DEVELOPMENT OF FUNCTIONAL MARKER FOR GRAIN WEIGHT GENE *GW2* IN RICE

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B.Sc .(Ag.)

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BY

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DECLARATION

I, **P. VENKAT REDDY** hereby declare that the thesis entitled **"DEVELOPMENT OF FUNCTIONAL MARKER FOR GRAIN WEIGHT GENE *GW2* IN RICE "** submitted to the **Acharya N. G. Ranga Agricultural University** for the degree of **MASTER OF SCIENCE IN AGRICULTURE** is the result of original research work done by me. I also declare that any material contained in the thesis has not been published earlier in any manner.

Date:

Place: Hyderabad **(P.VENKAT REDDY)**

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May the Divine protect us both, the teacher and the student,
May He nourish us both,
May we work together with great energy,
May our study be vigorous and fruitful
May we not hate each other.

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Needless to say omissions and errors are mine

VENKAT REDDY PONNA

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LIST OF SYMBOLS AND ABBREVIATIONS

> : Greater than

< : Less than

@ : At the rate of

^{o}C : Degree celsius

et al. : And others

Fig. : Figure

g : Gram

g/L : Gram per litre

h : Hours

i.e. : That is

L : Litre

m : Meter (s)

M : Molar

mg : Milligram (s)

µg : Microgram (s)

µl : Micro litre

ml : Millilitres (s)

min : Minute (s)

mm : Millimetre (s)

N : Normality

NaOH : Sodium hydroxide

% : Per cent

pH : Negative logarithm of hydrogen ion activity

pp : Page number

± : Plus or minus

rpm : Rotation per minute

Vol : Volume

viz., : Namely

Wt.	Weight
W/v	Weight/volume
TGW	Thousand-grain weight
PGWC	Percentage of grains with chalkiness
Vty	Variety
F.M	Functional marker
MAS	Marker assisted selection
QTL	Quantitative trait loci
SNP	Single nucleotide polymorphism
DNA	Deoxyribonucleic acid
TGW	Thousand grain weight
HGW	High grain weight
LGW	Low grain weight
PCR	Polymerase chain reaction
dNTPs	Deoxynucleoside triphosphates

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ABSTRACT

Rice is central to the lives of billions of people around the world. Rice is vital to global food security, being staple food for more than 60 % of world’s population. Rice is an excellent model system for genome studies and evolution of crop plants among monocot species, because of its small genome size and the availability of high-density molecular marker linkage maps. Significant progress in rice functional genomics is being made since the completion of the international rice genome sequencing project.

With an ever-increasing global population but rapid shrinking of fertile cultivable lands day by day and declining yield stability in cereals is one of the major issues, the India is facing at present. There is an urgent need to maintain the yield stability of rice and further improve the rice grain yield using molecular genetic breeding approaches.

Changes in a desirable variety can be achieved more rapidly through molecular breeding than with traditional breeding techniques. The presence of the desired gene controlling the trait can be tested for at any stage of growth, such as in small seedlings in a greenhouse tray. The precision and versatility of today’s biotechnology enable improvements in food quality and production to take place more rapidly than when using traditional breeding.

In traditional breeding, crosses are made in a relatively uncontrolled manner. The breeder chooses the parents to cross, but at the genetic level, the results are

unpredictable. DNA from the parents recombines randomly, and desirable traits are bundled with undesirable traits, such as lower yield or poor quality.

The present investigation is carried out with the objective of development of functional marker for grain weight (yield component). Of the three reported SNPs (Song *et al.* 2007), an SNP at 114 nucleotide position (C/T) was targeted and primers were designed and standardized at an annealing temperature of 64^{o}C. Validation was carried out in the total selected germplasm (124varieties) and observed the dominance of low grain weight allele “C”. The high grain weight allele “T” was present very rarely indicating that, it may have arisen recently from the process of rice domestication.

Since the marker (namely DRR-GW) showed very less association with the grain weight, an attempt was made to know the association between kernel length and grain weight, which showed a good association. Hence, it may be illated that, as the newly developed marker DRR-GW was targeted only one functional polymorphism i.e. SNP at 114 nucleotide position of *GW2* which may not be sufficient enough to explain the variation exist in the grain weight phenotype of selected germplasm.

Chapter I

INTRODUCTION

Rice is central to the lives of billions of people around the world and is vital to global food security, being staple food for more than 60 % of world's population especially for most of the people of South-East Asia. Rice cultivation is the largest single use of land for producing food, covering 9% of the earth's arable land. Asia accounts for over 90% of the world's production of rice, with China, India and Indonesia producing the most. India is number one in area with approximately 43.5 million hectares (23.3% of gross cropped area) of rice and ranks second in production next to China with approximately 89.1 million tones but the productivity of 2048 kg ha^{-1} is far below the world's average productivity (CMIE, Annual report 2010).

It appears that we have exhausted the potential of the 'green revolution' strategies, as we witness declining rice productivity in many countries. In the next 3 decades, farmers need new approaches and technologies to produce 50 to 60 percent more rice on existing or less land and water with limiting and/or expensive inputs. The three factors that could contribute to increased rice productivity are: (a) developing new rice varieties including hybrids with higher yield potential; (b) minimizing the yield gap between what is currently harvested by farmers and the achievable highest on-farm yield of varieties they grow; and (c) reducing the post-harvest losses and improving grain quality to enhance profitability (Bal Subramanian *et al.* 2000).

Rice is an excellent model system for genome studies and evolution of crop plants among monocot species, because of its small genome size and the availability of high-density molecular marker linkage maps (McCouch *et al.* 1997). Significant progress in rice functional genomics is being made since the completion of the international rice genome sequencing project (IRGSP, 2005), which offers tremendous opportunities for breeders to improve this important crop by molecular breeding.

Polygenic characters which were previously very difficult to analyze using traditional plant breeding methods, would now be easily tagged using molecular markers. Molecular markers have several advantages over the traditional phenotypic markers that were previously available to plant breeders. They offer great scope for improving the efficiency of conventional plant breeding by carrying out selection not directly on the trait of interest but on molecular markers linked to that trait. This, of course, would require a molecular marker to be tightly linked to the trait of interest. Besides, these markers are not environmentally regulated and are, therefore, unaffected by the conditions in which the plants are grown and are detectable in all stages of plant growth.

Marker assisted selection (MAS) hold great potential for plant breeding as it promises to expedite the time taken to produce crop varieties with desirable characters. With the use of molecular techniques it would now be possible to hasten the transfer of desirable genes among varieties and to introgress novel genes from related wild species. Polygenic characters which were previously very difficult to analyze using traditional plant breeding methods, would now be easily tagged using molecular markers. It would also be possible to establish genetic relationships between sexually incompatible crop plants. Techniques which are particularly promising in assisting selection for desirable characters involves the use of molecular markers such as random-amplified polymorphic DNAs (RAPDs), restriction fragment length polymorphisms (RFLPs), and PCR-based DNA markers such as simple sequence repeats (SSRs), sequence characterized amplified regions (SCARs), sequence-tagged sites (STS) and inter-simple sequence repeat amplification (ISSRs), amplified fragment length polymorphic DNAs (AFLPs) and amplicon length polymorphisms (ALPs). The use of cost-effective DNA markers derived from the fine mapped position of the genes for important agronomic traits and MAS strategies will provide opportunities for breeders to develop high-yielding, stress-resistant, and better-quality rice cultivars.

During the last two decades molecular markers have been successfully incorporated in various breeding and genetics programs in crop plants. DNA based markers are increasingly being recognized as useful tools for various applications in plant breeding. DNA markers detect genetic variation among genotypes and are useful as landmarks to tag functional genes.

The wealth of sequence databases and advanced bioinformatics tools have led to the improvement of the process of molecular marker development in terms of efficiency of time and cost. This maximizes the efficiency and effectiveness of marker development, marker utilization, and marker application.

Molecular markers associated with gene coding regions are useful tools for bridging functional and structural genomics. Functional marker development requires allele sequences of functionally characterized genes from which polymorphic, functional motifs affecting plant phenotype can be identified.

A successful application of molecular markers to assist breeding procedures rely on several factors: (i) a genetic map with molecular markers linked to the major gene(s) or QTLs of agronomic interest (ii) a tight association between the markers and the major gene(s) or the QTLs (iii) adequate recombinations between the markers associated to the trait(s) of interest and the rest of the genome and (iv) the possibility of analyzing a large number of individuals in a time and cost effective manner. The success of MAS also depends on the localization of the marker with respect to the target gene. (Francia *et al.* 2005).

Functional marker development requires (1) functionally characterized genes, (2) allele sequences from such genes, (3) identification of polymorphic, functional motifs affecting plant phenotype within these genes, and (4) validation of associations between DNA polymorphisms and trait variation. Finally, application of functional markers depends on the availability of robust marker assay technologies (Lubberstedt *et al*. 2005).

