A, Yordan C and Martienssen RA (2000) The essential Mcm7 protein PROLIFERA is localized to the nucleus of dividing cells during the G(1) phase and is required maternally for early *Arabidopsis* development. *Development* **127**:1815-1822.

Stachel SE and Zambryski PC (1986) virA and virG control the plant-induced activation of the T-DNA transfer process of *Agrobacterium tumefaciens*. *Cell* **46**:325-333.

Stam M, De Bruin R, Kenter S, Van Der Hoorn RAL, Van Blokland R, Mol JNM and Kooter JM (1997) Post-transcriptional silencing of chalcone synthase in *Petunia* by inverted transgene repeats. *The Plant Journal* **12**:63-82.

Sudakin V, Ganoth D, Dahan A, Heller H, Hershko J, Luca FC, Ruderman JV and Hershko A (1995) The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis. *Molecular Biology of the Cell* **6**:185-197.

Sun TP and Gubler F (2004) Molecular mechanism of gibberellin signaling in plants. *Annual Review of Plant Biology* **55**:197-223.

Sundaram RM, Viraktamath, BC, Rani NS and Sarla N. (2010) Rice genetics. *Current Science* **98**:1422-1426.

Sundaresan V (1996) Horizontal spread of transposon mutagenesis: new uses for old elements. *Trends in Plant Science* **1**:184-190.

Sundaresan V, Springer P, Volpe T, Haward S, Jones JD, Dean C, Ma H and Martienssen R (1995) Patterns of gene action in plant development revealed by enhancer trap and gene trap transposable elements. *Genes and Development* **9**:1797-1810.

Supartana P, Shimizu T, Shioiri H, Nogawa M, Nozue M, Kojima M (2005) Development of simple and efficient in planta transformation method for rice (*Oryza sativa* L.) using *Agrobacterium tumefaciens. Journal of Bioscience and Bioengineering* **100**(4): 391-397.

Suzuki Y, Uemura S, Saito Y, Murofushi N, Schmitz G, Theres K and Yamaguchi I (2001) A novel transposon tagging element for obtaining gain-of-function mutants based on a self-stabilizing Ac derivative. *Plant Molecular Biology* **45**:123-131.

Swain SM, Tseng TS and Olszewski NE (2001) Altered expression of SPINDLY affects gibberellin response and plant development. *Plant Physiology* **126**:1174-1185.

Swain SM, Tseng TS, Thornton TM, Gopalraj M and Olszewski NE (2002) SPINDLY is a nuclear-localized repressor of gibberellin signal transduction expressed throughout the plant. *Plant Physiology*.

Takahashi A, Casais C, Ichimura K and Shirasu K (2003) HSP90 interacts with RAR1 and SGT1 and is essential for RPS2-mediated disease

resistance in *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* **100**:11777-11782.

Talon M, Koornneef M and Zeevaart J (1990) Accumulation of C19-gibberellins in the gibberellin-insensitive dwarf mutant *gai* of *Arabidopsis thaliana* (L.) Heynh. *Planta* **182**:501-505.

Tang Z, Bharadwaj R, Li B and Yu H (2001a) Mad2-Independent Inhibition of APCCdc20 by the Mitotic Checkpoint Protein BubR1. *Developmental Cell* **1**:227-237.

Tang ZY, Li B, Bharadwaj R, Zhu HH, Ozkan E, Hakala K, Deisenhofer J and Yu HT (2001b) APC2 cullin protein and APC11 RING protein comprise the minimal ubiquitin ligase module of the anaphase-promoting complex. *Molecular Biology of the Cell* **12**:3839-3851.

Tavernarakis N, Wang SL, Dorovkov M, Ryazanov A and Driscoll M (2000) Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nature Genetics* **24**:180-183.

Thimm O, Bläsing O, Gibon Y, Nagel A, Meyer S, Krüger P, Selbig J, Müller LA, Rhee SY and Stitt M (2004) Mapman: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *The Plant Journal* **37**:914-939.

Thornton BR, Ng TM, Matyskiela ME, Carroll CW, Morgan DO and Toczyski DP (2006) An architectural map of the anaphase-promoting complex. *Genes and Development* **20**:449-460.

Thornton T, Kreppel L, Hart G and Olszewski N (1999.) Genetic and biochemical analysis of *Arabidopsis* SPY.. In Plant Biotechnology and in Vitro Biology in the 21st Century, A. Altman,M. Ziv, and S. Izhar, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), 445–448.

Tinland B (1996) The integration of T-DNA into plant genomes. *Trends in Plant Science* **1**:178-184.

Townsley FM and Ruderman JV (1998) Proteolytic ratchets that control progression through mitosis. *Trends in Cell Biology* **8**:238-244.

Tsugeki R, Kochieva EZ and Fedoroff NV (1996) A transposon insertion in the Arabidopsis SSR16 gene causes an embryo-defective lethal mutation. *The Plant Journal* **10**:479-489.

Tuoc TC and Stoykova A (2010) Roles of the ubiquitin-proteosome systems in neurogenesis. *Cell Cycle* **9**:3174-3180.

Tyagi AK and Mohanty A (2000) Rice transformation for crop improvement and functional genomics. *Plant Science* **158**:1-18.

Tzfira T and Citovsky V (2000) From host recognition to T-DNA integration: the function of bacterial and plant genes in the *Agrobacterium*–plant cell interaction. *Molecular Plant Pathology* **1**:201-212.

Ueguchi-Tanaka M, Ashikari M, Nakajima M, Itoh H, Katoh E, Kobayashi M, Chow TY, Hsing YIC, Kitano H, Yamaguchi I and Matsuoka M (2005) GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. *Nature* **437**:693-698.

Ueguchi-Tanaka M, Fujisawa Y, Kobayashi M, Ashikari M, Iwasaki Y, Kitano H and Matsuoka M (2000) Rice dwarf mutant d1, which is defective in the α subunit of the heterotrimeric G protein, affects gibberellin signal transduction. *Proceedings of the National Academy of Sciences, USA* **97**:11638-11643.

Vain P, Keen N, Murillo J, Rathus C, Nemes C and Finer J (1993) Development of the particle inflow gun. *Plant Cell, Tissue and Organ Culture* **33**:237-246.

Van Blokland R, Van der Geest N, Mol JNM and Kooter JM (1994) Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. *The Plant Journal* **6**:861-877.

Vaughan (2003) Diversity in the *Oryza* genus. *Current Opinion in Plant Biology* **6**:139-146.

Visintin R, Prinz S and Amon A (1997) CDC20 and CDH1: A family of substrate-specific activators of APC-dependent proteolysis. *Science* **278**:460-463.

Vodermaier HC (2001) Cell cycle: Waiters serving the destruction machinery. *Current Biology* **11**:R834-R837.

Vodermaier HC (2004) APC/C and SCF: Controlling each other and the cell cycle. *Current Biology* **14**:R787-R796.

Voges D, Zwickl P and Baumeister W (1999) The 26S Proteasome: a molecular machine designed for controlled proteolysis. *Annual Review of Biochemistry* **68**:1015-1068.

Wang JC (2005) Finding primary targets of transcriptional regulators. *Cell Cycle* 4:356-358.

Wang KLC, Yoshida H, Lurin C and Ecker JR (2004) Regulation of ethylene gas biosynthesis by the *Arabidopsis* ETO1 protein. *Nature* **428**:945-950.

Wang R, Okamoto M, Xing X and Crawford NM (2003) Microarray analysis of the nitrate response in *Arabidopsis* roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. *Plant Physiology* **132**:556-567.

Waterhouse PM and Helliwell CA (2003) Exploring plant genomes by RNA-induced gene silencing. *Nature Reviews Genetics* **4**:29-38.

Watson A, Mazumder A, Stewart M and Balasubramanian S (1998) Technology for microarray analysis of gene expression. *Current Opinion in Biotechnology* **9**:609-614.

Watson SJ, Meng F, Thompson RC and Akil H (2000) The "chip" as a specific genetic tool. *Biological Psychiatry* **48**:1147-1156.

Wei G, Tao Y, Liu G, Chen C, Luo R, Xia H, Gan Q, Zeng H, Lu Z, Han Y, Li X, Song G, Zhai H, Peng Y, Li D, Xu H, Wei X, Cao M, Deng H, Xin Y, Fu X, Yuan L, Yu J, Zhu Z and Zhu L (2009) A transcriptomic analysis of superhybrid rice LYP9 and its parents. *Proceedings of the National Academy of Sciences, USA* **106**:7695-7701.

Weindruch R, Kayo T, Lee CK and Prolla TA (2001) Microarray profiling of gene expression in aging and its alteration by caloric restriction in mice. *The Journal of Nutrition* **131**:918S-923S.

Weising K, Schell J and Kahl G (1988) Foreign genes in plants: transfer, structure, expression and applications. *Annual Review of Genetics* **22**:421-477.

Wen CK and Chang C (2002) *Arabidopsis* RGL1 encodes a negative regulator of gibberellin responses. *The Plant Cell* **14**:87-100.

Wesley SV, Helliwell CA, Smith NA, Wang M, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green

AG and Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *The Plant Journal* **27**:581-590.

White JG, Amos WB and Fordham M (1987) An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. *The Journal of Cell Biology* **105**:41-48.

Wiederstein M and Sippl MJ (2007) ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic Acids Research* **35**:W407-W410.

Wilhelm BT and Landry JR (2009) RNA-Seq-quantitative measurement of expression through massively parallel RNA-sequencing. *Methods* **48**:249-257.

Wilkinson KD (2000) Ubiquitination and deubiquitination: Targeting of proteins for degradation by the proteasome. *Seminars in Cell and Developmental Biology* **11**:141-148.

Willige BrC, Ghosh S, Nill C, Zourelidou M, Dohmann EMN, Maier A and Schwechheimer C (2007) The DELLA domain of GA insensitive mediates the interaction with the GA INSENSITIVE DWARF1A gibberellin receptor of *Arabidopsis*. *The Plant Cell* **19**:1209-1220.

Winans SC, Kerstetter RA and Nester EW (1988) Transcriptional regulation of the vir A and vir G genes of *Agrobacterium tumefaciens*. *Journal of Bacteriology* **170**:4047-4054.

Wisman E, Hartmann U, Sagasser M, Baumann E, Palme K, Hahlbrock K, Saedler H and Weisshaar B (1998) Knock-out mutants from an En-1 mutagenized *Arabidopsis thaliana* population generate phenylpropanoid biosynthesis phenotypes. *Proceedings of the National Academy of Sciences, USA* **95**:12432-12437.

Wu H, Lan Z, Li W, Wu S, Weinstein J, Sakamoto KM and Dai W (2000) *p55CDC/hCDC20* is associated with BUBR1 and may be a downstream target of the spindle checkpoint kinase. *Oncogene* **19**: 4557-4562.

Wu J, Yamagata H, Hayashi-Tsugane M, Hijishita S, Fujisawa M, Shibata M, Ito Y, Nakamura M, Sakaguchi M, Yoshihara R, Kobayashi H, Ito K, Karasawa W, Yamamoto M, Saji S, Katagiri S, Kanamori H, Namiki N, Katayose Y, Matsumoto T and Sasaki T (2004) Composition and

structure of the centromeric region of rice chromosome 8. *The Plant Cell* **16**:967-976.

Xu R and Li QQ (2008) Protocol: streamline cloning of genes into binary vectors in *Agrobacterium* via the Gateway® TOPO vector system. *Plant Methods* **4**:1-7.

