Received: 05 Jan 2023 | Accepted: 28 May 2024 | Online: 03 Jun 2024

Electronic Journal of Plant Breeding

Research Article

Association study of allelic variation identified at yield contributing loci in rice (*Oryza sativa* L.)

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Abstract
The knowledge of functionally characterized genes and linked markers that govern grain yield and its related traits were used to unravel the markers/genes that associate with diversity of yield traits in a panel 30 novel rice genotypes comprising of *indica*, *japonica*, aromatic, landraces, interspecific crosses (*O.glaberrima*/*O.sativa*) etc. The ANOVA showed significant differences (P=<0.001) for all traits among the genotypes. Molecular analysis was done by employing 37 gene-specific markers targeting 24 yield governing genes in rice. Two markers *viz*., RM223(YLD) on chromosome 8 and GW004(*GW2,* LOC_Os02g14720) on chromosome 2 were identified as polymorphic. Average PIC (polymorphic information content) value for these primers was 0.37, suggesting the availability of accountable variability at these loci. Cluster analysis grouped the genotypes into three clusters. Marker-trait-association analysis revealed significant association of the marker GW004 with panicle length, plant height and filled grains in rice. Pearson correlation study also supported the molecular associations through significant phenotypic associations for trait pairs plant height and filled grains, panicle length and plant height. The phenotypic variance explained (R^2%) by the marker across panicle length, plant height and filled grains was ≥15%, suggesting major role of the locus/gene in controlling respective traits. Thus, GW004 marker could be employed in breeding programs for simultaneous selection of three traits panicle length, plant height and filled grains. From the current study it can be concluded that allelic variation studies using novel genotypes can result in the identification of suitable donors along with trait associated markers for rice yield improvement.

Keywords: Rice, gene tagged markers, Allelic variation, Association study, GW2 gene

INTRODUCTION

Rice (*Oryza sativa* L.) is a fundamental food source for more than half of the global population and is a crucial crop worldwide. The majority of rice, around 90%, is grown and consumed in Asia (USDA, 2020). In Andhra Pradesh, rice is cultivated over an area of 2.32 million hectares, producing 7.88 million tonnes with a productivity of 4437 kg per hectare (Ministry of Agriculture & Farmers Welfare, Govt of India 2020-21). Improving the yield of food crops, including rice, is a constant concern due to the increasing global human population, decreasing arable land, and water resources. Enhancing grain yield in rice involves targeting various yield-contributing traits (Anh *et al*., 2015).

Rice varietal development programme relies equally on yield enhancement and on grain quality parameters. Thus, breeders practicing artificial selection during rice breeding for both yield and quality characters depending on the local consumer's preference. However, the yield contributing traits are controlled by many genes and are quantitatively inherited. Many of Quantitative Trait Loci (QTLs) studies showed the association of one genomic site with several traits, especially yield and its component traits, showing linkage and/or pleiotropic effects.

In the past two decades, a large number of QTLs responsible for rice yield and its component traits were

identified (Huang *et al*., 2013) and some genes were cloned *viz*., *sd1* on chromosome 1, *Gn1a*, *DEP1* and *GW2* (Song *et al.,* 2007) on chromosome 2, *GS3* (Fan *et al.,* 2006) and *qGL3* (Zhang *et al*., 2012) on chromosome 3, *Grain Incomplete Filling1* (*GIF1*) on chromosome 4, *Dwarf1* (*D1*), *GW5/qGW5*, *GS5* and *SRS3* on chromosome 5 etc. Grain weight is a typical quantitative trait as the GW is affected by grain length (GL), width (GW) and thickness.

Association analysis is a powerful tool used in genetics to identify genes associated with important traits. In the field of evolutionary biology, analysis of genetic diversity has huge importance for the effective use of genetic resources. Besides predicting potential genetic gain, it contributes significantly to plant breeders for monitoring the germplasm. Molecular markers are promising tool for analyzing genetic diversity in the germplasm. Until recently, majority of studies are based on usage of SSR (simple sequence repeat / microsatellite) markers that randomly spanned on entire genome, which may not directly link to a trait or genic region. With advent of cloned list of trait-oriented genes and with the help of gene tagged functional markers, it is encouraging to analyze novel allelic variants across the available rich genetic diversity of rice thereby to use in the yield or grain quality improvement (Bai *et al*., 2012). Hence, the research has to be hastened up for studying association of novel allelic variation on yield related traits.