Many agriculturally important traits such as yield, quality and some forms of disease resistance are controlled by many genes and are known as quantitative traits (also polygenic/ multi factorial /complex traits). The regions within genomes that contain genes associated with a particular quantitative trait are known as quantitative trait loci (QTLs). The identification of QTLs based only on conventional phenotypic evaluation is not possible. A major breakthrough in the characterization of quantitative traits that created opportunities to select for QTLs was initiated by the development of DNA (molecular) markers in the 1980s (Collard *et al.* 2005).

Quantitative trait loci (QTL) are marvelous genetic entities demonstrating greater utility in genetic understanding of complex traits and promise greater impact in crop improvement endeavors. They have given us clearer view of the genetics of agronomically important, and several other traits too. A quantitative trait could be now considered as effect of series of monogenes acting ‘in concert’ or unison so as to influence a single trait. Each gene pair (locus) interacts with the environment and causes effect/s that other/s add/s to or subtract/s from the effects of other gene pairs that influence the trait. The cumulative effect or trait manifestation of a QTL is thus a combination of the effects of each gene pairs (either in homozygous and in heterozygous condition) with the action of environment. It is now possible to map QTLs on rice genomic sequence and find out reading frames, overlapping genes, copies of genes at the locus. The most likely position of QTL may appear to be between pair of markers, but it could be as far as 20 cM away.

The QTLs classified based on phenotypic variance explained. The QTL responsible for more than 50% of the phenotypic variation are designated as ‘major genes’, where as loci controlling 25-50% of the variation would be minor QTL. In rice, a total of 7056 QTLs have been reported from 1994 to October 2004 (www.gramene.org).

The grain yield potential of rice can be dissected into four major components: grain weight, grain number per panicle, panicle number per plant (closely related to tiller number per plant), and ratio of filled grains. Grain weight is nearly completely governed by genetic factors, whereas the ratio of filled grains is strongly affected by environmental factors Grain weight is determined by grain length, width, and thickness. Grain size is a major target of breeding, not only as a component of grain yield but also as a quality trait, because increased grain size may reduce grain quality.

Many QTLs for grain weight have been detected, of which following were identified recently: *GS3*, located on the pericentromeric region of chromosome 3, is a major QTL for grain length and weight. *GW2* was identified from a QTL on the short arm of chromosome 2 that controls grain width and weight and *GW5* represents a major QTL underlying rice width and weight, and that it likely acts in the ubiquitin-proteasome pathway to regulate cell division during seed development. *G1F1* governs expression of grain filling ultimately grain weight also cloned and characterized using map based approach.

Single nucleotide polymorphisms (SNPs) are good candidates for marker development, as they constitute the most common DNA sequence variations found in genomes of most organisms. Molecular genetic research relies heavily on the ability to detect polymorphisms in DNA. Single nucleotide polymorphisms (SNPs) are the most frequent form of DNA variation in the genome. SNPs have become an important type of marker for genotyping applications as automated genotyping systems have been developed that yield accurate genotypes.

Thus in the last few years there are many reports of amalgamation of classical breeding and modern biotechnological approaches which have unlimited scope in Indian agriculture.

Song *et al.* (2007) reported that a QTL for rice grain width which encodes RING-type E3 ubiquitin ligase. They have reported that *GW2* is the one of the major QTLs for grain width and minor for grain weight. The *GW2* gene was cloned and reported that *GW2* gene negatively regulates the grain weight i.e. loss of *GW2* resulting in enhanced grain width, weight thus ultimately yield. Three SNPs were reported at 114, 316 and 1116 nucleotide positions. Out of which SNP at 114 nucleotide position has been targeted where presence of nucleotide “T” allele resulting in high grain weight while presence of “C” allele is resulting in low grain weight.

Based on this background we hypothesized to check the presence of such functional SNP at 114 nucleotide position in a wide range of *indica* varieties setting the following objectives to select better parents in crop improvement program.

The present study aims at reporting a development of PCR based DNA markers designed to facilitate an appropriate marker-assisted selection in rice breeding for yield.

Objectives of the Research:

1) To design PCR primers for functional nucleotide polymorphism associated with grain weight

2) To analyze the amplification pattern of designed primer in a set of contrasting genotypes

Chapter II

REVIEW OF LITERATURE

Grain weight is the major determinant of crop grain yield and is controlled by naturally occurring quantitative trait loci (QTL). With the advent of molecular markers, identification of genomic regions associated with specific traits has been possible. Use of DNA based markers and linkage map helps us to understand the genetic architecture of complex quantitative characters, which are governed by many genes with small cumulative effects. Quantitative trait loci analysis provides a powerful tool for identifying marker defined genomic regions for any quantitative character. QTLs are the genomic regions harboring genes with major effect on quantitative traits and which forms the basis for marker assisted selection.

Review of relevant literature on grain related QTLs, SNPs and marker technology, including reports on molecular markers, mapping, statistical techniques for QTL mapping, marker assisted selection are presented here under following headings.

2.1. Grain related QTLs and QTL based markers in Rice.
2.2. Grain related SNPs and SNP based markers in Rice.
2.3. Markers Developed Related to other traits in plants / Crops.
2.4. Markers developed in fauna.

2.1 Grain related QTLs and QTL based markers in Rice

Redona *et al.* (1998) identified the QTLs controlling six panicle and grain characteristics using composite interval mapping. Two QTLs were identified for panicle size, with one on chromosome 3 accounting for 16% of the phenotypic variation. Four loci controlling spikelet fertility accounted for 23% of the phenotypic variation. Several QTLs were detected, of which seven for grain length, four for breadth, three for shape and two for weight, with the most prominent QTLs being on chromosomes 3, 4 and 7.

Yoshida *et al.* (2002) identified six QTLs for the increase of GW (grain weight) on five chromosomes in rice. Each of these QTLs accounted for 10 to 18 % of the phenotypic variance, and the cumulative effect was about 75 %.

Hittalmani *et al.* (2003) reported nineteen QTLs significantly affecting TGW (1000-grain weight). The number of QTLs detected at each location ranged from four (CNNRI) to none (UAS, IARI). Of these QTLs, one QTL was identified between the two markers RG134 and RZ500 on chromosome 10 was detected in five environments and explained 25.10% of total phenotypic variation in RRI.

Thomson *et al.* (2003) reported that introgressions from *O. rufipogon* in six QTLs on chromosomes 2, 3, 9, 10 and 12 as decreased grain weight (more exactly seed weight), whereas those in two QTLs on chromosomes 1 and 5 as increased seed weight

Li *et al.* (2004) reported the centromeric intervals on rice chromosome 3 were defined by molecular markers flanking the location of *gw*3.1.

Fan *et al.* (2006) isolated *GS3* by map-based cloning. Analysis of a random population of 201 individuals from the BC_3F_2 progeny confirmed that the *GS3* locus explained 80–90% of the variation for grain weight and length in this population. In addition, this locus was resolved as a minor QTL for grain width and thickness.

Fan *et al.* (2006) revealed that the *GS3* locus located in the pericentromeric region of rice chromosome 3 has been frequently identified as a major QTL for both grain weight (a yield trait) and grain length (a quality trait). They mapped the *GS3* locus and determined its candidate gene using advanced populations.

Xie *et al.* (2007) identified QTL for 1000 grain weight (TGW), *gw9.1*, within a cluster of yield-related QTLs on the long arm of chromosome 9. Each of these lines contained a 4.0 cM heterozygous introgression in the target region on chromosome 9, defined by markers RM242-RM215, as well as a second introgression on the short arm of chromosome 12 .

Wan *et al.* (2006) found two QTLs i.e. *qGL-3* for grain length and *qGW-5* for grain weight using chromosomal segment substitution lines (CSSLs) derived from the cross Asominori × IR24.

Fotokian (2008) detected four QTLs for grain weight and three of them (*QGw2a*, *QGw4*, and *QGwl2*) had relatively large effects on grain width, each explaining more than 12% of the variation. The effect of the remaining QTL was relatively small, accounting for 8%i of the variation.

Wan *et al.* (2008) identified a major, stable QTL, *qGW-5*, consistently decreased rice grain width in both the Asominori/IR24 RIL and CSSL populations with the genetic background Asominori. By investigating the distorted segregation of phenotypic values of rice grain width and genotypes of molecular markers in BC_4F_2 and BC_4F_3 populations, *qGW-5* was dissected into a single recessive gene, *gw-5*, which controlled both grain width and length–width ratio.