Yadav P. K., Singh V. K., Yadav S., Yadav K. D. S., Yadav D. (2009) In silico Analysis of Pectin Lyase and Pectinase Sequences, *Russian in Biokhimiya:* **74** (9) 1286-1293.

Yamaguchi S (2008) Gibberellin metabolism and its regulation. *Annual Review of Plant Biology* **59**:225-251.

Yamamoto K and Sasaki T (1997) Large-scale EST sequencing in rice. *Plant Molecular Biology* **35**:135-144.

Yamamoto Y, Hirai T, Yamamoto E, Kawamura M, Sato T, Kitano H, Matsuoka M and Ueguchi-Tanaka M (2010) A rice *gid1* suppressor mutant reveals that gibberellin is not always required for interaction between its receptor, GID1 and DELLA Proteins. *The Plant Cell* **22**: 3589-3602.

Yanagida M, Yamashita YM, Tatebe H, Ishii K, Kumada K, Nakaseko Y, Andrews PD, Hershko A, Toda T and Raff J (1999) Control of metaphase-anaphase progression by proteolysis: cyclosome function regulated by the protein kinase a pathway, ubiquitination and localization. *Philosophical Transactions: Biological Sciences* **354**:1559-1570.

Yang P, Fu H, Walker J, Papa CM, Smalle J, Ju YM and Vierstra RD (2004) Purification of the *Arabidopsis* 26S proteasome. *Journal of Biological Chemistry* **279**:6401-6413.

Yoshida H, Nagata M, Saito K, Wang KL and Ecker JR (2005) *Arabidopsis* ETO1 specifically interacts with and negatively regulates type 2 1-aminocyclopropane-1-carboxylate synthases. *BMC Plant Biology* **5**:14.

Yu J, Wang J, Lin W, Li S, Li H, Zhou J, Ni P, Dong W, Hu S, Zeng C, Zhang J, Zhang Y, Li R, Xu Z, Li S, Li X, Zheng H, Cong L, Lin L, Yin J, Geng J, Li G, Shi J, Liu J, Lv H, Li J, Wang J, Deng Y, Ran L, Shi X, Wang X, Wu Q, Li C, Ren X, Wang J, Wang X, Li D, Liu D, Zhang X, Ji Z, Zhao W, Sun Y, Zhang Z, Bao J, Han Y, Dong L, Ji J, Chen P, Wu S, Liu J, Xiao Y, Bu D, Tan J, Yang L, Ye C, Zhang J, Xu J,

Zhou Y, Yu Y, Zhang B, Zhuang S, Wei H, Liu B, Lei M, Yu H, Li Y, Xu H, Wei S, He X, Fang L, Zhang Z, Zhang Y, Huang X, Su Z, Tong W, Li J, Tong Z, Li S, Ye J, Wang L, Fang L, Lei T, Chen C, Chen H, Xu Z, Li H, Huang H, Zhang F, Xu H, Li N, Zhao C, Li S, Dong L, Huang Y, Li L, Xi Y, Qi Q, Li W, Zhang B, Hu W, Zhang Y, Tian X, Jiao Y, Liang X, Jin J, Gao L, Zheng W, Hao B, Liu S, Wang W, Yuan L, Cao M, McDermott J, Samudrala R, Wang J, Wong GK-S and Yang H (2005) The Genomes of *Oryza sativa*: A History of Duplications. *PLoS Biology* 3:38.

Zeng YX, Hu CY, Lu YG, Li JQ and Liu XD (2007) Diversity of abnormal embryo sacs in indica/japonica hybrids in rice demonstrated by confocal microscopy of ovaries. *Plant Breeding* **126**:574-580.

Zhang S, Chen L, Qu R, Marmey P, Beachy R and Fauquet C (1996) Regeneration of fertile transgenic indica (group 1) rice plants following microprojectile transformation of embryogenic suspension culture cells. *Plant Cell Reports* **15**:465-469.

Zheng N, Schulman BA, Song LZ, Miller JJ, Jeffrey PD, Wang P, Chu C, Koepp DM, Elledge SJ, Pagano M, Conaway RC, Conaway JW, Harper JW and Pavletich NP (2002) Structure of the Cul1-Rbx1-Skp1-F box(Skp2) SCF ubiquitin ligase complex. *Nature* **416**:703-709.

Zupan JR, Ward D and Zambryski P (1998) Assembly of the VirB transport complex for DNA transfer from *Agrobacterium tumefaciens* to plant cells. *Current Opinion in Microbiology* **1**:649-655.

APPENDICES

APPENDIX 1

Table 1: List of up-regulated genes showing differential expression in *OsAPC6* mutant in comparison to Basmati 370

S. No	Probe set ID	TIGR ID	Fold Change	Gene Function
1	Os.16233.1.S1_a_at	LOC_Os04g09900	15.8 +	GA Metabolism
2	Os.21776.1.S1_at	LOC_Os02g36140	13.1 +	Terpene Synthase
3	Os.54291.1.S1_at	LOC_Os11g42200.1	11.8 +	Laccase
4	Os.33625.4.S1_at	LOC_Os01g70850	11.8 +	Carboxylesterase activity
5	Os.26732.1.S1_at	LOC_Os01g08080	11.7 +	UDP-Glucoronosyl transferase
6	Os.10166.1.S1_at	LOC_Os04g41680.1	11.1 +	Hydrolase and Chitinase activity
7	Os.14951.1.S1_at	LOC_Os08g39730.1	7.54 +	Cytochrome P450
8	Os.26732.1.S1_at	LOC_Os01g08020.1	7.22 +	Boron Transporter
9	Os.7991.1.S1_at	LOC_Os04g41620.1	6.34 +	Chitinase Activity
10	Os.11991.1.S1_at	LOC_Os11g10520.2	6.2 +	Dehydrogenase
11	Os.43596.1.S1_at	LOC_Os01g10110.1	6.20 +	Cytokinin dehydrogenase
12	Os.12191.1.S1_x_at	LOC_Os03g13140.1	5.78 +	Non-Symbiotic hemoglobin 2
13	Os.13008.1.S1_at	LOC_Os08g39850.1	5.76 +	Lipoxygenase
14	Os.57506.1.S1_at	LOC_Os06g37300.1	5.68 +	Cytochrome P450
15	Os.5031.1.S1_at	LOC_Os12g36830.1	5.65 +	Pathogenesis-Related Bet V I Family Protein
16	Os.12191.1.S1_at	LOC_Os03g13140.1	5.63 +	Non-Symbiotic hemoglobin 2
17	Os.15537.1.S1_at	LOC_Os08g39660.1	4.8 +	Cytochrome P450
18	Os.47761.1.S1_at	LOC_Os03g13300.1	4.65 +	Glutamate decarboxylase Calmodulin-Binding
19	Os.7678.1.S1_at	LOC_Os03g12500.1	4.5 +	Cytochrome P450
20	Os.165.1.S1_at	LOC_Os12g36880.1	3.8 +	Pathogenesis-Related Bet V I Family Protein
21	Os.470.1.S1_s_at	LOC_Os01g42410.1	4.66 +	Pleiotropic Drug Resistance Protein
22	Os.3406.1.S1_at	LOC_Os04g51160.1	3.71 +	Transposon Protein
23	Os.2289.1.S1_at	LOC_Os12g39310.1	3.7 +	Cytochrome P450
24	Os.53660.1.S1_at	LOC_Os02g43790.1	3.58 +	Ethylene-responsive Transcription Factor
25	Os.22000.1.S1_at	LOC_Os06g51060.1	3.52 +	CHIT8 - Chitinase Precursor
26	Os.2678.1.S1_at	LOC_Os02g13870.1	3.51 +	Aquaporin Protein
27	OsAffx.28731.1.S1_s_at	LOC_Os07g33780.1	3.42 +	Pleiotropic Drug Resistance Protein
28	Os.27751.1.S1_at	LOC_Os02g36210.1	3.4 +	Ent-Kaurene Synthase, Chloroplast Precursor
29	Os.405.1.S1_a_at	LOC_Os12g37260.1	3.39 +	Lipoxygenase 2.1, Chloroplast Precursor
30	Os.32714.1.S1_at	LOC_Os03g17870.1	3.37 +	Metallothionein

31	Os.24952.1.S1_at	LOC_Os04g57720.1	3.37 +	Osrr6 Type-A Response Regulator
32	Os.26761.1.S1_s_at	LOC_Os02g24600	3.28 +	Unknown function
33	Os.11313.1.S1_at	LOC_Os03g18850.1	3.23 +	Pathogenesis-Related Bet V I Family Protein
34	Os.9145.1.S1_at	LOC_Os05g09440.1	3.19 +	NADP-Dependent Malic enzyme
35	Os.11851.1.S1_at	LOC_Os03g11900.1	3.18 +	Transporter protein
36	Os.11262.2.S1_x_at	LOC_Os04g32920.3	3.13 +	Potassium Transporter protein
37	Os.8178.1.S1_at	LOC_Os11g37970.1	3.10 +	WIP5 - Wound-Induced protein precursor
38	OsAffx.30475.1.S1_s_at	LOC_Os10g21270.1	3.05 +	Unknown function
39	Os.1385.1.S1_at	LOC_Os01g71670.1	2.97 +	Glycosyl Hydrolases
40	Os.3415.1.S1_at	LOC_Os10g39680.1	2.95 +	CHIT14 - Chitinase Family Protein Precursor
41	Os.15171.1.S1_s_at	LOC_Os12g37260.1	2.92 +	Lipoxygenase 2.1, Chloroplast Precursor
42	Os.38984.1.S1_s_at	LOC_Os01g23580.1	2.91 +	Inorganic H+ Pyrophosphatase
43	Os.47303.1.S1_s_at	LOC_Os01g54030.1	2.91 +	NADP-Dependent Malic Enzyme
44	Os.8684.1.S1_a_at	LOC_Os02g09490.1	2.88 +	Dehydrogenase
45	Os.7989.1.S1_at	LOC_Os02g20360.1	2.86 +	Tyrosine Aminotransferase
46	Os.4380.1.S1_at	LOC_Os12g02300.1	2.85 +	LTPL26 - Seed Storage/LTP protein
47	Os.57547.1.S1_at	LOC_Os07g48050.1	2.83 +	Peroxidase precursor
48	Os.22076.1.S1_a_at	LOC_Os01g62430.2	2.79 +	C2 Domain Containing Protein
49	Os.57569.2.A1_s_at	LOC_Os08g04500.1	2.77 +	Terpene Synthase, Signal Transduction
50	Os.11262.1.S1_a_at	LOC_Os04g32920.3	2.76 +	Potassium Transporter
51	Os.21629.1.S1_at	LOC_Os09g39940.1	2.74 +	Plastocyanin-like domain containing protein
52	Os.4807.1.S1_at	LOC_Os04g54200.1	2.63 +	Diacylglycerol Kinase
53	Os.15732.1.S1_s_at	LOC_Os03g29850.1	2.62 +	Metal Cation Transporter
54	Os.3710.1.S1_at	LOC_Os02g35329.1	2.57 +	RING-H2 Finger Protein
55	Os.49507.1.S1_at	LOC_Os04g48850.1	2.56 +	Aminotransferase
56	Os.773.1.S1_s_at	LOC_Os01g43750.1	2.53 +	Cytochrome P450
57	Os.51172.1.S1_x_at	LOC_Os06g51050.1	2.51 +	CHIT7 - Chitinase family protein precursor
58	Os.46813.1.S1_at	LOC_Os10g20610.1	2.43 +	Laccase-15 Precursor
59	Os.53604.1.S1_at	LOC_Os03g52860.1	2.42 +	Lipoxygenase
60	Os.3415.1.S1_s_at	LOC_Os10g39680.1	2.41 +	CHIT14 - Chitinase
61	Os.12738.1.S1_a_at	LOC_Os01g48960.1	2.4 +	Glutamate Synthase
62	Os.12191.1.S1_s_at	LOC_Os03g13140.2	2.4 +	Non-Symbiotic hemoglobin 2
63	Os.24822.1.A1_at	LOC_Os04g10060.1	2.38 +	Ent-Kaurene Synthase
64	Os.4416.1.S1_at	LOC_Os08g39840.1	2.36 +	Lipoxygenase
65	Os.2692.1.S1_x_at	LOC_Os06g51050.1	2.31 +	CHIT7 - Chitinase