MATERIALS AND METHODS

Phenotypic Analysis: A total of 30 rice genotypes, carefully selected from a pool of approximately 250 genotypes maintained by the Department of Genetics and Plant Breeding at S.V. Agricultural College, Tirupati, based on their diversity in yield component traits and categorized into different subgroups including *indica, japonica* and aromatic, were included in this study (**Table 1**). Phenotypic evaluation was carried out at Wetland Farm, S.V. Agricultural College, Tirupati, ANGRAU during *Kharif*, 2019. The experiment was laid out in three replications in Randomized Block Design (RBD) with spacing of 20cm×15cm and two rows of 4m length. The agronomic management was done as per the standard recommendations. The phenotypic data was recorded for 12 yield contributing traits namely number of productive per plant, panicle length, plant height, number of filled grains per panicle, number of chaffy grains per panicle, total grains number per panicle, spikelet fertility, grain length, grain width, grain length to width ratio,1000-grain weight and yield per plant from five plants per replication per genotype employing standard procedures. The data was analyzed using Microsoft Office Excel program to calculate various statistical parameters such as mean, maximum, and minimum values, standard deviation, skewness, and kurtosis. The significance of the measured traits was assessed using IBM-SPSS software, version 20. Frequency distribution curves were plotted for all phenotypic traits. Pearson's correlation analysis was conducted in Microsoft Excel using the average trait values for trait pairs among genotypes, with significance levels set at $p<0.05$ and $p<0.01$.

Molecular Screening of the genotypes: Genomic DNA was extracted from rice leaf samples using a modified version of the Cetyl Tri Methyl Ammonium Bromide (CTAB) method as described by Lin *et al*. (2001). The quality and quantity of the isolated DNA were confirmed by running 0.8% Agarose gel electrophoresis and using a Nanodrop reader. The DNA samples were diluted to a final concentration of 30 ng/µl for subsequent polymerase chain reaction (PCR) analysis.

Table 1. List of diverse rice genotypes used in the present study

S.No.	Genotype	Subspecies/Group	S.No.	Genotype	Subspecies/Group
1	Haryana Basmati	Aromatic, Indica	16	Mrunalini	*Indica*
2	Pant Sugandh Dhan 15	Aromatic, Indica	17	Numali	*Indica*
3	Taramati	Aromatic, Indica	18	Pathariya	*Indica*
4	Azucena	Aromatic, Japonica	19	SannaJajula	*Indica*
5	Abhaya	*Indica*	20	Savithri	*Indica*
6	Aditya	*Indica*	21	Sharbati	*Indica*
7	AMO	*Indica*	22	Solumpiket	*Indica*
8	Anjali	*Indica*	23	TKM 6	*Indica*
9	Badshabhog	*Indica*	24	Burma Black	*Indica* (Landrace)
10	Daddiga	*Indica*	25	RPBio 248	*Indica*/*Indica* wild
11	Disang	*Indica*	26	NL 1 (Nerica line 1)	*Og/Os*
12	HIM 799	*Indica*	27	NL 16 (Nerica line 16)	*Og/Os*
13	Kesari	*Indica*	28	NL 3 (Nerica line 3)	*Og/Os*
14	Krishna	*Indica*	29	NL 9 (Nerica line 9)	*Og/Os*
15	Luit	*Indica*	30	WAB450-24-32-P18-HB	*Og/Os*

Og/Os: Oryza glaberrima/Oryza sativa

The genotype panel was mined for the presence of variation in 24 grain yield related genes using 37 reported gene tagged/linked markers (Monna *et al.,* 2002; Song *et al*., 2007; Shomura *et al.,* 2008; Huang *et al.,* 2009; Wang *et al.,* 2008; Gaafar, 2010; Miura, 2010; Li *et al*., 2011; Wang *et al.,* 2012; Zhao *et al*., 2015; Kim *et al*., 2016 and Liu *et al.,* 2017). The genomic DNA of the rice genotypes was subjected to PCR amplification using a programmable thermal cycler (Eppendorf, Germany). PCR was performed with a reaction volume of 10µl, consisting of 2µl of template DNA at the concentration of 30ng/µl, 0.5µl (2.5pmol) of each forward and reverse primers, 1µl of 2.0mM deoxynucleotide triphosphates (dNTPs), 1µl of 10X assay buffer (10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% Gelatin) and 0.1µl of 5U/µl *Taq* DNA polymerase (Himedia). The PCR was performed with the initial denaturation at 94˚C for 5min followed by 35 cycles of repeated PCR amplification with the denaturation at 94˚C for 1min, annealing at 54-65˚C for 30sec, primer extension at 72˚C for 1min and with final extension at 72˚C for 10min. The amplified PCR products were resolved on a 4% agarose gel prepared in 1X TAE buffer stained with ethidium bromide (10mg/ml).The gel was allowed to run for about two hours at 80-120V. The resolved PCR bands were documented using Bio-Rad Molecular Image-Gel Doc XR System. Allele scoring was done in relative to the molecular weight of known size, *i.e*. 50 or 100 base pairs (bp) DNA ladder and expressed in base pairs.