Li *et al.* (2009) reported that there is a significantly positive correlation between TGW (thousand-grainweight) and PGWC (percentage of grains with chalkiness) analyzing the epistatic effect and the QTL × environment interaction (QE) in rice using a backcross inbred line (BIL) population (Koshihikari (*O s.*subsp. *japonica*) X Kasalath (*O.s.* subsp. *indica*), the main-effect QTLs for TGW and PGWC and inferred that *qTGW-3c*, *qTGW-4a*, and *qTGW-6b*, only controlled TGW and could be used in breeding programs.

Bai *et al.* (2010) identified four QTLs for grain length and these were detected on chromosomes 3 and 7 and 10 QTLs for grain width and 9 QTLs for grain thickness were identified on chromosomes 2, 3, 5, 7, 9 and 10, respectively.

Oh *et al.* (2010) indicated that *tgw2* was located in the 384-kb interval between two SSR markers, RM12813 and RM12836. Four QTLs for grain length, grain width, and grain thickness were also located in the same interval suggesting that a single gene is involved in controlling these four traits. Substitution mapping also indicated that two QTLs for grain weight and culm length, *tgw2* and *cl2*, were tightly linked.

Liu *et al.* (2010) identified four and five quantitative trait loci (QTLs) for 1,000-grain weight (TGW) and spikelets per panicle (SPP), respectively, using rice recombinant inbred lines (RILs). According to liu *et al.* grain shape was strongly associated with TGW. Grain length, grain width, and grain thickness were also largely controlled by *TGW3b*.

Liu *et al.* (2010) showed that QTLs conferring bigger TGW (Thousand grain weight) and higher YP (Yield per Panicle) were genetically stable in different populations with different genetic backgrounds. They have chosen the previously identified quantitative trait loci (QTLs) conferring 1000-grain weight (TGW) and grain yield per plant (YP), on the same location of chromosome 6, designated tgw6.1 and yp6.1 respectively, as target QTLs to verify the QTL effects by using three segregating populations through marker-assisted selection (MAS).

Vikram *et al.* (2011) identified a major QTL for GY under RS(reproductive-stage drought stress), qDTY1.1, on rice chromosome 1 flanked by RM11943 and RM431 in all three populations. In combined analysis over two years, qDTY1.1 showed an additive effect of 29.3%, 24.3%, and 16.1% of mean yield in N22/Swarna, N22/IR64, and N22/MTU1010, respectively, under RS. They reported that this is the first QTL in rice with a major and consistent effect in multiple elite genetic backgrounds under both RS and NS(Non stress) situations.

Heang *et al.* (2012) reported an antagonistic pair of basic helix-loop-helix (bHLH) proteins is involved in determining rice grain length by controlling cell length in the lemma/palea. As expected, silencing of ANTAGONIST OF PGL1 (APG) produced the same phenotype as over expression of POSITIVE REGULATOR OF GRAIN LENGTH 1 (PGL1), suggesting antagonistic roles for the two genes. Transcription of two known grain-length-related genes, *GS3* and SRS3, was largely unaffected in the PGL1-overexpressing and APG-silenced plants. Observation of the inner epidermal cells of lemma revealed that are caused by increased cell length. PGL1-APG represents a new grain length and weight-controlling pathway in which APG is a negative regulator whose function is inhibited by PGL1.

Li *et al.* (2012) reported the isolation of a hemizygous mutation, heading and grain weight (hgw), which delays heading and reduces grain weight in rice. Molecular cloning and characterization of the HGW gene showed that it encodes a novel plant-specific ubiquitin-associated (UBA) domain protein localized in the cytoplasm and nucleus, and functions as a key upstream regulator to promote expressions of heading date- and grain weight-related genes.

2.2. Grain related SNPs and SNP based markers in Rice.

Hittalmani *et al.* (1995) developed a PCR based molecular marker for the genomic clone RG64, which is tightly linked to the blast resistance gene *Pi-2(t)* in rice. These results demonstrate the utility of specific amplicon polymorphism (SAP) markers as simple and yet reliable landmarks for use in marker-assisted selection and breeding within cultivated rice.

Williams *et al.* (1996) developed PCR-based allele-specific RFLP marker linked to resistance to cereal cyst nematode in wheat. A dual- PCR system was developed in which the primer sets AWP2 and AWP3 produced allele-specific amplification while AWP1 was not polymorphic. The concentration of the oligo-nucleotide primers and the sequence of the primer-template mismatches were critical to the success of discriminatory allele amplification.

Nasu *et al.* (2002) established 213 co-dominants SNP markers distributed throughout the genome, illustrates the immense potential of SNPs as molecular markers not only for genome research but also for molecular breeding of rice. They found 2800 SNPs in approximately 250,000 aligned bases for an average of one SNP every 89 bp, or one SNP every 232 bp between two randomly selected strains and reported that 94 SNPs that were found between the closely related cultivars Nipponbare and Koshihikari can be converted into molecular markers.

Lang *et al.* (1993) developed a PCR based STS Markers for thermo sensitive genetic male sterility gene *tms3* (t) in rice and estimated the marker efficiency in MAS (Marker assisted Selection) as 84.6%. Besides recommending these PCR-based markers for *tms3* (t) can now be used in selecting TGMS plants at seeding stage in the segregating populations in environment independent of controlled temperature regime.

Hayashi *et al.* (2004) developed allele-specific PCR based SNP markers in the vicinity of the rice blast resistant genes. Of the 49 candidate allele-specific markers, 33 unambiguously and reproducibly discriminated between the two alleles. They have suggested that, because of its ability to generate numerous markers within a target region and its simplicity in assaying genotypes, SNP genotyping with allele-specific PCR is a valuable tool for gene mapping, map-based cloning, and marker-assisted selection in crops, especially rice.

Cordeiro *et al.* (2005) developed an SNP Based marker to allow quantitative SNP allelotyping in sugarcane that will allow mapping of single dose markers and potentially identify gene haplotypes linked to phenotypes

Song *et al.* (2007) reported that the *GW2*, a new QTL controls rice grain width and weight and showed that *GW2* encodes a previously unknown RING-type protein with E3 ubiquitin ligase activity, which is known to function in the degradation by the ubiquitin-proteasome pathway. Loss of *GW2* function increased cell numbers, resulting in a larger (wider) spikelet hull, and it accelerated the grain milk filling rate, resulting in enhanced grain width, weight and yield.

Chagne *et al.* (2008) identified a total of 71,482 putative SNPs. A total of 93 new markers containing 210 coding SNPs were successfully mapped using the high-resolution melting (HRM) technique. This new set of SNP markers for the apple offers new opportunities for understanding the genetic control of important horticultural traits using quantitative trait loci (QTL) or linkage disequilibrium analysis.

Lang *et al.* (2008) developed PCR-based DNA marker for precise and efficient transfer of the *fgr* gene into new elite improved lines. This was done with an informative RFLP marker RG28, and RM223, which showed the closest linkage to *fgr* gene in rice. They have identified two polymorphic markers, amplified with RG28 FL-RL (STS) and

RM223 (SSR). They have demonstrated the utility of STS and microsatellite markers for use in marker-assisted selection and breeding within cultivated rice.

Sakthivel *et al.* (2009) developed a simple, co-dominant, functional marker for fragrance trait, which can be resolved in an agarose gel and validated in Basmati and non-Basmati aromatic rice varieties and in a mapping population segregated for fragrance trait. The marker targets the InDel polymorphism in *badh2* gene and amplifies 95 and 103 bp fragments in fragrant and non-fragrant genotypes, respectively. This marker was highly efficient in discriminating all fragrant and non-fragrant genotypes and showed perfect co-segregation with the trait of fragrance in the mapping population.

Ram Kumar *et al.* (2010) reported the development of functional marker targeting the *GS3* gene. Kernel length (KL) is an important agronomic and grain quality trait in breeding of rice (*Oryza sativa* L). The trait of kernel length is controlled by quantitative trait loci (QTL) and various studies carried out across different genetic backgrounds and environments have identified a QTL with major effect for kernel length in the vicinity of the centromeric region of chromosome 3 (This QTL, later named *GS3*, located in the pericentromeric region of chromosome 3) is reported to be responsible for 80–90% of the variation in kernel length.

Chen *et al.* (2010) developed two molecular markers designated as InDel-*Lgc1-1* and InDel-*Lgc1-2* to detect the low glutelin-content gene *Lgc1* thus to improve the selection efficiency in low glutelin-content rice breeding and reported that combined use of the two markers could easily distinguish the genotypes of *Lgc1* from different rice varieties.

Zhang *et al.* (2010) reported the discovery and characterization of 46 SNPs in the eastern oyster by mining expressed sequence tags (ESTs) and resequencing.

Ihara *et al.* (2010) developed a total of 574 sequence tagged sites (STSs) from *Cryptomeria japonica* and HRM analysis was used to screen for polymorphisms in these STS markers. They reported that the combination of SNP screening from an EST database combined with HRM analysis is a highly efficient way to develop SNP markers for expressed genes. This method will contribute to both genetic mapping and the identification of SNPs in non-model organisms.