				family protein precursor
66	Os.53276.1.S1_at	LOC_Os06g37224.1	2.3 +	Cytochrome P450
67	Os.2416.1.S1_a_at	LOC_Os01g71340.1	2.29 +	Glycosyl Hydrolases
68	Os.17509.1.S1_at	LOC_Os02g50490.1	2.26 +	Endoglucanase
69	Os.49111.1.S1_at	LOC_Os04g48930.2	2.23 +	Ferric-Chelate Reductase
70	Os.12163.1.S1_at	LOC_Os03g12510.1	2.2 +	Non-Symbiotic Hemoglobin 2,
71	Os.29866.1.S1_at	LOC_Os01g52530.1	2.21 +	Phosphoribosyl Transferase
72	Os.20289.1.S1_at	LOC_Os11g37960.1	2.2 +	WIP4 - Wound-Induced Protein Precursor
73	Os.38984.1.S1_at	LOC_Os01g23580.1	2.19 +	H+ Pyrophosphatase
74	Os.15830.1.S1_at	LOC_Os05g39770.1	2.18 +	Aminotransferase
75	Os.20541.1.S1_at	LOC_Os03g37840.1	2.18 +	Potassium Transporter
76	OsAffx.30475.5.S1_x_at	LOC_Os12g10570.1	2.17 +	ATP Synthase Subunit
77	Os.6037.1.S1_at	LOC_Os09g27580.2	2.16 +	Potassium Transporter
78	Os.48057.1.S1_at	LOC_Os03g13390.2	2.09 +	Oxidoreductase, Aldo/Keto Reductase
79	Os.313.1.S1_a_at	LOC_Os02g49720.6	2.07 +	Aldehyde Dehydrogenase
80	Os.25557.1.S1_at	LOC_Os06g46310.3	2.06 +	Metal Transporter Nramp6
81	Os.2210.1.S1_at	LOC_Os08g08970.1	2.01 +	Cupin Domain Containing Protein

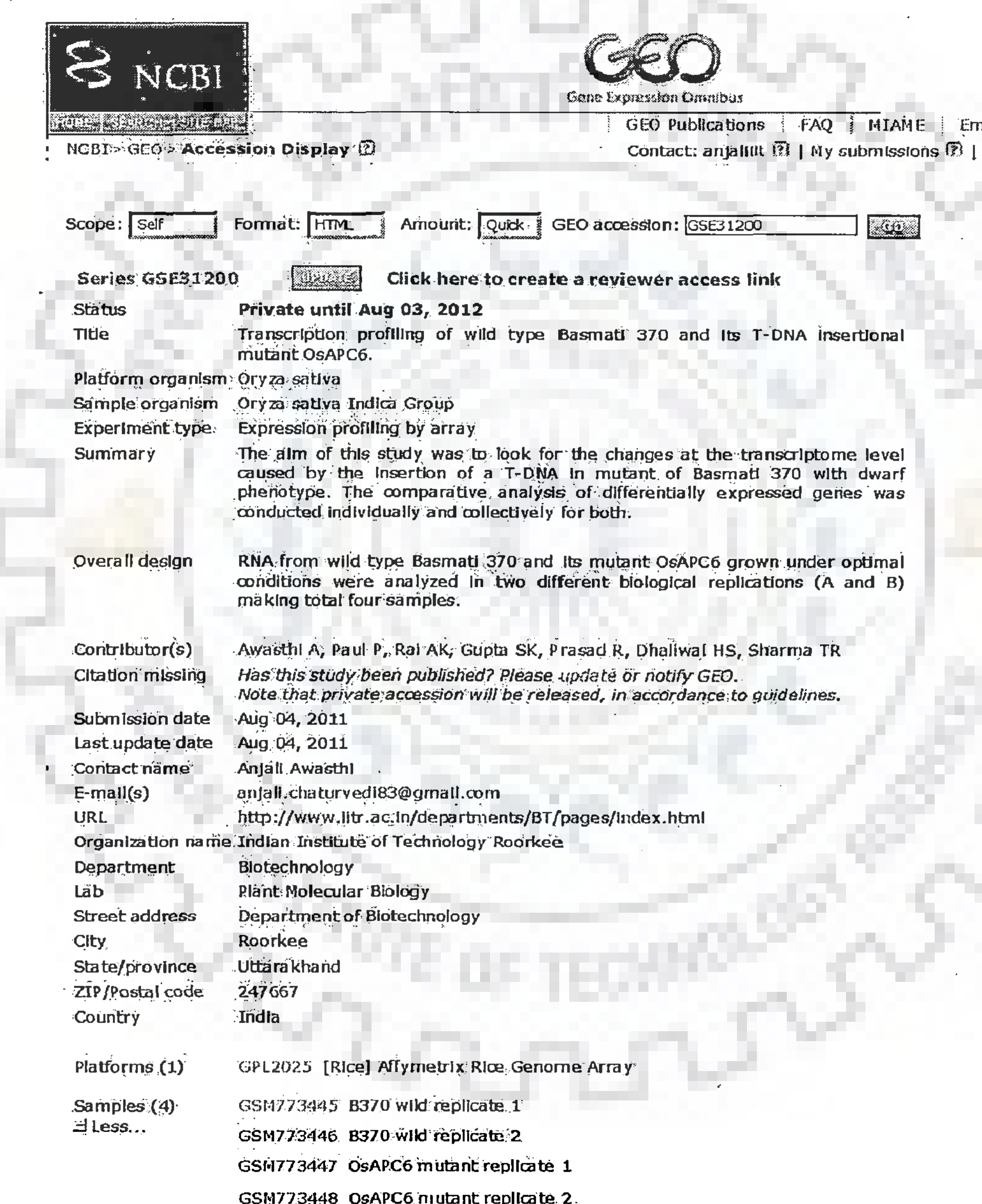
Table 2: A list of down-regulated genes showing differential expression in *OsAPC6* mutant in comparison to Basmati 370

S. No	Probe set ID	TIGR ID	Fold Change	Gene Function
1	Os.11450.1.S2_a_at	LOC_Os01g52110.9	3.23 -	RING finger domain-containing protein
2	Os.5638.1.S1_at	LOC_Os03g16610.1	3.04 -	Laccase precursor protein
3	Os.12201.1.S1_at	LOC_Os09g27750.1	2.44 -	1-Aminocyclopropane-1-Carboxylate Oxidase
4	Os.50175.2.S1_at	LOC_Os04g51820.1	2.34 -	Na+ Transporter
5	Os.37893.1.S1_at	LOC_Os04g43760.1	2.30 -	Phenylalanine Ammonia-Lyase (PAL)
6	Os.50053.1.A1_at	LOC_Os07g05940.1	2.23 -	9-Cis-Epoxycarotenoid Dioxygenase 1
7	Os.27520.1.S1_at	LOC_Os11g02379	2.18 -	LTPL6 –seed storage/LTP family protein precursor
8	Os.2617.2.S1_a_at	LOC_Os06g48810.1	2.10 -	Na+ Transporter
9	Os.12724.1.S1_a_at	LOC_Os08g36320.3	2.02 -	Decarboxylase
10	Os.46690.1.S1_x_at	LOC_Os10g25090.1	2.02 -	Strubbelig-receptor family 6 Precursor, Kinases
11	Os.15729.2.S1_at	LOC_Os04g49570.1	2.00 -	Glutamate Receptor

APPENDIX 2

MIAME DATABASE FILES

GEO Accession viewer http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31200



NCBI > GEO > Accession Display

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Contact: anjaliiit | My submissions | Logout

Scope: Self Format: HTML Amount: Quick GEO accession: GSE31200

Series GSE31200 Click here to create a reviewer access link

Status	**Private until Aug 03, 2012**
Title	Transcription profiling of wild type Basmati 370 and its T-DNA insertional mutant OsAPC6.
Platform organism	Oryza sativa
Sample organism	Oryza sativa Indica Group
Experiment type	Expression profiling by array
Summary	The aim of this study was to look for the changes at the transcriptome level caused by the insertion of a T-DNA in mutant of Basmati 370 with dwarf phenotype. The comparative analysis of differentially expressed genes was conducted individually and collectively for both.
Overall design	RNA from wild type Basmati 370 and its mutant OsAPC6 grown under optimal conditions were analyzed in two different biological replications (A and B) making total four samples.
Contributor(s)	Awasthi A, Paul P, Rai AK, Gupta SK, Prasad R, Dhaliwal HS, Sharma TR
Citation missing	*Has this study been published? Please update or notify GEO. Note that private accession will be released, in accordance to guidelines.*
Submission date	Aug 04, 2011
Last update date	Aug 04, 2011
Contact name	Anjali Awasthi
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URL	http://www.iitr.ac.in/departments/BT/pages/index.html
Organization name	Indian Institute of Technology Roorkee
Department	Biotechnology
Lab	Plant Molecular Biology
Street address	Department of Biotechnology
City	Roorkee
State/province	Uttarakhand
ZIP/Postal code	247667
Country	India
Platforms (1)	GPL2025 [Rice] Affymetrix Rice Genome Array
Samples (4) Less...	GSM773445 B370 wild replicate 1
	GSM773446 B370 wild replicate 2
	GSM773447 OsAPC6 mutant replicate 1
	GSM773448 OsAPC6 mutant replicate 2

The following supplementary file types are provided: CEL, CHP
Raw data provided as supplementary file
Processed data included within Sample table
Processed data provided as supplementary file

Scope: Self Format: HTML Amount: Quick GEO accession: GSE31248

Series GSE31248 Click here to create a reviewer access link

Status	**Private until Aug 04, 2012**
Title	Transcription profiling of Basmati T-DNA insertional mutant lines
Platform organism	Oryza sativa
Sample organism	Oryza sativa Indica Group
Experiment type	Expression profiling by array
Summary	The aim of this study was to look for the changes at the transcriptome level caused by the same T-DNA cassette in three mutants of Basmati 370 with different phenotypes. The comparative analysis of differentially expressed genes was conducted individually and collectively for all three mutants.
Overall design	RNA from Basmati mutants OsPE, Osoc and Ossl grown under optimal conditions were analyzed in two different biological replications (A and B) making total six samples.
Contributor(s)	Paul P, Awasthi A, Rai AK, Gupta SK, Prasad R, Dhaliwal HS, Sharma TR
Citation missing	*Has this study been published? Please update or notify GEO.* *Note that private accession will be released, in accordance to guidelines.*
Submission date	Aug 08, 2011
Last update date	Aug 08, 2011
Contact name	Anjali Awasthi
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URL	http://www.iitr.ac.in/departments/BT/pages/Index.html
Organization name	Indian Institute of Technology Roorkee
Department	Biotechnology
Lab	Plant Molecular Biology
Street address	Department of Biotechnology
City	Roorkee
State/province	Uttarakhand
ZIP/Postal code	247667
Country	India
Platforms (1)	GPL2025 [Rice] Affymetrix Rice Genome Array
Samples (6) Less...	GSM774603 OsPE mutant replicate 1
	GSM774604 OsPE mutant replicate 2
	GSM774605 Osoc mutant replicate 1
	GSM774606 Osoc mutant replicate 2
	GSM774607 Ossl mutant replicate 1
	GSM774608 Ossl mutant replicate 2