Molecular Data Analysis: Estimation of the polymorphic information of the markers: The Polymorphism Information Content (PIC) value for each of the polymorphic SSR markers was determined by estimating the number of alleles (Na) present for each marker. The formula used to calculate the PIC value for each marker is as follows: PIC = 1 - Σ(Pi^2) - ΣPj^2, where 'i' is the total number of alleles detected for the SSR marker and 'Pi' is the frequency of the ith allele in the set of thirty genotypes analyzed. In this formula, 'j' is equal to 'i+1'(Botstein *et al.,* 1980).

Cluster Analysis: The DARWIN software version 6.0.010 (http://darwin.cirad.fr/) was utilized to calculate a dissimilarity index based on the allele size data set, which contained single allele data. The resulting dissimilarity matrix was then subjected to Neighbour-Joining analysis, and the dendrogram was constructed using the unweighted pair group method with arithmetic averages (UPGMA) algorithm.

Association analysis: The association between marker alleles and yield and yield-related traits was analyzed using TASSEL (Trait Analysis by aSSociation, Evaluation and Linkage) Version 2.1 software (http://www.maizegenetics.net/). The analysis took into consideration the population structure (Q) and kinship (K) values, following the approach described by Bradbury *et al.* (2007). Both GLM (General Linear Model) and MLM (Mixed Linear Model) methods were used for association analysis. The markers tested and sub-population data (Q matrix) were considered as fixed-effect factors, while the kinship matrix was considered as a random-effect factor. The results were reported in terms of 'P value', which indicates the significance of the association between the marker and the trait, and 'R2', which represents the fraction of the total variation explained by the marker.

RESULTS AND DISCUSSION

The detailed description of phenotypic observations on grain yield and its related traits, their means, maximum value, minimum value, standard deviation, ANOVA (*P*-value), skewness and kurtosis are furnished in **Table 2**. Genotype's ranking for respective traits was assigned based on Duncan's Multiple Range Test (DMRT) performed in IBM-SPSS version 20, which shows wide variation in the phenotypic performance for all the traits.
Rice genotypes possessed vast variation for the yield and grain quality traits under study and which is evident from the ranks assigned to mean *perse* of each genotype under each trait (**Table 2**). The analysis of variance (ANOVA) also showed highly significant differences (*P*=<0.001) for all traits among the genotypes (**Table 2**) and thus the suitability of the genotypes to conduct association analysis at the molecular level is explained. It further strengthens about the availability of rich genetic diversity in rice which can be used in rice improvement programmes after its validation (Bashir *et al*., 2010; Anh *et al*., 2015)

The details of five best performed genotypes for yield and its related traits are provided in the **Table 3**. Four genotypes namely Burma Black, SannaJajula, Mrunalini and Azucena recorded better performance for multitude of traits *i.e.* about five yield contributing traits, when compared to other genotypes. Therefore, these genotypes can be utilized as donors for improvement of yield and grain quality in rice.

The frequency distribution pattern is not following the normal distribution for the traits, productive tillers per plant (skewness,1.56; kurtosis,3.71) (**Table 2**), panicle length (skewness,0.62; kurtosis,0.47), plant height (skewness,1.31; kurtosis,2.23), number of filled grains per panicle (skewness,0.44; kurtosis,-0.67), number of chaffy grains per panicle (skewness,0.87; kurtosis,0.56), and grain length/width ratio (skewness,1.45; kurtosis,3.68), which exhibited skewness towards more positive values from mean, and along with positive kurtosis nature that denotes peaked distribution except for chaffy grains per panicle. Percent spikelet fertility (skewness,-0.76; kurtosis,0.06) exhibited negative skewness, explaining that in many genotypes under study the trait is controlled by low performing genes. The traits, grains per panicle (skewness,0.15; kurtosis,-0.81), grain length (skewness,0.37; kurtosis,-0.48), grain width (skewness,0.36; kurtosis, 0.76), 1000-grain weight (skewness,-0.22; kurtosis,-0.40) and YP (skewness,0.35;