Liu *et al.* (2011) uncovered the well-known *Indica-Japonica* subspecific differentiation and geographic differentiations within *Indica* and *Japonica* using 372 sets of SNPs. They revealed some common and contrasting patterns of the haplotype diversity along different rice chromosomes in the Indica and Japonica accessions, which suggest different evolutionary forces possibly acting in specific regions of the rice genome during domestication and evolution of rice.

2.3. Markers Developed Related to other traits in plants / Crops.

Paran *et al.* (1993) developed a PCR based Cleaved Amplified Polymorphic Regions (CAPS) in lettuce which were derived from eight random amplified polymorphic DNA (RAPD) markers linked to disease resistance genes in lettuce. This study provided information on the molecular basis of RAPD markers.

Venter *et al.* (2000) identified a putative marker linked to the *Dn5* resistance gene through RAPD in wheat. This marker was converted to a more reliable sequence-characterized-amplified regions (SCAR) marker. This marker was tested in a segregating F_2 population carrying the *Dn5* resistance gene and proved able to differentiate between the segregating individuals.

Zhang *et al.* (2003) confirmed two SNPs and one SNP was discovered for genes at the Gli-A1, Gli-B1 and Gli-D1 loci. They have generated dominant DNA markers based on these three SNPs using modified allele-specific PCR procedure. Markers GliA1.2, GliB1.2 and GliD1.2 were found to be correlated with the Glu-A3 d or e, Glu-B3 a, g or h and Glu-D3 c alleles, respectively. These results indicated that the gamma-gliadin SNP markers could be used for detecting linked LMW glutenin subunit alleles that are important in determining the quality attributes of wheat products.

Zhang *et al.* (2004) designed allele-specific PCR markers based on the DNA polymorphisms identified between the LMW glutenin genes, and these markers were validated against a panel of cultivars containing different Glu-A3 alleles. PCR was used to amplify low-molecular-weight (LMW) glutenin genes from the Glu-A3 loci of hexaploid wheat cultivars containing different Glu-A3 alleles. The complete coding sequence of one LMW glutenin gene was obtained for each of the seven alleles Glu-A3a to Glu-A3g. This collection of markers represents a valuable resource for use in marker-assisted breeding to select for specific alleles of this important quality-determining locus in bread wheat.

Garland *et al.* (2005) developed a polymerase chain reaction (PCR)-based marker system that consists of a dominant marker from the available gene sequence representing the *Sw-5b* gene (resistant to Tomato spotted wilt virus -TSWV) sequence and incorporates the modified CAPS marker as a positive control and potential indicator of genotype (homozygous resistant *v.* heterozygote).

Peter *et al.* (2005) developed markers for 40 SNP sites in 28 barley genes based on the nested allele specific PCR method. For primer synthesis, only the best one or two allele specific primers were chosen for synthesis and obtained a 77% success rate and reported that, this method of allele specific PCR shows promise for the widespread development of cheap transferable SNP based markers for plant genomics.

Garland *et al.* (2005) developed an improved PCR based marker system for TSWV resistant gene Sw-5 namely Sw-5b LRR (modified CAPS Marker of CT220) and this marker is more successful when rapid DNA extraction method is used.

Atienza *et al.* (2006) developed a diagnostic cleaved amplified polymorphism (CAP) marker which is able to differentiate between *H. chilense* and durum wheat *PSY1* and demonstrated for the first time that *PSY* (Phyto synthase gene) is duplicated in *H. chilense*, *Triticum urartu*, and durum wheat (*PSY1* and *PSY2*).

Chagne *et al.* (2007) identified the *Rni* locus, a major genetic determinant of the red foliage and red color in the core of apple fruit. They have reported that red colourations in the fruit core as well as red foliage are both controlled by a single locus named *Rni*. They have shown that the transcription factor MdMYB10 may be the gene underlying *Rni* as there were no recombinants between the marker for this gene and the red phenotype in a population of 516 individuals.

Wang *et al.* (2007) developed Got-2 CAPS marker from the tomato glutamate oxaloacetate transaminase isozyme gene family as a means of revitalising old isozyme markers and recruiting new ones that offers a facile PCR-based alternative to the isozyme marker for the marker assisted breeding of I-3. They have also generated nine CAPS markers, two SCAR markers and one SSR marker, which were bin-mapped using a set of tomato introgression lines.

Brennan *et al.* (2008) developed and validated a PCR based marker based on sequence-specific primer E41M88-280 against mite in the black current by converting AFLP into easy and reproducible PCR based marker .It was reported that gall mite is closely associated Ce gene.

Dianese *et al.* (2010) developed a locus-specific, co-dominant SCAR marker for *Sw-5* (Tospovirus resistance) gene by evaluating a panel of seven PCR primer pairs matching different sequences within a genomic region spanning the Sw-5a and Sw-5b gene cluster. Complete correlation was observed between resistance under greenhouse conditions and the presence of the marker.

Su *et al.* (2011) reported that the ortholog of OsGW2 (gene is involved in rice grain development, influencing grain width and weight) in wheat, TaGW2, was considered as a candidate gene related to grain development They found that TaGW2 is constitutively expressed, with three orthologs expressing simultaneously and concluded that there is a positive association.

Huang *et al.* (2011) developed nine pina-STS markers (Pina-STS1 to Pina-STS9) using three primer PCR system and temperatures witch (TS) PCR was used to develop six co-dominant SNP markers for genotyping the Pinb-D1a, Pinb-D1b, Pinb-D1c and Pinb-D1d alleles to genotype puroindoline a and b alleles for grain hardness in bread wheat

Redona *et al.* (2012) developed a microsatellite marker for the first time for *Masdevallia solomonii* genus to serve as a tool for in future population genetic studies and reported that this marker can assess genetic diversity and structure of this species and will serve to propose effective conservation actions.

2.4. Markers developed in fauna.

Rohrer *et al.* (2007) reported that parental exclusion probabilities for SNP and microsatellite marker panels were similar, but the SNP panel was much more sensitive for individual identification. Of 80 SNPs that were evaluated, 60 SNPs were selected for the final panel of markers. This panel of SNP markers is theoretically sufficient for individual identification of any pig in the world and is publicly available.

Hara *et al.* (2010) developed efficient SNP markers for individual identification and parentage tests in a japanese black cattle population. An amplified fragment length polymorphism (AFLP) method was employed to detect informative candidate markers, and yielded 44 SNP markers from 220 primer combinations. The allelic frequencies for each marker were estimated by using PCR-RFLP in the japanese black population and inferred that these SNP markers are theoretically sufficient for individual identification, and would also be a powerful tool for a parentage test in japanese black cattle.

Chapter III

MATERIALS & METHODS

The material used for the study and the methods followed are presented here.

3.1. PHENOTYPING METHODS

3.1.1. Plant materials

124 prominent local rice varieties were selected for the current study and the plants were multiplied during kharif 2011 for further studies.

3.1.2. Location of work

Individual variety seeds were sown in the seed bed as separate lines and were transplanted to main field after 25 days in college farm during Kharif 2011 at College of Agriculture, Rajendranager, ANGRAU, Hyderabad-30 and Laboratory work was done at Directorate of Rice Research, Rajendranager.

3.1.3. Selection of Controls

Based on phenotypic data i.e. thousand grain weight (TGW) following controls were selected for contrasting genotypes. A graph was constructed using phenotypic (TGW) data then varieties which have TGW (1000 Grain weight) <22g were considered as low grain weight (LGW) and whose TGW is > 22 g were considered as high grain weight (HGW) varieties. Controls selected for *GW2* based on phenotypic data were

S.No.	Name of the Variety	Thousand grain weight (g)
1	NLR 3041	11.2
2	BPT 5204	12.9
3	DRR- 1265	26.7

3.2 MOLECULAR ANALYSIS.

3.2.1. Isolation of genomic DNA.

Fresh Leaves of 35-dayold seedlings were collected during early hours of the of day and used for DNA extraction. Entire leaf material was stored at -80^0C for long storage. Genomic DNA was extracted from stored leaf using cetyltrymethyl ammonium bromide (CTAB) method.

Method has been described below

1. A piece of young fresh rice leaf (2 cm) sample was taken into a well of a spot test plate (Thomas Scientific) and ground using a polished glass rod after adding 400 µl of prewarmed (65^0C) extraction buffer (0.5ml). Grinding was done until the buffer turned green, which is an indication of cell breakage and release of chloroplasts and cell contents. Another 400 ul of the extraction buffer was added and mixed into the well by pipetting.