The following supplementary file types are provided: CEL
Raw data provided as supplementary file
Processed data included within Sample table
Processed data provided as supplementary file

APPENDIX 3

Components used for media preparation

Medium	Components
***Luria-Bertani* (LB) medium**	100 ml
	1% Triptone
	0.5% Sodium Chloride (NaCl)
	0.5% Yeast extract
	pH 7.0
YEP medium	100 ml
	1% Peptone
	1% Yeast extract
	0.5% Sodium Chloride (NaCl)
	pH 7.2
Murashige and Skoog (MS) medium	1L
	1650 mg NH_4NO_3
	1900 mg KNO_3
	440mg $CaCl_2.2H_2O$
	370 mg $MgSO_4.7H_2O$
	170 mg KH_2PO_4
	6.2 mg H_3BO_3
	16.9 mg $MnSO_4.H_2O$
	8.6 mg $ZnSO_4.H_2O$
	0.83 mg KI
	0.25 mg $NaMoO_4.2\ H_2O$
	0.025 mg $CuSO_4.5H_2O$
	0.025 mg $CoCl_2.6H_2O$
	27.8 mg $FeSO_4.7H_2O$
	37.3 mg $Na_2EDTA.2H_2O$
	100 mg myo-inositol
	0.5 mg nicotinic acid
	0.1 mg thiamine.HCl
	0.5 mg pyridoxine.HCl
	2 mg glycine
	pH 5.8
	30 gm sucrose
Regeneration medium	1L
	MS medium
	0.5 mg kinetin
	5mg NAA
	30 mg sucrose
Rooting medium	1L
	MS medium
	5 mg/l NAA
	70 mg sucrose
Winans AB Minimal medium	100 ml
AB glucose	45 ml

	0.25 gm Glucose (0.5% w/v)
AB buffer	100 ml
	6 gm Potassium dihydrogen orthophosphate
	2 gm Sodium dihydrogen orthophosphate
	pH 7.0
AB salt	100 ml
	2.0 gm Ammonium chloride
	0.6 gm Magnesium sulphate hepta hydrate
	0.3 gm Potassium chloride
	0.3 gm Calcium chloride
	0.005 gm Ferrous sulphate hepta hydrate

APPENDIX 4

PUBLICATIONS

A. ***In Journals***

1. **Anjali Awasthi,** Priyanka Paul, Satish Kumar, Shailender Kumar Verma, R. Prasad, H.S.Dhaliwal **(2012),** Abnormal endosperm development causes female sterility in rice insertional mutant *OsAPC6,* Plant Science, **183**:167-174.

2. **Anjali Awasthi,** Priyanka Paul, Amit Kumar Rai, Santosh Kumar Gupta, Ramasare Prasad, T.R.Sharma, H.S.Dhaliwal **(2011),** Transcriptome analysis of a Basmati rice mutant OsAPC6 provides an insight into the role of APC6 in GA-signaling, Communicated with Journal of Experimental Botany

3. **Anjali Awasthi**, Priyanka Paul, Ramasare Prasad, T.R.Sharma, H.S.Dhaliwal **(2011),** Effect of single T-DNA insertion at different positions leads to different phenotype in rice, Communicated with Molecular Plant.

4. **Anjali Awasthi**, Sonali Dhindwal, Priyanka Paul, Amit Kumar Rai, Ramasare Prasad, Parvindra Kumar, H.S.Dhaliwal, T.R.Sharma **(2011),** Prediction of protein interaction between APC6 and its substrates using bioinformatics, Manuscript under preparation.

B. ***In conference proceeding:***

5. **Anjali Awasthi,** Priyanka Paul, R. Prasad, H. S.Dhaliwal, **(2010)**, Study of the functional domains of proteins by using bioinformatics, *"Complexity in Periodically Structured Systems"* Max Planck Institute, Dresden, Germany

C. ***MIAME Database:***

8. **Anjali Awasthi,** Priyanka Paul, Amit Kumar Rai, Santosh Kumar Gupta, R. Prasad, H.S.Dhaliwal, T.R.Sharma (2011), Transcription profiling of wild type Basmati 370 and its T-DNA insertional mutant OsAPC6 (Accession number:GSE31200) http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31200

9. Priyanka Paul, **Anjali Awasthi,** Amit Kumar Rai, Santosh Kumar Gupta, R. Prasad, H.S.Dhaliwal, T.R.Sharma (2011), Transcription profiling of T-DNA insertional mutant lines of Basmati 370 (Accession number:GSE31248) http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31248

D. Material Registration:

10. Deepak Rajpurohit, **Anjali Awasthi**, Priyanka Paul, Satish Kumar, Rahul Kumar, Kuldeep Singh, Harcharan S. Dhaliwal **(2011),** Registration of high yielding pyramid lines of Type 3 Basmati with two bacterial leaf blight resistance and a semidwarfing gene using marker-assisted backcross breeding, Registration of Plant Germplasm, NBPGR, New Delhi

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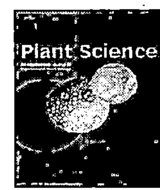

Abnormal endosperm development causes female sterility in rice insertional mutant *OsAPC6*

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ABSTRACT

A T-DNA insertional mutant *OsAPC6* of rice, with gibberellic acid insensitivity and reduced height, had up to 45% reduced seed set. The insertion occurred on chromosome 3 of rice in the gene encoding one of the subunits of anaphase promoting complex/Cyclosome APC6. The primary mother cells of the mutant plants had normal meiosis, male gametophyte development and pollen viability. Confocal laser scanning microscopic (CLSM) studies of megagametophyte development showed abnormal mitotic divisions with reduced number or total absence of polar nuclei in about 30–35% megagametophytes of *OsAPC6* mutant leading to failure of endosperm and hence embryo and seed development. Abnormal female gametophyte development, high sterility and segregation of tall and gibberellic acid sensitive plants without selectable marker *Hpt* in the selfed progeny of *OsAPC6* mutant plants indicate that the mutant could be maintained in heterozygous condition. The abnormal mitotic divisions during megagametogenesis could be attributed to the inactivation of the APC6/CDC16 of anaphase promoting complex of rice responsible for cell cycle progression during megagametogenesis. Functional validation of the candidate gene through transcriptome profiling and RNAi is in progress.

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1. Introduction

Rice (*Oryza sativa* L., $2n = 24$) is the second most important cereal and the staple food for more than one-third of the world's population. After the complete genome sequencing of *Oryza sativa* L. [1], it is highly imperative to functionally characterize the annotated genes. Several approaches of reverse genetics have been used for functional genomics [2,3] and a large number of insertional mutants have been generated [4] in *Arabidopsis* and rice for gene annotation [5–7].

In about 70% angiosperms including *Arabidopsis* and rice, embryo sac development is of polygonum type [8,9] having two stages of development, megasporogenesis followed by megagametogenesis, occurring inside the ovule. An archesporial cell from nucellus undergoes one mitotic division to produce a megasporocyte and a somatic cell. The megasporocyte (or megaspore mother cell) undergoes meiosis to form four megaspores out of which the megaspore closest to the chalaza enlarges while the three non-functional megaspores degenerate and are eventually crushed by the expanding functional megaspore [8]. This chalazal megaspore undergoes three successive mitotic divisions to form seven-celled coenocytic megagametophyte. Wall formation, nuclear migration and differentiation follow further, forming a mature seven-celled and eight-nucleated embryo sac [10]. Three cells opposite to the micropylar end form antipodal cells, three cells at the micropylar end form the egg apparatus consisting of an egg cell and two synergids. During cellularization one nucleus from each pole migrates towards the centre forming the polar nuclei or the central cell. In angiosperms, double fertilization occurs where one sperm cell fuses with egg cell forming zygote while the second with central cell resulting in the formation of endosperm [11].

A number of mutants defective in gametogenesis have been isolated and described in model plants [12]. Several mutants in *Arabidopsis* with defective cell cycle progression such as mutations affecting division initiation and regulation during the three rounds of mitotic nuclear divisions have been reported [13,14]. In rice defective male gametogenesis has been reported in a T-DNA insertional mutant *rip1* where the insertion was in the *rice immature pollen 1*, a pollen preferrential gene [15].

The cell cycle in eukaryotes is regulated by coordinated destruction of cell cycle regulatory proteins through ubiquitin mediated 26S proteosome. The key component of the proteolytic system is a multi-subunit ubiquitin ligase and anaphase-promoting complex/cyclosome (APC/C) that provides a platform and specificity for the ubiquitination reactions [16]. The APC/C is essential for the ubiquitin-dependent degradation of cell cycle regulatory proteins. APC/C regulates mitosis including metaphase–anaphase transition and mitotic exit and maintains G1 phase. The structure of APC is conserved among eukaryotes and phylogenetic analyses

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indicate that some APC subunits have experienced gene duplication events in plants, in contrast to animals [17]. In general, plant APC/C has 11 core subunits, where APC2 which is a distant member of the cullin protein family that functions as a scaffold in SCF (SKP1, Cullin/CDC53, F-box protein) assembly [18]. APC11 is similar to the RING-H2 finger protein Rbx1, which plays a key function in the ubiquitylation reaction [19]. The largest APC/C subunit (APC1) also shares some homology with two subunits of the 26S proteosome. The TPR-repeat-containing APC/C subunits (APC3, APC6, APC7 and APC8), the Doc-domain protein APC10/DOC1 and the RING-H2 domain protein APC11 are some other subunits identified in plants. The function of some of these subunits has been characterized. Initial evidence supporting a particular role for the APC/C in plants came from the characterization of the *CCS52* gene, a functional homolog of the yeast CDH1 activator [20] and its role in cell division comes from characterization of cohesin mutants [21,22]. In *Arabidopsis*, expression of several subunits of APC has been detected suggesting that in plants the complex might be regulated by subunit availibility and that different subunits could play unique regulatory roles [23]. Mutations in *HOBBIT/CDC27b* gene in *Arabidopsis* strongly affects cell division and differentiation after embryogenesis [24]. The CDC27a subunit of APC in *Arabidopsis* was ectopically expressed in *Nicotiana tabacum* and was found that the overexpression of *AtCDC27a* exhibits increased growth rate and organ size indicating multiple roles during plant development [25]. The role of plant APC/C in hormone regulation has been studied in *Arabidopsis* where a T-DNA insertion in 5′ regulatory region of *APC10* gene results in its overexpression, thereby indicating its role in auxin and ethylene signalling [26]. Also a link between APC/C and processes associated with auxin regulation and vascular development in stems and cotyledons has been documented where reduced levels of APC6 and APC10 subunits have been reported, indicating a complex role of APC/C in plant development [27]. In yeast specific mutation in either CDC16 or CDC23 results in stabilization of cyclin B leading to anaphase arrest [28]. Importantly, *cdc16* and *cdc23* mutants are not only defective in exit from mitosis but are also defective for separation of sister chromatids at the metaphase-to-anaphase transition [29]. In another mutant *MtCDC16* of *Medicgo truncatula* reduced number of lateral roots, a fourfold increase in nodules and reduced sensitivity to auxin was observed as a result of partial suppression of CDC16 subunit of APC indicating a potential function of CDC16 in auxin signalling [30].