Table 2. Phenotypic analysis performed for the observations recorded among 30 genotypes on grain yield and its related traits

S.No.	Genotype	PT	PL(cm)	PH(cm)	FG	CG	GN	SF(%)	GL(mm)	GW(mm)	GR	TGW(g)	YP(g)
1	Abhaya	10.60^{bcde}	25.27^{defg}	120.80^{cdef}	123.43^{fghijkl}	16.38^{hij}	139.82^{ijklmn}	89^{abcd}	8.71^{jklmn}	2.41^{jkl}	3.61^{efghi}	22.58^{bcdef}	22.18^{bcdefg}
2	Aditya	10.60^{bcde}	21.93^{hi}	**81.20^{n}**	**58.58^{o}**	11.60^{ij}	**70.18^{q}**	84^{abcdef}	8.81^{jklm}	2.77^{def}	3.18^{ijklm}	24.69^{abcde}	**7.90^{i}**
3	AMO	11.58^{bcde}	24.07^{efgh}	101.60^{hijk}	158.73^{bcdef}	34.00^{cdefghi}	192.73^{cdefg}	83^{abcdef}	8.16^{nop}	2.55^{ghijk}	3.21^{ijkl}	21.40^{cdefgh}	25.49^{abcde}
4	Anjali	**6.80^{e}**	26.33^{bcde}	113.60^{efgh}	78.53^{mno}	12.67^{ij}	91.20^{opq}	86^{abcde}	8.03^{op}	3.02^{c}	2.66^{mn}	24.34^{abcde}	**8.48^{i}**
5	Azucena	15.00^{bc}	26.13^{bcde}	98.42^{ijkl}	125.58^{fghijk}	23.92^{ghij}	149.50^{ghijklmn}	84^{abcdef}	10.41^{b}	2.91^{cd}	3.57^{fghi}	27.71^{abc}	29.47^{abc}
6	Badshabhog	10.33^{bcde}	28.73^{ab}	118.87^{cdefg}	147.27^{cdefg}	29.87^{defghij}	177.13^{efghij}	83^{abcdef}	10.03^{bcde}	2.41^{jkl}	4.16^{cde}	21.74^{cdefgh}	22.82^{bcdef}
7	Burma Black	7.02^{e}	28.33^{abc}	**175.33^{a}**	190.47^{ab}	18.33^{ghij}	208.80^{abcde}	**91^{a}**	9.57^{defghi}	**3.49^{a}**	2.74^{lm}	28.71^{ab}	28.14^{abcd}
8	Daddiga	7.20^{e}	23.60^{efghi}	128.73^{cd}	180.17^{bc}	21.67^{ghij}	201.83^{bcdef}	89^{abc}	**7.36^{q}**	3.31^{b}	**2.22^{n}**	24.65^{abcde}	21.42^{bcdefg}
9	Disang	11.47^{bcde}	23.93^{efgh}	95.80^{jkl}	86.85^{lmno}	15.88^{hij}	102.73^{nopq}	85^{abcdef}	8.09^{op}	2.58^{ghij}	3.14^{ijklm}	24.43^{abcde}	10.22^{hi}
10	Haryana Basmati	8.93^{de}	22.07^{hi}	87.87^{lmn}	137.60^{efghi}	29.87^{defghij}	167.47^{efghij}	82^{abcdefg}	8.48^{lmno}	2.17^{mn}	3.91^{efg}	**15.00^{i}**	19.03^{defgh}
11	HIM799	15.00^{bc}	25.87^{cdef}	106.60^{ghij}	103.00^{hijklmn}	41.00^{bcdefg}	144.00^{hijklmn}	74^{efgh}	9.00^{ijkl}	2.55^{ghijk}	3.54^{fghi}	**30.03^{a}**	21.63^{bcdefg}
12	Kesari	15.53^{b}	**21.13^{i}**	97.60^{ijkl}	148.80^{cdefg}	16.67^{hij}	165.47^{efghijk}	90^{ab}	8.14^{nop}	2.53^{ghijk}	3.22^{ijkl}	18.54^{efghi}	29.93^{ab}