2. Total amount of the lysate was transferred to the 1.5 ml labeled centrifuge tubes.

3. Kept in water bath at 65^0C for 30 min with intermittent mixing

4. Samples cooled to room temperature.

5. The lysate was deproteinized using equal volume of chloroform: isoamyl-alcohol (24:1).

6. Spun at 12,000 rpm for 10 min.

7. The aqueous supernatant was transferred to a labeled fresh centrifuge 1.5 ml tube carefully.

8. Equal volume of prechilled isoproponal added and mixed gently.

9. Allowed for 30 min at -20^oC/overnight.

10. DNA was spun down at 12,000 rpm for 5-10 min.

11. Obtained DNA Pellet was washed with 70% Ethanol for three times.

12. DNA was air dried and resuspended in 50 µl of T_{10}-E_1 buffer (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0). The DNA was stored in -20^0C for any further use.

3.2.2. Quality-check for DNA.

Extracted DNA samples were run through 0.8% agarose gel (0.5X TBE) and after series of dilutions all samples were brought to uniform concentration of 20-25 ng/ µl. Then these samples were tested for amplification using designed primers and run through 1% agarose gel (0.5X TBE).

3.2.3. PCR Protocols.

The standard PCR reagents in total volume of 15 µl. were

For *GS3*

S. No.	Component	Vol(µl)
1	Deionised distilled Water	8.0
2	10X PCR Buffer	1.5
3	dNTP (mix) (10 mM each)	0.5
4	EFP –External Forward primer (5 pM)	0.5
5	ERP- External Reverse primer (5 pM)	0.5

For *GW2*

S. No.	Component	Vol(µl)
1	Deionised distilled Water	8.0
2	10X PCR Buffer	1.5
3	dNTP (mix) (10 mM each)	0.5
4	EFP- External Forward primer (5 pM)	0.5
5	ERP- External Reverse primer (5 pM)	0.5

6	IFLP- Forward primer (5 pM)-Long	0.5
7	ISRP- Reverse primer (5 pM)-Short	0.5
8	Taq Polymerase (5U/µl)	1.0
9	DNA Template (20-25 ng/L)	2.0

6	TSR-- Reverse primer (5 pM)-High	0.5
7	CSF- Forward primer (5 pM)-Low	0.5
8	Taq Polymerase (5U/ µl)	1.0
9	DNA Template (20-25 ng/L)	2.0

Table 3.1.PCR master mix.

The primer pairs and their sequences are listed below.

Table.3.2.List of primers of DRR- GW marker system

Primer name	Sequence	Specific to	Pro Size
E3_114T_R	CACGGCGCGAGCTTGGCCTCGAGA	allele T(high)	573
E3_114C_F	CAGAAGAAGCTCCGGAAGCTGATC	allele C(low)	307
E3_114_EF:	GCGGCTATGTGGCTCTGGCAC		
E3_114_ER	CCTAATCGAAACGCTCCACGCC		

Table.3.3.List of primers of DRR- GL marker system

Primer Name	Sequence	specific to	Pro size
EFP	AGGCTAAACACATGCCCATCTC		
ERP	CCCAACGTTCAGAAATTAAATGTGCTG		
IRSP	AACAGCAGGCTGGCTTACTCTCTG	allele C	147
IFLP	ACGCTGCCTCCAGATGCTGA	allele A	262

PCR reaction and conditions for the amplification of designed primers as follows and PCR reaction was carried out using Eppendorf thermal cycler (USA).

Initially primers were tested at 55^0C. Amplification was good but non specific bands also were produced hence the annealing temperature for the primer pairs was adjusted through gradient PCR to avoid non-specific allele amplification The PCR products separated on 1% agarose gels according to the lengths of amplified fragments.

The cycler was programmed as under.

Programme for *GW2*

Step	Temp(°C)	Duration HH:MM:SS	Cycles
Initial denaturation	94	00:04:00	1
Denaturation	94	00:00:30	30
Primer annealing	64	00:00:30	30
Primer Extension	72	00:01:00	30
Final Extension	72	00:07:00	1
Store	12	infinite	1

Programme for *GS3*

Step	Temp(°C)	Duration HH:MM:SS	Cycles
Initial denaturation	94	00:04:00	1
Denaturation	94	00:00:30	30
Primer annealing	69	00:01:00	30
Final Extension	72	00:07:00	1
Store	12	Infinite	1

Table 3.4. PCR programme

3.2.4. Visualization of amplified products in agarose gel

Separation and visualization of PCR products was done on 1% agarose. Agarose gels were used to check amplification and polymorphism among rice accessions.

Agarose gel electrophoresis

Agarose was casted in 1% gels in TBE buffer (0.5X). Slabs were casted in a horizontal gel frame and products were visualized by incorporating 0.5 µl (10 mg/ml) ethidium bromide per 10 ml of gel solution and viewed in a gel documentation system. The procedure of gel electrophoresis is as follows

1. The Perspex tray and comb were thoroughly was cleaned with 70% alcohol with tissue paper.
2. The ends of perspex tray were sealed with cellophane tape.
3. Agarose gel (1 %) was prepared by adding 1 g agarose to 100 ml of TBE (0.5X) buffer (EDTA 0.5 M at pH 8).
4. The solution was boiled by putting the flask in microwave oven and ethidium bromide (5 µl of conc. 10 mg/ml) was added to the gel when temperature of gelis approximately 45^0C and mixed gently.
5. The gel was poured into the tray and air bubbles were removed by using pipette. After the gel was completely set, tape was removed and the gel was placed into the electrophoresis tank.

6. 0.5X TBE buffer was poured into the electrophoresis tank enough to cover the gel to a depth of 5 mm.
7. Comb was removed carefully after completed solidification.
8. About 3µl of loading buffer (3X) bromophenol blue dye was added to PCR samples and mixed by gentle tapping.
9. PCR samples were loaded onto the wells and the power supply of about 120 Volts was provided to run the gel.
10. The power supply was switched off when was about 2 cm from positive end, and the gel was removed from the gel apparatus.
11. The gel was viewed and photographed by using gel documentation system (Alpha Innotech,USA).

Chapter IV

RESULTS AND DISCUSSIONS

4.1. Grain Weight distribution among the genotypes.

In the present study 127 rice varieties with varied grain weights were selected and thousand grain weight (TGW) was measured. These germplasm was phenotyped and a graph was developed from the phenotypic data of thousand grain weight. Distribution pattern of grain weight among the varieties is given below.

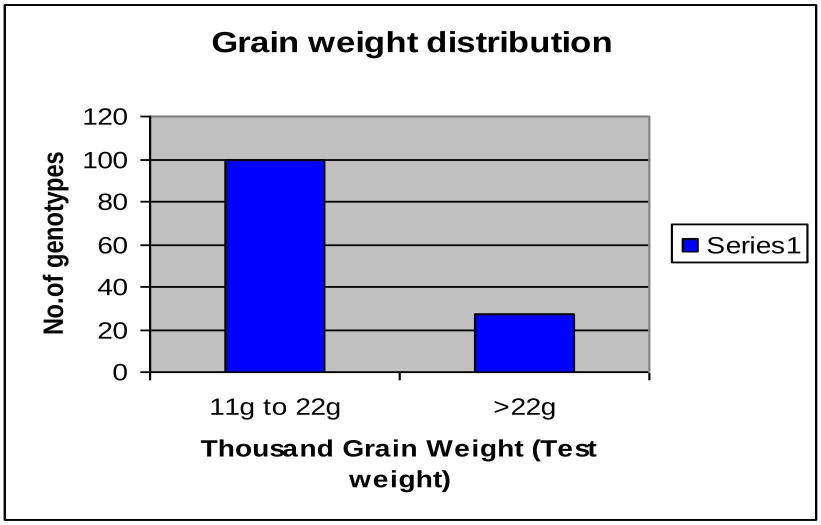

Fig.4.1. Grain weight distribution among the selected germplasm

In the selected material, 100 varieties have shown low grain weight (TGW<22g), 27 varieties have shown high grain weight (TGW >= 22g).

Song *et al.* (2007) identified *GW2*, a major QTL for grain weight on second chromosome using WY3 and FAZ1 (*japonica* cultivars). Later, that QTL was fine mapped and cloned the gene for *GW2* which codes for E3 Ubiquitin ligase. Further they compared between WY3 (High TGW variety) and FAZ1 (Low TGW variety) alleles and identified three SNPs i.e. one at 114 nucleotide position (presence of “T” allele in high TGW cultivars/presence of “C” allele in low TGW cultivars), second one at 316 nucleotide position (“A” deleted) and third one at 1116 nucleotide position (presence of “G” allele in high TGW cultivars/presence of “C” allele in low TGW cultivars).

To design a functional marker targeting the functional nucleotide polymorphism, the genomic sequence of the *GW2* gene was obtained from GenBank (accession number EF447275) and an SNP at nucleotide position of 114 was targeted. At 114 nucleotide position presence of “T” was reported in high TGW genotype (WY3) and presence of “C” was reported in low TGW genotype (FAZ1). One pair of primers targeting each “T” and “C” allele of *GW2* and one pair of common primers were designed for this purpose using the software tool i.e. FastPCR (Kalendar 2009).