A superfine quality *indica* rice cultivar Basmati 370 was transformed through *Agrobacterium tumefaciens* strain EHA101 [31] and a T-DNA insertional mutant *OsAPC6* of Basmati 370 was isolated [5]. Single copy insertion was confirmed using Southern hybridization and TAIL-PCR. The T-DNA flanking region sequenced through thermal asymmetric interlaced polymerase chain reaction showed a single hit on chromosome 3 of japonica rice cultivar Nipponbare in the second exonic region of a gene which encodes for one of the subunits *APC6* of anaphase promoting complex. The insertion led to knock-down of the *APC6* gene. The mutant with dark green leaves and normal pollen viability had reduced plant height (nearly 50%) and seed set (up to 40–45%) and insensitivity to exogeneous GA_3.

The present article deals with the confocal laser scanning microscopic (CLSM) studies of female gametophyte development in the rice *OsAPC6* mutant with high female sterility.

2. Materials and methods

2.1. Plant material

The development of T-DNA insertion lines by Dhaliwal et al. [31] and isolation and characterization of gibberellic acid insensitive dwarf insertional mutant *OsAPC6* in Basmati rice have already been described in detail by Kumar et al. [5]. From the selfed progeny of *OsAPC6* mutant, dwarf plants having partially fertile panicles were taken to investigate the cause of sterility in the mutant. The isogenic wild type Basmati 370 panicles having normal fertility was taken as control. All the plant material was sown in the green house at the Indian Institute of Technology, Roorkee, Uttarakhand, India.

2.2. Cytological analysis

For meiotic analysis, panicles from the mutant plants were fixed in Carnoy's solution (6 ethanol: 3 chloroform: 1 acetic acid) for 24 h and transferred to 70% ethanol. Anthers at various stages of meiotic division were squashed in 2% acetocarmine, and pollen mother cells (PMCs) were screened for chromosome number and pairing. Photographs were taken with a digital camera (Canon PC1049, No. 6934108049).

2.3. Pollen viability

Pollen grains from the mutant and wild type Basmati 370 plants were collected from freshly dehiscing florets and anthers. Pollen viability in the mutant was recorded after I_2-KI staining. The stained pollen grains were observed by the Zeiss Aristoplus (Germany) microscope. Well developed and round pollen grains with deep blue coloured starch grains were classified as fertile while the smaller grains with pale yellowish colour as sterile.

2.4. Fixation of material for CLSM

Rice panicles were collected at different developmental stages from Basmati 370 and its *OsAPC6* mutant. Florets with open glumes with mature embryo sacs ready for fertilization were collected at noon. All the samples were fixed in FAA (formaldehyde, acetic acid and 50% ethanol in 5:6:89 ratio) for at least 24 h, then washed with 50% ethanol and stored in 70% ethanol at 4 °C.

2.5. Staining and mounting of material

The ovaries were dissected from the florets in 70% ethanol under a binocular dissecting microscope, and hydrated sequentially in 50% ethanol, 30% ethanol and distilled water. The staining procedure for ovaries was according to [32] but the florescent dye used here was Eosin Y ($C_{20}H_6Br_4Na_2O_5$, FW 691.9), a tissue stain. The ovaries were pretreated in 2% aluminium potassium sulphate for 20 min to allow the dye to enter the embryo sac more readily. The ovaries were then stained with 1 g/l Eosin Y solution (dissolved with 1.6 ml glacial acetic acid) for 10–12 h at room temperature. The samples were post-treated in 2% aluminium potassium sulphate for 20 min in order to remove excess dye from the ovary walls. The samples were rinsed with distilled water three times, and dehydrated with a series of ethanol solutions (30%, 50%, 70%, 90% and 100%). Subsequently, the dehydrated samples were transferred to a mixture of absolute ethanol and methyl salicylate (1:1) for 1 h, and then cleared in pure methyl salicylate solution for at least 1 h. The mounting was done in 80% glycerol and slides prepared were sealed with nail paint.

2.6. Scanning of embryo sacs

The mounted ovaries were scanned under Leica TCS SP5 (AOBS-Acousto optical beam Splitter based) Laser Scanning Confocal Microscope (CLSM). CLSM was equipped with Ar Laser and AOBS filter set for illumination. The excitation wavelength was 514 nm and the emission wavelength was detected to be between 525 and 600 nm. The images were collected at 0.2–0.4 μm optical sections using 20× and 40×. The images were collected using LAS AF version

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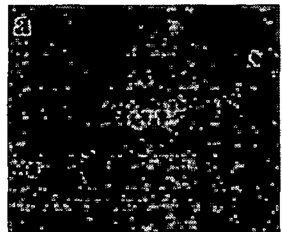

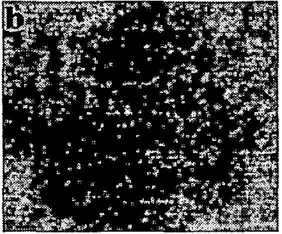

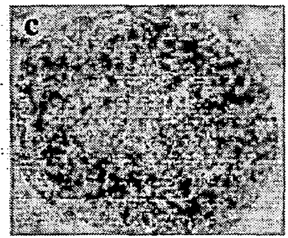

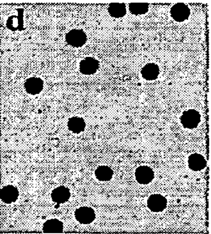

Fig. 1. Chromosome pairing and pollen grain stainability in *OsAPC6* mutant. (a) 12 bivalents ($2n = 24$ chromosomes) at metaphase I. (b) Normal chromosome disjunction to the opposite poles during anaphase I. (c) Early telophase I where the chromosomes have moved to the opposite poles. (d) Nearly normal pollen stainability/viability.

2.3.5. build 5379 software (Leica). Analysis and processing of the images was done using LAS AF version 2.0.2. build 2038 software (Leica).

2.7. RT (reverse transcription) PCR

The seeds of the mutants and wild type Basmati 370 were grown till three-leave seedling stage. The total RNA was isolated from these seedlings using the Spectrum^{TM} Plant Total RNA Kit obtained from Sigma Aldrich. RNA concentration was measured spectrophotometrically and quality of RNA was determined using denaturing formaldehyde gel. The mRNA was reverse transcribed using the superscriptTM II RT Kit (Invitrogen) and 2 µl of it was used for further PCR amplification using gene specific primers. Normalization of the signals obtained for the gene of interest was done using a housekeeping gene Elongation factor 1α (*Ef1α*) as a reference gene. Two sets of gene specific primers based on the coding sequence of the candidate gene were used for RT-PCR. Primer A was designed flanking the region of insertion and primer B was designed to amplify the 3′ region of the gene. The sequence of the primers used is as follows:

Primer A-F: 5′-AAAATTCGGTTTCCCTCCAG-3′
Primer A-R: 5′-ACGCGTACAGGTGCTTCC-3′
Primer B-F: 5′-AGCGCGTTTGCTGGTCTTGCTT-3′
Primer B-R: 5′-GTCCGAGCAGTGATTTGACAGCTGG-3′

3. Results

3.1. Chromosome pairing and pollen viability in OsAPC6

Meiotic analysis at metaphase, anaphase and early telophase I of the pollen mother cells of the mutant from the fixed florets showed normal chromosome pairing with 12 bivalents, normal movement of homologous chromosomes to two poles during anaphase I, normal telophase without any micronuclei (Fig. 1a–c) and normal pollen tetrads. On an average 92–98% pollen grains were stainable and hence viable in most of the florets (Fig. 1d). The results of normal meiotic chromosome pairing and high pollen stainability similar to that of the wild parent Basmati 370 indicated that microsporogenesis and male gametophyte development were normal in *OsAPC6* mutant.

3.2. Maintenance of OsAPC6 mutant

The *OsAPC6* insertional mutant plants with reduced height, dark green leaves, partial female sterility and gibberellic acid insensitivity could not be maintained as true breeding homozygous mutant after several generations of selfing and selection since its original isolation [31]. A few tall, light green and fertile plants without gibberellic acid insensitivity and the *Hpt* selectable marker similar to the wild type Basmati 370 parent appeared in the selfed progeny of all the *OsAPC6* plants. Normal pollen viability and failure to achieve homozygosity could be attributed to abnormal meiosis or female gametophyte development in the *OsAPC6* mutant.

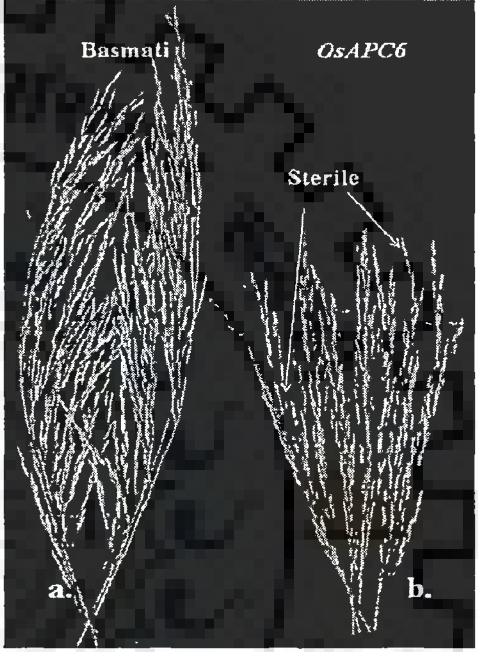

Fig. 2. Female fertility in rice panicles (a) wild type Basmati 370, (b) *OsAPC6* dwarf mutant (with reduced seed set and height) at maturity. The sterile florets have green coloured hulls. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.3. Female fertility

There was reduced seed set up to 40–45% in the mature panicles of *OsAPC6* as compared to the wild type parent Basmati 370 (Table 1). Significantly less than 50% sterility in the mutant plants indicates incomplete penetrance of *OsAPC6* in causing female sterility due to which some of the female gametophytes with the lethal mutant allele developed normally. The sterile florets with empty hulls stayed green while the fertile florets had well filled grains with brown hulls (Fig. 2). Furthermore, the panicles were more compact due to dwarfness and gibberellic acid insentivity in *OsAPC6* as reported previously by Kumar et al. [5]. More than 50% germination of seeds in the selfed progeny of *OsAPC6* mutant plants at 80 ppm hygromycin (Table 2), further indicates incomplete penetrance of the mutation.

3.4. Female gametophyte development

Normal male gametogenesis of the mutant indicated that the sterility could be due to defective megagametogenesis in the

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Table 1
Female sterility observed in mature panicles of mutant *OsAPC6* and wild type Basmati 370 plants.