This marker assay named DRR-GW consists of two common primers (External forward Primer- EFP and External Reverse Primer-ERP) flanking the SNP and one specific primer targeting the “C” allele (C specific forward Primer-CSF), while the other specific primer targets the “T” allele (T specific reverse primer-TSR). The common primers EFP/ERP amplify at a region of 881 bp amplicon as a common fragment, while the “C” allele-specific primer pairs, CSF and ERP amplify 307bp amplicon whenever “C” allele exist. Where as “T” allele-specific primer, i.e TSR and ESF amplify 573 bp, amplicon whenever “T” allele exist (Fig. 4.1).The primers have been designed in such a way that each allele-specific primer produces a PCR product size of 100 bp more or less than that of any other and hence PCR amplicons can be separated in a lesser amount of agarose gels.

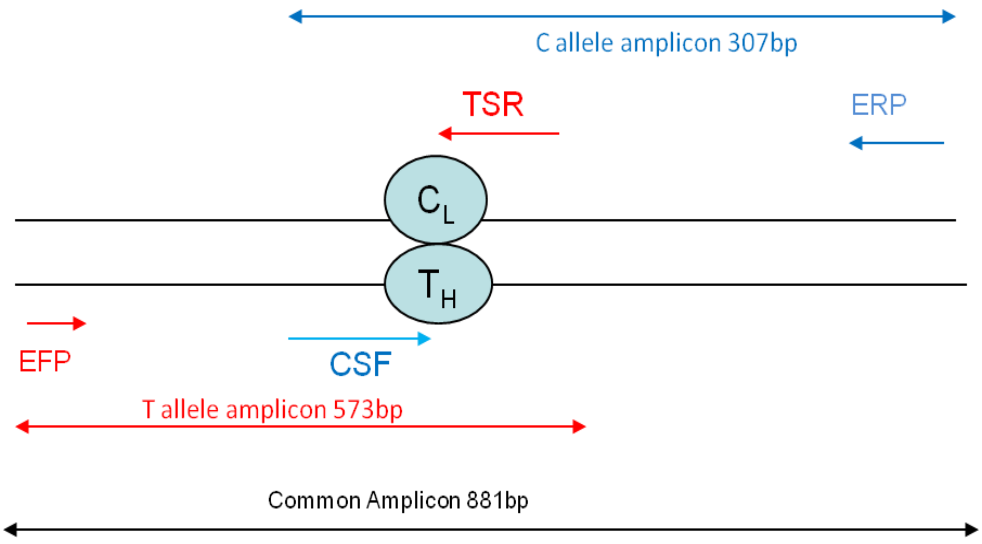

Fig.4.2 Schematic illustration of the development of PCR based marker DRR-GW targeting an SNP at 114 nucleotide position of *GW2* gene.

4.2 PARENTAL POLYMORPHISM.

For standardization of the designed marker system (DRR-GW) NLR-3041and BPT 5204 were selected as low TGW parents and DRR-1265 was selected as high TGW parent (Table.4.1).

S.No.	Name of the variety	Test Weight(TGW)(g)
L_1	NLR 3041	11.2
L_2	BPT 5204	12.9
H	DRR-1265	26.3

Table. 4.1. Parents. L_1, L_2 are low grain weight varieties and H represents high grain weight variety.

Designed primers have been standardized at an annealing temperature of 64°C by gradient PCR.Using the DRR-GW assay, in NLR 3041 and NLR 34449 (both low grain weight varieties) have showed expected common amplicon of 881 bp and LGW allele i.e. "C" allele specific amplicon size of 307 bp. In case of DRR-1265 (HGW cultivar) showed expected "T" allele specific amplicon size of 573 bp. But the expected common amplicon size of 881 bp was not observed in DRR-1265 (HGW variety)(Fig.4.1). Since the both allele specific amplicons were distinct enough, the further validation was carried out.

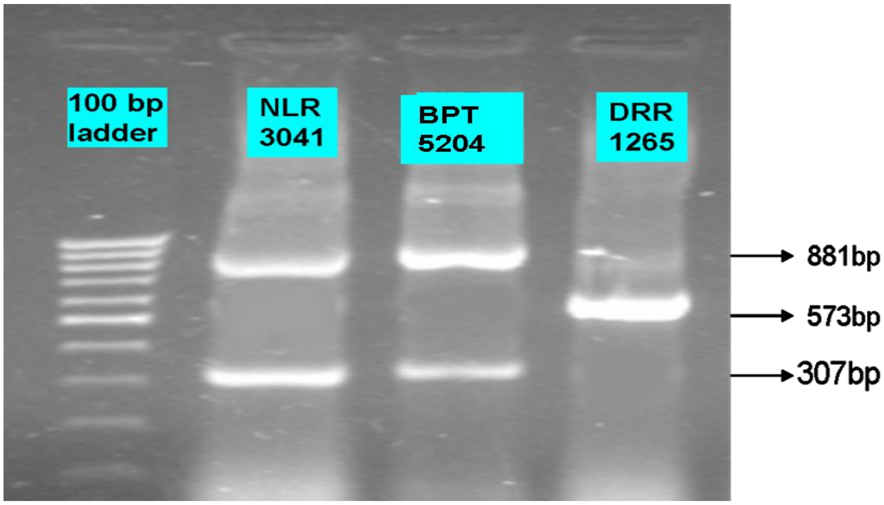

Fig: 4.3. Parents showing expected amplification pattern

4.3. VALIDATION OF MARKER SYSTEM (DRR-GW).

Among 127 varieties, 100 varieties which have low TGW were selected initially and used for screening. In the screening, the marker system (DRR-GW) showed the expected amplicon size of 307bp in all the low TGW varieties. Later, all the 27 varieties which have high TGW were screened. Out of 27, only seven (7) have shown the expected amplicon size of 573 bp corresponding to the “T” allele i.e. high TGW, since these varieties have also showed high grain width.

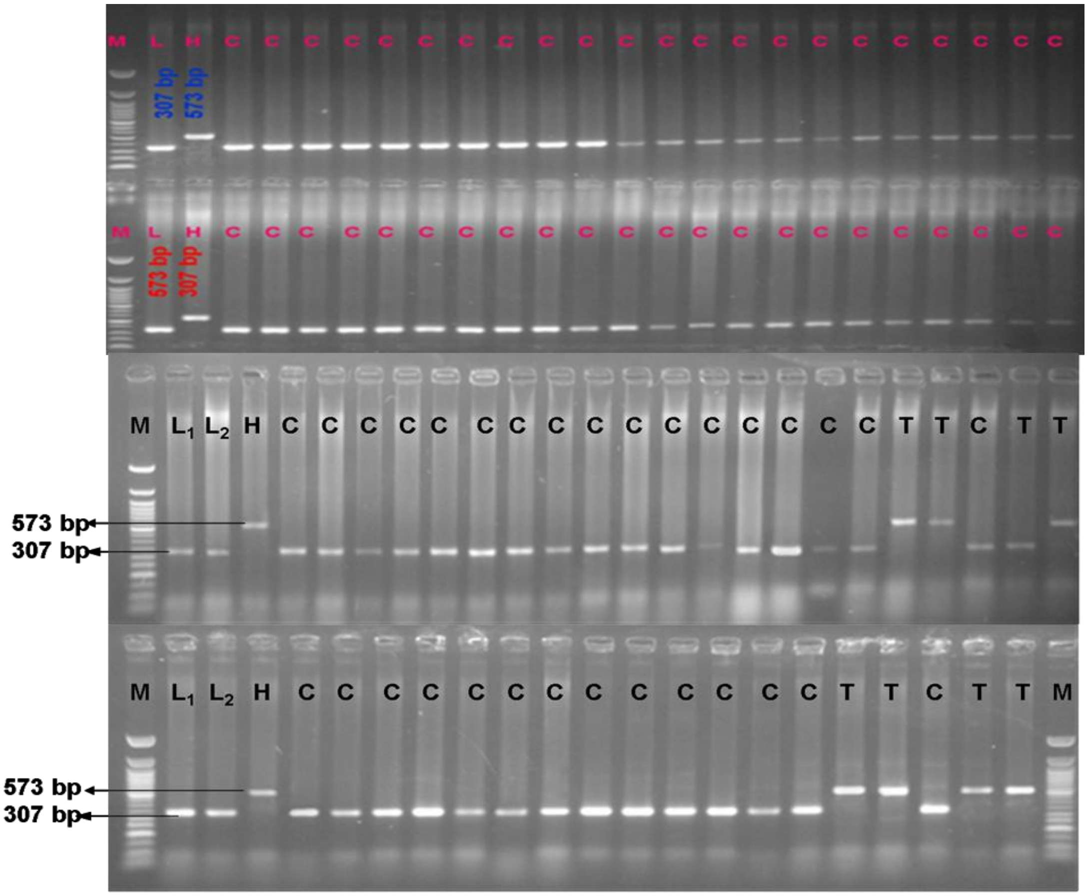

Fig. 4.4. (Representative) genotyping with DRR-GW and dominance of "C” allele in the screened germplasm. L in first and second rows represents NLR-3041(a low TGW variety) and H represents DRR-1265 (a high TGW variety). $L_{1,}$ $L_{2,}$ H in third and fourth rows represents NLR 3041, BPT 5204, DRR-1265 respectively, C and T are the observed alleles in the screened germplasm.

Name of the Variety	Test weight(g)	Observed allele in *GW2*	Name of the Variety	Test weight(g)	Observed allele in *GW2*
NLR 3041	11.2	C	NLR 20083	16.9	C
BPT 5204	12.9	C	MTU 1032	17.0	C
NLR34449	13.0	C	MTU 20084	17.0	C
PL 25	13.1	C	NLR 20128	17.0	C
MTU 1006	13.2	C	DRR 2711	17.1	C
PE 14	13.3	C	NRL 33626	17.1	C
AL 11	13.4	C	NLR - 3061	17.2	C
BPT 5204	13.5	C	MDT 8	17.2	C
PL 24	13.6	C	PE - 3	17.3	C
JGL - 1798	13.9	C	AE 4	17.4	C
PL 23	14.0	C	PE 7	17.5	C
NLR 2014	14.3	C	DRR 1218	17.5	C
PE - 1	14.6	C	NRL 20127	17.5	C
NLR 20106	14.6	C	NLR 34242	17.5	C
JGL 3449	14.7	C	NLR 30491	17.6	C
NLR 40024	14.7	C	PL - 4	17.8	C
NLR 2146	15.0	C	NLR 40058	17.8	C
NLR 40024	15.1	C	NLR 34303	17.9	C
NRL 38892	15.2	C	PLA 1100	18.0	C
AE 14	15.3	C	NRL 400058	18.1	C
NRL 20131	15.6	C	MTU 1031	18.2	C
NLR - 34303	15.8	C	NLR 33671	18.2	C
NLR 33671	15.8	C	DRR 1065	18.5	C
PE 11	16.1	C	MTU 5249	18.5	C
DRR 1865	16.1	C	MTU 2077	18.5	C
NRL 20017	16.2	C	NRL 28600	18.5	C
PL 18	16.3	C	PE - 10	18.6	C
PE 8	16.4	C	NLR 34242	18.6	C
AE 12	16.5	C	AE 14	18.6	C
NRL 3042	16.5	C	PE 19	18.6	C
NRL 30491	16.8	C	NLR 20136	18.6	C
MTU 1064	18.7	C	DRR 1424	21	C
NRL 28523	18.7	C	NRL 20002	21	C
AE 7	18.9	C	NRR - 40062	21.2	C
MTU 1075	18.9	C	DRR 1819	21.2	C
NTU 7029	19.0	C	IMP SAMBA MASHURI	21.2	C
NRL 20022	19.2	C	NLR 34242	21.7	C
NLR 33641	19.2	C	MTU 1010	21.9	C
SWARNA SUB 1A	19.2	C	NLR 33359	22.2	T
NLR 3058	19.3	C	NLR 34452	22.3	T
DRR 2898	19.3	C	NLR 27999	22.3	C
NLR 33654	19.4	C	DRR 126	22.5	C
NLR 145	19.4	C	PUSA SUG 2	22.6	T
MTU 2067	19.6	C	MTU1001	22.6	C
NLR 9676 - 96	19.6	C	DRR 1899	22.9	C
DRR 3750	19.7	C	DRR 1304	23.1	C
MTU 1061	19.7	C	MTU 3626	23.1	C
MTU 1120	19.9	C	TELLA HAMSA	23.2	C

RAJENDRA	20.0	C	PUSASUG 5	23.4	C
RRL 9674	20.0	C	PUSA 1460	23.4	C
NRR 53359	20.1	C	BO 160	23.4	C
AE 5	20.1	C	PE 6	23.5	C
NRL 33358	20.1	C	BO 163	23.5	C
NLR 33654	20.1	C	DRR 65(S)	23.7	C
NRL 20083	20.2	C	DRR1765	23.9	C
DRR 1209	20.4	C	NRL 33057	24.0	C
NRL 3010	20.4	C	NLR 3064	24.2	T
DRR 1847	20.5	C	PUSA SUG 3	24.2	T
DRR 2064	20.5	C	DRR 3757	24.5	C
DRR 3386	20.6	C	PUSA 33	25.0	T
DRR 1405	20.9	C	I6 64	26.2	T
MTU 4870	20.9	C	DRR 1265	26.7	T

Table 4.2. Genotyping pattern with DRR-GW among different varieties

Genotyping with DRR-GW revealed that most of the varieties showed the presence of "C" allele which is specific to the low grain weight varieties and very less frequency of presence of "T" allele was observed in case of high grain weight varieties. Generally presence of "T" allele is expected in all high grain weight varieties, but only seven genotypes out of 27 (26%) showed the presence "T" allele. This indicates the dominance of "C" allele in the screened germplasm and it also indicates that, in the evolution the high grain weight allele "T" might have arisen recently in the process of rice domestication.

DRR-GW marker assay showed the presence "C" allele at 114 nucleotide position of *GW2* in all low grain weight varieties. In seven of high grain weight varieties showed presence of "T" allele, since these varieties also showed high grain width (>2mm). The rest of high grain weight verities doesn't have high grain width compared to those seven varieties. *GW2* earlier reported the major QTL for grain weight and width. But in present study it appeared that, *GW2* may have more correlation to grain width rather than grain weight.

4.4. Association of grain size major QTL, GS3 with grain weight.

In the earlier marker assay (DRR-GW), less significance association was observed between marker and grain weight. So we have selected another functional marker assay (DRR-GL) which was developed based on the SNP present on the second exon of *GS3* gene. *GS3* is a major gene identified by Fan *et al.* (2006) and later, gene was cloned which codes for Transmembrane protein. In exon 2 of *GS3*, the presence of C/A SNP was reported. In most of the short grain genotypes the presence of "C" allele was reported where as in long grain varieties reported the presence of "A" allele. To standardize this marker assay (DRR-GL), varieties like Swarnadhan and Jaya were selected since these varieties showed short kernel length (<8 mm) and also have low TGW (TGW < 15g). Similarly Phalguna and Mugadsugandh were selected since they have long kernel length (>9mm) and also high TGW (>22g).

Upon marker assay (DRR-GL) with these four varieties Swarnadhan(S_1) and Jaya(S_2) showed expected common amplicon of 365 bp and 147 bp amplicon which is specific to "C" allele. While Phalguana(L_1) and Mugadsugandh (L_2) also shown expected amplicon of 365 bp and a specific amplicon of 262 bp. The amplification pattern in these four varieties was matched with reported allele sizes of parents used in the assay of Ramkumar *et al.* (2010).To further test this marker assay (DRR-GL), 52 varieties of the germplasm were selected.