Mutant plant	Fertile florets	Sterile florets	Percentage sterility	χ^2 value at df = 1	
				Observed χ^2 at fertile: sterile 1:1 ratio	Level of significance
OsAPC6-1	170	118	40.9	9.38	$p \leq 0.005$
OsAPC6-2	166	128	43.3	4.90	$p \leq 0.05$
OsAPC6-4	121	91	42.9	4.24	$p \leq 0.05$
OsAPC6-5	115	85	42.5	4.50	$p \leq 0.05$
Basmati 370	221	05	97.7	–	–

Table 2
Hygromycin resistance in well-developed seeds of four plants of *OsAPC6* mutant and the wild type Basmati 370.

Plant	Resistant seeds	Susceptible seeds	Percentage resistance	χ^2 value at df = 1	
				Observed χ^2 at resistant: susceptible 1:1 ratio	Level of significance
OsAPC6-1	69	51	57.5	3.33	$p \leq 0.10$
OsAPC6-2	81	49	62.3	7.87	$p \leq 0.05$
OsAPC6-4	77	53	59.2	4.40	$p \leq 0.05$
OsAPC6-5	78	52	60.0	3.80	$p \leq 0.05$
Basmati 370	0	50	0.0	–	–

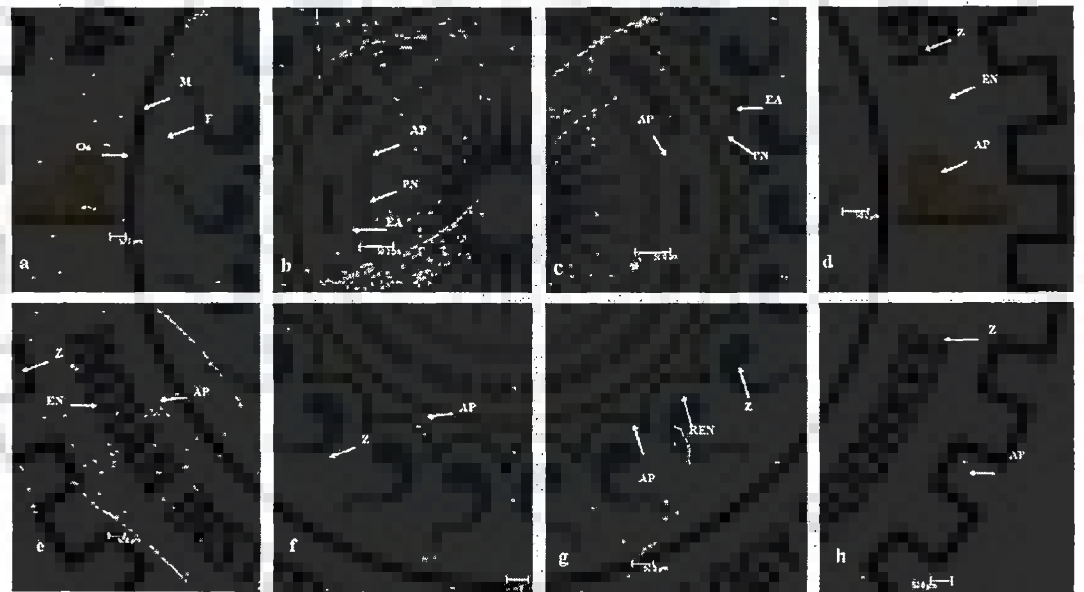

Fig. 3. Female gametophyte at different stages of development in wild type Basmati 370 and *OsAPC6* mutant. (a) Anatropous ovule. (b and c) Mature embryo sacs at different stages of development in WT. (d) Mature embryo sac in WT showing endosperm formed soon after fertilization. (e) Mature embryo sac in WT showing fully developed endosperm. (f) Mature embryo sac in mutant with no endosperm. (g) Mutant embryo sac showing fertilized zygote but limited endosperm development. (h) Zygote is developed normally but endosperm is absent in the mutant. AP = antipodal cell, EA = egg apparatus, EC = embryo sac cavity, EN = endosperm, F = funiculus, Oe = ovule, M = micropyle, PN = polar nucleus, REN = reduced endosperm, Z = zygote. Bar = 50 μm.

mutant. To determine the nature of defect in *OsAPC6* female gametophyte, we analyzed the embryo sac formation at different developmental stages using CLSM. Each floret observed in the mutant and wild type had an ovary where the ovule was bent back on itself at nearly 180° (Fig. 3a). An embryo sac with seven-cell and eight-nuclei structure within a large embryo sac cavity was also observed in wild type Basmati 370 (Fig. 3b). A mature and fully receptive embryo sac of the wild type Basmati 370 with three antipodal cells, two polar nuclei and an egg apparatus was observed (Fig. 3c). After double fertilization of the egg cell and the polar nuclei with the two respective sperm nuclei, zygote was formed and endosperm formation and the degeneration of the antipodal cells were initiated (Fig. 3d). A fully developed syncytial endosperm was formed in the wild type which filled the embryo sac cavity completely (Fig. 3e).

CLSM studies in case of *OsAPC6* mutant showed that the fertilization was normal as zygote was formed and the degenerating antipodal cells were also observed (Fig. 3f, g and h). On further analysis of immature embryo sacs we found that female gametophytes of wild type Basmati 370 had normal mitotic division (Fig. 4a and b) while in the mutant gametophyte a cell at the micropylar end with brighter nuclei and a dividing cell at the chalazal end with two nuclei was observed (Fig. 4c). The chalazal end cell was probably undergoing second mitosis whereas in the micropylar end cell the division was delayed or arrested. In Fig. 4d vacuole formation by the degradation of the nucellar tissue can be observed but towards the

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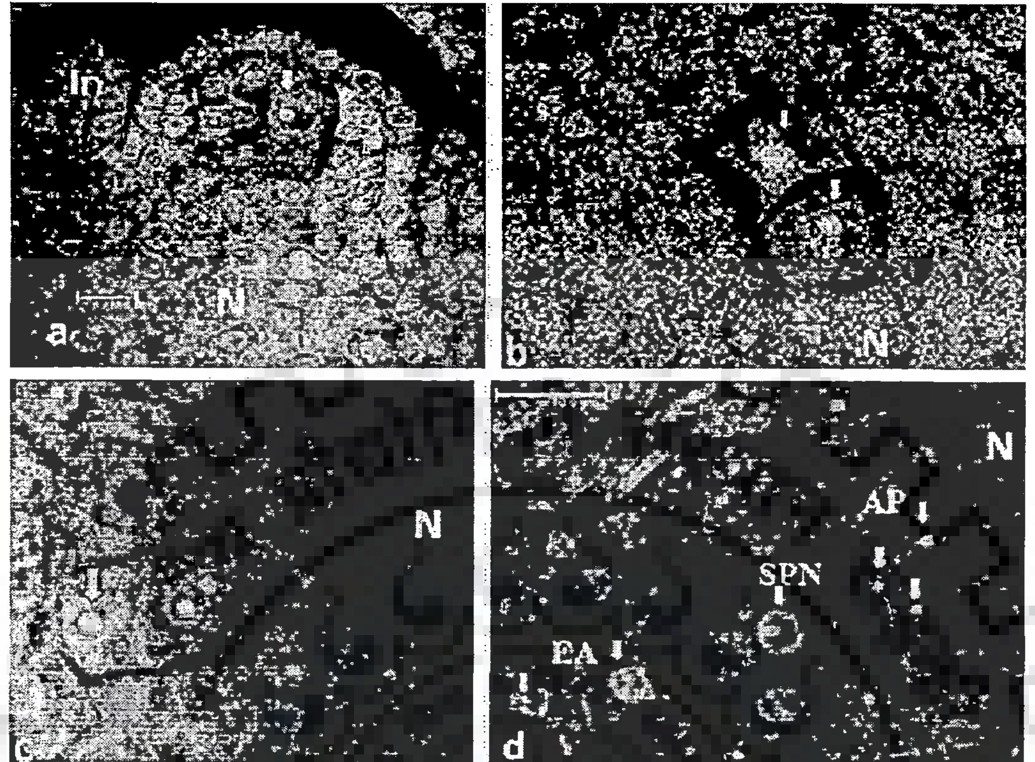

Fig. 4. Megagametogenesis in *OsAPC6*. (a) A megaspore ready to enter mitotic-phase (arrowhead). (b) Two nuclei formed after normal first mitotic division. (c) Nucleus (arrowhead) with arrested second mitosis in the mutant. (d) Seven nuclei (arrowhead) after three rounds of mitosis in the mutant and single nucleus instead of two nuclei in the secondary polar cell. AP = Antipodal cell, In = integuments, EA = egg apparatus, N = nucellar tissue, SPN = single polar nucleus. Bar = 10 μm.

central part of the gametophyte single nucleus instead of two polar nuclei was observed. This could be probably due to the abnormal third mitotic division in one of nuclei formed after second mitosis which led to formation of seven nuclei in place of eight. We found that the female gametophytes were arrested at three-nucleate or seven nucleate stages, indicating that *APC6* loss-of-function impairs megagametogenesis after first or second mitotic division. Abnormal gametophyte development was observed in about 30–35% florets (Table 3). Significantly reduced abnormality in female gametophyte development than the expected 50% in a heterozygous mutant progeny further substantiates the association between the mutant and female sterility and reduced penetrance of the *OsAP6* mutant.

3.5. Expression of APC6 in the wild type and mutant plants

Reverse transcription PCR was performed to check the expression of the *APC6* gene in the wild type and the mutant plant mRNA. Normalization of the signals obtained for the gene of interest was done using a housekeeping gene Elongation factor 1α (*Ef1α*) as a reference gene (Fig. 5a). The mutant progeny segregating for height consisted of tall and dwarf plants. The expression of *APC6* gene was studied in two dwarf and a homozygous tall plant of the mutant progeny together with the wild type Basmati 370 using two primers. The primer pair A was designed flanking the region of insertion while the second primer pair B was used to amplify the 3′end region of the mRNA as shown in Fig. 6. There was reduced expression for the *APC6* gene in the two dwarf plants while the tall plant had similar expression pattern for *APC6* gene as that of the wild type Basmati 370 for both the primer pairs (Fig. 5b and c). Further amplification of *Hpt* gene was checked and it was found that the dwarf plants were positive for *Hpt* while the tall and wild type had no *Hpt* amplification (Fig. 5d). These results indicate that the mutant dwarf plants with *Hpt* selectable marker and the T-DNA insertion had significantly reduced expression of *OsAPC6* transcript. The mutant phenotype was due to knock-down of the *APC6* gene and mutant dwarf plants with reduced seed set could be maintained only in heterozygous condition.

Table 3
CLSM analysis of megagametophyte development in fixed florets from panicles of *OsAPC6* mutant plants.