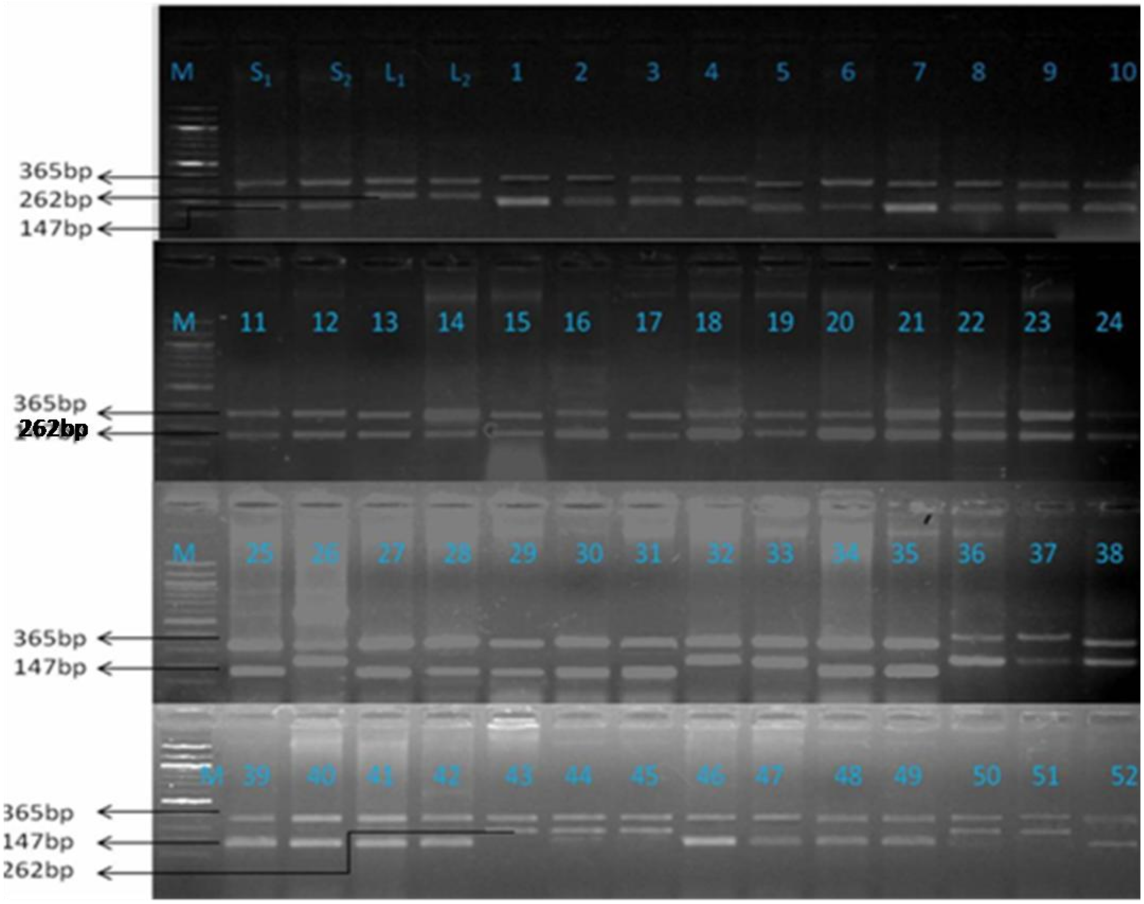

Fig: 4.5. Genotyping with DRR-GL among 52 varieties. S_1, S2, L_1 and L_2 represent Swarnadhan, Jaya, Phalguan and Mugadsugandh respectively. 1-52 represents local varieties used in screening of *GS3*. M represents 100 bp ladder.

Upon genotyping with DRR-GL in 52 varieties, this marker assay showed more correlation with grain weight. The "C" allele of *GS3* which was reported to the associated with short grain length was also present in low grain weight varieties. Similarly "A" allele of *GS3* which was reported the associated with long grain length varieties was also present in high grain weight varieties.

Among 52 varieties, the "A" allele corresponding to the high grain weight was observed in 16 varieties where grain length is also long. Where as "C" allele corresponds to the low grain weight present in 10 varieties where grain length is short. In another class, 18 varieties where grain length is long, grain weight is low, but showed presence of "C" allele, which corresponds to the low grain weight. In one variety, which has small grain length but showed high grain weight, in this case also DRR-GL showed presence "A" allele, which corresponds to high grain weight. Totally 45 varieties have showed corresponding allele with DRR-GL. This correspondence was around 86 %. Only seven varieties which have short grain length but have high grain weight, in this case DRR-GL showed presence of non corresponding allele "C". Hence in the presence study, the DRR-GL showed more correspondence (86 %) to the grain weight than the GRR-GW (26%). The newly developed functional marker DRR-GW was targeted only one functional polymorphism i.e. SNP at 114 nucleotide position of *GW2* which may not be sufficient enough to explain the variation exists in the grain weight phenotype of selected germplasm.

It is expected that another two reported functional polymorphism together with this 114 nucleotide SNP may be responsible for creation variation in grain weight phenotype. Hence, the development of functional markers targeting another two polymorphic sites may be required to use effectively in molecular breeding programmes.

Chapter V

SUMMARY AND CONCLUSIONS

With an ever-increasing global population but rapidly decreasing farmland and changing environment, there is an urgent need to maintain the yield stability of rice and further improve the rice grain yield using molecular genetic breeding approaches.

Advances in rice biotechnology and genomics have paved the way to meeting the challenges and major agronomic traits have been systematically identified using DNA markers. For some genes, functional molecular markers derived from fully characterized sequence motifs have been identified showing complete linkage with the target traits (Andersen and Lubberstedt 2003). Agronomically valuable genes can be efficiently incorporated into the genetic background of elite *indica* and *japonica* cultivars without linkage drag through an appropriate breeding strategy combined with biotechnology and bioinformatics tools by MAS.

In this work, a gene approach based on the ubiquitin ligase gene (*GW2*) was followed to investigate whether *GW2* may be responsible for the high grain weight in *Oryza sativa*.sub.*indica* and to develop a functional marker for *GW2* in *indica* varieties. This gene was reported to code for the first step in the ubiquitin ligase synthetic pathway.

Development of simple and reliable PCR-based markers is an important component of marker-aided selection (MAS) activities for agronomically important genes in rice breeding. In order to develop PCR-based markers for a rice grain weight gene *GW2*, located on chromosome 2, the nucleotide sequence of *GW2* was used to design and synthesize primers.

A Simple SNP Based PCR Marker development for grain weight and width was taken up in the Department of Plant Molecular Biology & Biotechnology, college of agriculture, Rajendranagar, Acharya N.G. Ranga Agricultural University in Collaboration with Directorate of Rice Research, Rajendranagar, Hyderabad.

The present experiment was aimed at studying the association between grain weight and other yield components and to develop the functional marker for grain weight, which can be used in molecular breeding programme for selection of favorable allele of grain weight and subsequent development of varieties with more grain weight to enhance the yield.

The major reported QTL, *GW2* was selected and the SNP (C/T) at 114 nucleotide position was targeted for developement of PCR based co-dominant functional marker. This marker assay was named as DRR-GW and consists of two primers, which are common to the two alleles and one pair of primer specific to the SNP, these primer were designed in such a way to amplify the amplicons in varied length, specific to each allele.

This maker assay DRR-GW was standardized using very high and very low grain weight genotypes. Upon validation of this marker system all the low grain weight varieties were showed the corresponding “C” allele, where as only seven high grain weight varieties showed the corresponding “T” allele.

To test the correlation of grain length to the grain weight the previously developed functional marker i.e. DRR-GL (which was developed based on the SNP of *GS3* gene) was tested. The DRR-GL showed the presence the “C” allele (which is short grain allele) in most of low grain weight varieties. Similarly, presence of “A” allele (which is long grain allele) also showed in most of the high grain weight varieties. Though some varieties showed non correspondence of grain length to the weight, the DRR-GL showed the corresponding allele to the grain weight. Overall, in the presence study an attempt was made to determine the proportion of *GW2/GS3* allele and their association to the grain weight trait.

The information generated in the present study could be used in development of more refined functional marker to be used precisely in molecular breeding programme.

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APPENDICES

0.5 % EDTA -100 ml, pH-8

Take 18.61g of EDTA and initially dissolve it in 40 ml of distilled water .Mix it well by stirring with a magnetic stirrer. Adjust the pH to 8.0 with NaOH or HCl. Allow it to dissolve completely, and then make the volume up to 100 ml with dist.water. Sterilize by autoclaving and check pH by using pH paper .Store at room temperature.

Preparation of ethedium bromide
One gram of ethedium bromide for one ml sterile distilled water

1X TE Buffer (10:1) – 100 ml

Take 1 ml of 1M Tris, 200 ul of 0.5 M EDTA (pH 8) and make the volume up to 100 ml with sterile distilled water

1M Tris HCl MW=121.4gm 1M solution = 121.4 gm in 1000ml
0.5 M EDTA MW=372.2gm 1M solution = 372.2gm in 1000ml

5M NaCl – 1000ml
Dissolve 29.22 gm of NaCl in 80ml of SDW. Sterilize by autoclaving and store at room temperature in transparent bottles

Loading dye – 100 ml 6X
40% sucrose = 40 gm
0.0025% bromophenol = 0.25gm
0.0025% xylene cynol = 0.25 gm
Take 40gm of sucrose and dissolve in SDW add BPB and xylene cynol Allow it dissolve completely. Make the volume up to 100 ml and store it at room temperature

0.5X TBE buffer – 2L

Take 100 ml of 10X TBE buffer in measuring cylinder and add 19 ml of SDW to make the volume 2 liters.

10X TBE buffer – 1L

Add 108gm of Tris base in 500ml of DDW then add 55 gm of boric acad. Mix well by stirring with magnetic stirrer and then add 40ml of 0.5 M EDTA. After complete dissolving, water filter using 0.45 uM filter paper. Sterilize by autoclaving store at room temperature

1M Tris Cn – 1L pH 8

Take 12.114gm of Tris base in 40ml of SDW of mix it by stirring with a magnatac stirrer heat it slightly if required. Then make the volume up to 80ml before adjusting the pH. Adjust the pH to 8 by adding approximately 30ml of 0.1N HCl. Then make the volume of 100ml with SDW. Autoclave it then store at room temperature.