Florets per panicle	Abnormal florets	Normal florets	Percentage abnormality	χ^2 value at df = 1	
				Observed χ^2 at normal: abnormal 1:1 ratio	Level of significance
93	30	63	32.2	11.6	$p \leq 0.05$
88	28	60	31.8	11.6	$p \leq 0.05$
105	36	69	34.9	11.6	$p \leq 0.05$
103	36	67	34.9	11.6	$p \leq 0.05$
105	33	72	31.4	9.3	$p \leq 0.05$
94	31	63	32.9	14.4	$p \leq 0.05$

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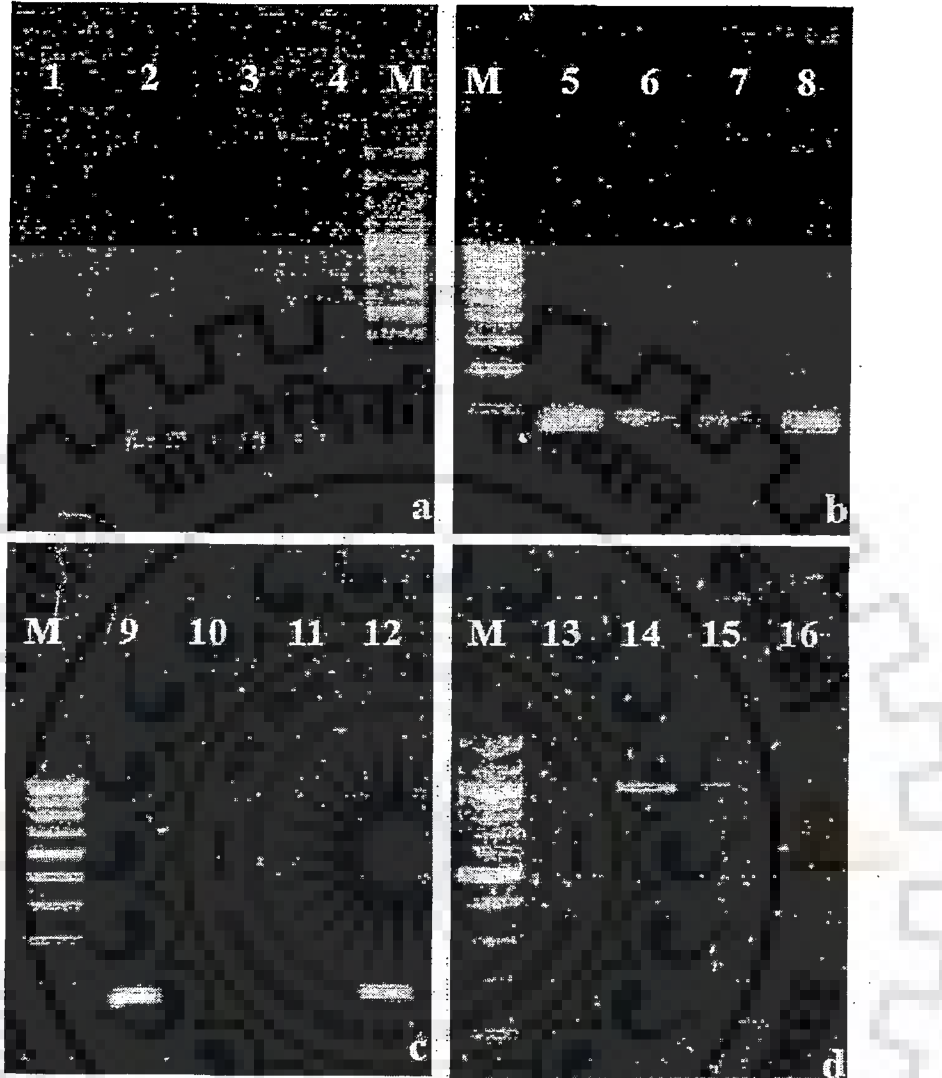

Fig. 5. RT-PCR results and *Hpt* amplification in wild type and mutant plants. (a) RT-PCR result using *EF1α* (house keeping gene). (b) RT-PCR using *OsAPC6* gene specific primer pair A. (c) RT-PCR using *OsAPC6* gene specific primer pair B. (d) *Hpt* PCR. Lane 1, 5, 9 and 13 wild type Basmati 370. Lane 2, 3,6,7,10,11, 14 and 15 heterozygous dwarf mutant plants and Lane 4, 8, 12 and 16 homozygous tall plants obtained from segregating mutant population. *M* = 100 bp ladder.

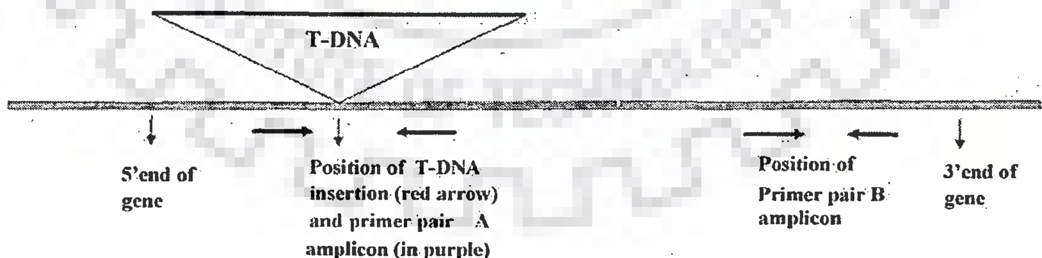

Fig. 6. T-DNA insertion in the *APC6* gene and position of primer pairs A and B used for RT-PCR.

4. Discussion

Normal meiosis and pollen development and slightly higher than 50% fertility and hygromycin resistance in the selfed progeny of the *OsAPC6* mutant plants strongly suggest female gametophyte lethality and incomplete penetrance of the mutant allele. Normal male gametophyte and pollen development have been reported in several mutants like *apc2* and *nomega*, defective in megagametogenesis [33,34]. The expression analysis of the *APC6* gene using RT-PCR showed reduced expression in the mutant indicating that the T-DNA insertion knocked out the candidate gene expression. The tall plants without the *Hpt* selectable marker used for transformation were found to be normal with almost similar expression of *APC6* gene as that of wild type. All these results unequivocally indicate that the mutant with female gametophyte lethality could be maintained as heterozygous.

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The CLSM results showed that the fertilization and the zygote development in both the wild type and mutant were normal. There was, however, no endosperm development in a high proportion of female gametophytes of the *OsAPC6* mutant due to abnormal polar nuclei formation. During double fertilization in angiosperms, one sperm nucleus from the pollen grain fuses with the egg cell and the resultant zygote develops into an embryo while the central cell with two polar nuclei fuses with the second sperm nucleus to form a triploid primary endosperm cell which develops into the endosperm [35]. Endosperm, as a nutritive source in plants, is generally utilized for embryo development and germination [36]. In *OsAPC6* mutant the fertilization was found to be normal but there was reduced seed set up to 40–45% which could be attributed to the absence of endosperm.

Further megagametogenesis studies showed abnormal second mitosis as one of the nucleus at the micropylar end was found to be more prominent and bright indicating an arrest or delayed division. Also in one of the observed cases there were seven nuclei in the mutant embryo sac. Normally it is expected to have eight nuclei after three consecutive mitotic divisions but since seven nuclei were observed, indicating that there was an abnormal progression of cell division where one of the nucleus did not either enter third mitotic division or the division was delayed thus the polar nuclei of the central cell in the mutant were found to be either reduced in number or were completely absent. As it is known that in the Polygonum type embryo sac development, the nuclear migration occurs during cellularization and one nucleus from each of the poles moves towards centre thereby forming two polar nuclei and a central cell [37]. In case of *OsAPC6* mutant there was reduction in total number of nuclei at the end of megametogenesis, reduced number or complete absence of the polar nuclei and abnormal development of central cell and endosperm in the mutant.

Abnormal central cell development during megagametogenesis due to arrested or delayed mitotic divisions led to failure of endosperm development and reduced seed set. Several mutations affecting nuclear division, polar nuclear fusion and cell specification associated with megagametogenesis have been described. The majority of the mutants show defects in nuclear division. These include defects in the first division as in female gametophyte 2 (*fem2*), *fem3*, *gametophytic factor* (*gf*), *gametophytic factor 4* (*gfa4*) and *gfa5* of *Arabidopsis* [38], in the second or third division as in *cell division cycle 16* (*cdc16*) [34] and *prolifera* (*prl*) [39] or all the three divisions as in *hadad* (*hdd*) [13]. Mutation in PRL, a homologue of DNA replication factor Mcm7, causes embryo sac arrest at the four nucleate stage [39]. A case of slow and arrested cell division in female gametophyte at 2–8 nucleate stage was reported in the *slow walker 2 (swa2)* [40] and the *slow walker 3 (swa3)* [41] mutant of *Arabidopsis* with female sterility. Confocal laser scanning microscopy in another *Arabidopsis* mutant *Gf*, defective in female gametogenesis, showed that the *Gf* mutant female gametophyte was arrested at the uninucleate stage [42]. There are several other mutants with of loss-of-function in CYTOKININ INDEPENDENT 1 [43], DIANA/AGAMOUS-LIKE 61 [44] and Nuclear fusion DEFECTIVE1 [45] which affect polar nuclei fusion and central cell development in *Arabidopsis*.

The development of female gametophyte involves several rounds of nuclear division during which key proteins like cyclin and securin are needed to be degraded in order to facilitate the metaphase–anaphase transition. This is regulated by ubiqutin mediated 26S proteolytic system. The key component of this proteolytic system is a multi-subunit ubiquitin E3 ligase, the anaphase-promoting complex (APC/C) that provides a platform and specificity for the ubiquitination reactions [16]. In yeast several genes have been reported, including *CDC16* and *CDC23*, which, when mutated, blocked mitotic cyclin degradation during G1 [28]. In plants, the APC2, APC/NOMEGA, and APC3/HOBBIT (HBT) proteins have been recently characterized. Both *apc2* and *apc6/nomega* mutant plants exhibit defects in gametogenesis [33,34]. In *Arabidopsis* mutant *apc2* impaired female gametogenesis with cell division arreseted at one or two nuclei stage was observed [33]. APC2 is a subunit of APC/C complex which plays a role in cell cycle regulation. The embryo sac development in *nomega* mutant of *Arabidopsis* was found to be arrested at the two-nucleate stage leading to female sterility. The NOMEGA gene product had high homolgy to the CDC16 subunit of APC [34]. The *nomega* mutant embryo sacs were unable to degrade cyclin B, an important APC/C substrate as the mutation was in the APC6/CDC16 subunit of APC/C complex [34]. Thus the role of APC/C in plant cell cycle progression is evident.

Since in *OsAPC6* the mutation is found to be in the same gene encoding the anaphase promoting complex APC6/CDC16 [5] of *Arabidopsis thaliana* [34], the abnormal embryo sac development and female sterility could be attributed to this gene. *OsAPC6* had other pleiotropic phenotypic traits such as reduced height, dark green and broad leaves and insensitivity to gibberellic acid which have not been reported in other APC mutants. These pleiotropic effects of the mutant are dominant and hence it was abbreviated as *OsAPC6* [5]. Gibberellic acid insensitivity associated with dwarfness has been invariably found to be dominant in wheat, maize [46], and rice [47]. High female sterility due to abnormal endosperm development in the *OsAPC6* mutant is clearly a lethal pleiotropic effect responsible for its maintenance mostly as heterozygous. Further functional validation of the candidate gene *APC6* through trancriptome profiling and RNAi is in progress.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2011.08.007.

References

[1] IRGSP, The map-based sequence of the rice genome, Nature 436 (2005) 793–800.
[2] S. Parinov, V. Sundaresan, Functional genomics in *Arabidopsis*: large-scale insertional mutagenesis complements the genome sequencing project, Curr. Opin. Biotechnol. 11 (2000) 157–161.
[3] A. Krishnan, E. Guiderdoni, G. An, Y.C. Hsing, C. Han, M.C. Lee, S.M. Yu, N. Upadhyaya, S. Ramachandran, Q. Zhang, V. Sundaresan, H. Hirochika, H. Leung, A. Pereira, Mutant resources in rice for functional genomics of the grasses, Plant Physiol. 149 (2009) 165–170.
[4] E. Gilchrist, G. Haughn, Reverse genetics techniques: engineering loss and gain of gene function in plants, Brief. Funct. Genomics 9 (2010) 103–110.
[5] M. Kumar, O.P. Basha, A. Puri, D. Rajpurohit, G.S. Randhawa, T.R. Sharma, H.S. Dhaliwal, A candidate gene *OsAPC6* of anaphase-promoting complex of rice identified through T-DNA insertion, Funct. Integr. Genomics 10 (2010) 349–358.
[6] S. Filleur, M.F. Dorbe, M. Cerezo, M. Orsel, F. Granier, A. Gojon, F. Daniel-Vedele, An *Arabidopsis* T-DNA mutant affected in *Nrt2* genes is impaired in nitrate uptake, FEBS Lett. 489 (2001) 220–224.
[7] A. Puri, O.P. Basha, M. Kumar, D. Rajpurohit, G.S. Randhawa, S. Kianian, A. Rishi, H.S. Dhaliwal, The polyembryony gene (*OsPE*) in rice, Funct. Integr. Genomics 10 (2010) 359–366.
[8] L. Reiser, R.L. Fischer, The ovule and the embryo sac, Plant Cell 5 (1993) 1291–1301.
[9] P. Maheshwari, An Introduction to the Embryology of Angiosperms, McGraw-Hill, New York, 1950.

Author's personal copy

[10] Lersten, Flowering Plant Embryology, Blackwell Publishing Ltd., Iowa, USA, 2004.
[11] V. Raghavan, Some reflections on double fertilization, from its discovery to the present, New Phytol. 159 (2003) 565–583.
[12] J. Liu, L.J. Qu, Meiotic and mitotic cell cycle mutants involved in gametophyte development in *Arabidopsis*, Mol. Plant. 1 (2008) 564–574.
[13] J.M. Moore, J.P. Vielle Calzada, W. Gagliano, U. Grossniklaus, Genetic characterization of *hadad*, a mutant disrupting female gametogenesis in *Arabidopsis thaliana*, Cold Spring Harbor Symp. Quant. Biol. 62 (1997) 35–47.
[14] G.C. Pagnussat, H.J. Yu, Q.A. Ngo, S. Rajani, S. Mayalagu, C.S. Johnson, A. Capron, L.F. Xie, D. Ye, V. Sundaresan, Genetic and molecular identification of genes required for female gametophyte development and function in *Arabidopsis*, Development 132 (2005) 603–614.
[15] M.J. Han, K.H. Jung, G. Yi, D.Y. Lee, G. An, Rice immature pollen 1 (*RIP1*) is a regulator of late pollen development, Plant Cell Physiol. 47 (2006) 1457–1472.
[16] M. Pal, O. Nagy, D. Menesi, A. Udvardy, P. Deak, Structurally related TPR subunits contribute differently to the function of the anaphase-promoting complex in *Drosophila melanogaster*, J. Cell Sci. 120 (2007) 3238–3248.
[17] M.D. Lima, N. Eloy, C. Pegoraro, R. Sagit, C. Rojas, T. Bretz, L. Vargas, A. Elofsson, A. de Oliveira, A. Hemerly, P. Ferreira, Genomic evolution and complexity of the Anaphase-promoting Complex (APC) in land plants, BMC Plant Biol. 10 (2010) 254.
[18] C. Gieffers, A. Schleiffer, J.M. Peters, Cullins and cell cycle control, Protoplasma 211 (2000) 20–28.
[19] M. Gmachl, C. Gieffers, A.V. Podtelejnikov, M. Mann, J.M. Peters, The RING-H2 finger protein APC11 and the E2 enzyme UBC4 are sufficient to ubiquitinate substrates of the anaphase-promoting complex, PNAS 97 (2000) 8973–8978.
[20] A. Cebolla, J. Maria Vinardell, E. Kiss, B. Olah, F. Roudier, A. Kondorosi, E. Kondorosi, The mitotic inhibitor ccs52 is required for endoreduplication and ploidy-dependent cell enlargement in plants, EMBO J. 18 (1999) 4476–4484.
[21] A.M. Bhatt, C. Lister, T. Page, P. Fransz, K. Findlay, G.H. Jones, H.G. Dickinson, C. Dean, The *DIF1* gene of *Arabidopsis* is required for meiotic chromosome segregation and belongs to the REC8/RAD21 cohesin gene family, Plant J. 19 (1999) 463–472.
[22] X. Bai, B.N. Peirson, F. Dong, C. Xue, C.A. Makaroff, Isolation and characterization of *SYN1*, a *RAD21-like* gene essential for meiosis in *Arabidopsis*, Plant Cell 11 (1999) 417–430.
[23] N.B. Eloy, F. Coppens, G.T. Beemster, A.S. Hemerly, P.C. Ferreira, The *Arabidopsis* anaphase promoting complex (APC): regulation through subunit availability in plant tissues, Cell Cycle 5 (2006) 957–965.
[24] I. Blilou, F. Frugier, S. Folmer, O. Serralbo, V. Willemsen, H.N. Wolkenfelt, b.B. Eloy, P.C.G. Ferreira, P. Weisbeek, B. Scheres, The *Arabidopsis HOBBIT* gene encodes a *CDC27* homolog that links the plant cell cycle to progression of cell differentiation, Genes Dev. 16 (2002) 2566–2575.
[25] C. Rojas, N. Eloy, M. de Freitas Lima, R. Rodrigues, L. Franco, K. Himanen, G. Beemster, A. Hemerly, P. Ferreira, Overexpression of the *Arabidopsis* anaphase promoting complex subunit *CDC27a* increases growth rate and organ size, Plant Mol. Biol. 71 (2009) 307–318.
[26] D. Lindsay, P. Bonham-Smith, S. Postnikoff, G. Gray, T. Harkness, A role for the anaphase promoting complex in hormone regulation, Planta 233 (2011) 1223–1235.
[27] K. Marrocco, A. Thomann, Y. Parmentier, P. Genschik, M.C. Criqui, The APC/C E3 ligase remains active in most post-mitotic *Arabidopsis* cells and is required for proper vasculature development and organization, Development 136 (2009) 1475–1485.
[28] S. Irniger, S. Piatti, C. Michaelis, K. Nasmyth, Genes involved in sister chromatid separation are needed for B-type cyclin proteolysis in budding yeast, Cell 81 (1995) 269–277.
[29] J.W. Harper, A phosphorylation-driven ubiquitination switch for cell-cycle control, Trends Cell Biol. 12 (2002) 104–107.
[30] K.T. Kuppusamy, S. Ivashuta, B. Bucciarelli, C.P. Vance, J.S. Gantt, K.A. VandenBosch, Knockdown of *CELL DIVISION CYCLE16* Reveals an Inverse Relationship between Lateral Root and Nodule Numbers and a Link to Auxin in *Medicago truncatula*, Plant Physiol. 151 (2009) 1155–1166.
[31] H.S. Dhaliwal, A. Das, A. Singh, V.K. Gupta, Isolation of insertional mutants in indica rice using Ds transposable element of maize, Rice Genet. Newslett. 18 (2001) 98–99.
[32] Y.X. Zeng, C.Y. Hu, Y.G. Lu, J.Q. Li, X.D. Liu, Diversity of abnormal embryo sacs in indica/japonica hybrids in rice demonstrated by confocal microscopy of ovaries, Plant Breeding 126 (2007) 574–580.
[33] A. Capron, O. Serralbo, K. Fulop, F. Frugier, Y. Parmentier, A. Dong, A. Lecureuil, P. Guerche, E. Kondorosi, B. Scheres, P. Genschik, The *Arabidopsis* anaphase-promoting complex or cyclosome: molecular and genetic characterization of the APC2 subunit, Plant Cell 15 (2003) 2370–2382.
[34] H.S. Kwee, V. Sundaresan, The NOMEGA gene required for female gametophyte development encodes the putative APC6/CDC16 component of the Anaphase Promoting Complex in *Arabidopsis*, Plant J. 36 (2003) 853–866.
[35] S.D. Russell, The egg cell: development and role in fertilization and early embryogenesis, Plant Cell 5 (1993) 1349–1359.
[36] X. Mu, B. Jin, N. Teng, Studies on the early development of zygotic and synergid embryo and endosperm in polyembryonic rice *ApIII*, Flora 205 (2010) 404–410.
[37] C.A. Christensen, S. Subramanian, G.N. Drews, Identification of gametophytic mutations affecting female gametophyte development in *Arabidopsis*, Dev. Biol. 202 (1998) 136–151.
[38] G.N. Drews, D. Lee, C.A. Christensen, Genetic analysis of female gametophyte development and function, Plant Cell 10 (1998) 5–18.
[39] P.S. Springer, D.R. Holding, A. Groover, C. Yordan, R.A. Martienssen, The essential Mcm7 protein PROLIFERA is localized to the nucleus of dividing cells during the G(1) phase and is required maternally for early *Arabidopsis* development, Development 127 (2000) 1815–1822.
[40] N. Li, L. Yuan, N. Liu, D. Shi, X. Li, Z. Tang, J. Liu, V. Sundaresan, W.-C. Yang, SLOW WALKER2, a NOC1/MAK21 Homologue, is essential for coordinated cell cycle progression during female gametophyte development in *Arabidopsis*, Plant Physiol. 151 (2009) 1486–1497.
[41] M. Liu, D.Q. Shi, L. Yuan, J. Liu, W.C. Yang, SLOW WALKER3, encoding a putative DEAD-box RNA Helicase, is essential for female gametogenesis in *Arabidopsis*, J. Integr. Plant Biol. 52 (2010) 817–828.
[42] C.A. Christensen, E.J. King, J.R. Jordan, G.N. Drews, Megagametogenesis in *Arabidopsis* wild type and the *Gf* mutant, Sex. Plant Reprod. 10 (1997) 49–64.
[43] J. Hejátko, M. Pernisová, T. Eneva, K. Palme, B. Brzobohatý, The putative sensor histidine kinase CKI1 is involved in female gametophyte development in *Arabidopsis*, Mol. Genet. Genomics 269 (2003) 443–453.
[44] M. Bemer, M. WoltersArts, U. Grossniklaus, G.C. Angenent, The MADS domain protein DIANA acts together with AGAMOUS-LIKE80 to specify the central cell in *Arabidopsis* ovules, Plant Cell 20 (2008) 2088–2101.
[45] M.F. Portereiko, A. Lloyd, J.G. Steffen, J.A. Punwani, D. Otsuga, G.N. Drews, AGL80 is required for central cell and endosperm development in *Arabidopsis*, Plant Cell 18 (2006) 1862–1872.
[46] J. Peng, D.E. Richards, N.M. Hartley, G.P. Murphy, K.M. Devos, J.E. Flintham, J. Beales, L.J. Fish, A.J. Worland, F. Pelica, D. Sudhakar, P. Christou, J.W. Snape, M.D. Gale, N.P. Harberd, 'Green revolution' genes encode mutant gibberellin response modulators, Nature 400 (1999) 256–261.
[47] H. Itoh, M. Ueguchi-Tanaka, Y. Sato, M. Ashikari, M. Matsuoka, The Gibberellin signaling pathway is regulated by the appearance and disappearance of SLENDER RICE1 in nuclei, Plant Cell 14 (2002) 57–70.