13	Krishna	8.13^{de}	24.20^{efgh}	101.40^{hijk}	175.27^{bcd}	51.07^{bcd}	226.33^{abcd}	78^{bcdefgh}	8.57^{klmno}	2.16^{mn}	3.98^{def}	15.94^{hi}	22.39^{bcdefg}
14	Luit	7.67^{de}	23.13^{fghi}	95.13^{jklm}	106.87^{hijklmn}	25.87^{fghij}	132.73^{jklmno}	80^{abcdefg}	9.14^{hijk}	2.59^{ghi}	3.53^{fghi}	22.27^{cdefg}	11.03^{hi}
15	Mrunalini	10.87^{bcde}	28.73^{ab}	130.47^{c}	189.49^{ab}	49.89^{bcde}	239.37^{ab}	79^{abcdefg}	9.44^{efghi}	2.39^{kl}	3.95^{defg}	22.57^{bcdef}	**32.71^{a}**
16	NL1	10.67^{bcde}	22.87^{ghi}	82.47^{mn}	78.87^{mno}	34.87^{cdefghi}	113.73^{lmnopq}	69^{ghi}	9.80^{cdef}	2.45^{ijk}	3.99^{def}	22.78^{bcdef}	13.59^{fghi}
17	NL3	10.53^{bcde}	26.13^{bcde}	92.67^{klmn}	89.25^{klmno}	29.50^{defghij}	118.75^{klmnop}	76^{defgh}	10.36^{bc}	2.68^{efg}	3.87^{efgh}	25.35^{abcd}	10.74^{hi}
18	NL9	11.00^{bcde}	26.07^{bcde}	82.33^{mn}	75.00^{no}	27.20^{efghij}	102.20^{nopq}	74^{efgh}	10.11^{bcd}	2.52^{ghijk}	4.01^{def}	26.15^{abcd}	14.27^{fghi}
19	NL16	9.87^{cde}	25.67^{cdefg}	109.20^{fghi}	101.55^{ijklmn}	28.80^{defghilj}	130.35^{jklmno}	78^{bcdefgh}	9.75^{defg}	2.52^{ghijk}	3.87^{efgh}	26.21^{abcd}	22.18^{bcdefg}
20	Numali	7.00^{e}	24.33^{efgh}	129.60^{cd}	**221.63^{a}**	30.58^{defghij}	**252.22^{a}**	88^{abcd}	8.14^{nop}	2.81^{de}	2.90^{klm}	21.23^{defgh}	22.39^{bcdefg}
21	Pant Sugandh 15	11.58^{bbcde}	29.20^{a}	99.93^{ijkl}	95.82^{jklmno}	48.97^{bcdef}	144.78^{hijklmn}	66^{hi}	10.46^{b}	2.27^{lmn}	4.61^{bc}	21.01^{defghi}	19.68^{cdefgh}
22	Pathariya	**21.60^{a}**	24.07^{efgh}	126.00^{cde}	75.22^{no}	**7.11^{j}**	82.33^{pq}	**91^{a}**	8.41^{lmno}	3.18^{b}	2.65^{mn}	24.45^{abcde}	19.91^{bcdefgh}
23	RPBio/248	12.11^{bcde}	7.53^{abcd}	117.27^{defg}	128.27^{fghij}	23.93^{ghij}	152.20^{ghijklm}	84^{abcdef}	8.29^{mnop}	2.79^{def}	2.97^{jklm}	26.77^{abcd}	25.01^{abcde}
24	SannaJajula	9.53^{cde}	**32.33^{a}**	157.73^{b}	170.77^{bcde}	38.30^{bcdefgh}	209.07^{abcde}	81^{abcdefg}	**11.49^{a}**	**1.77^{o}**	**6.51^{a}**	17.86^{fghi}	22.82^{bcdef}
25	Savitri	7.13^{e}	22.93^{ghi}	93.73^{jklmn}	140.17^{defgh}	42.28^{bcdefg}	182.44^{defghi}	77^{cdefgh}	9.16^{ghijk}	2.66^{efgh}	3.45^{fghij}	23.22^{bcdef}	21.63^{bcdefg}
26	Sharbati	13.16^{bcd}	25.60^{cdefg}	109.27^{fghi}	126.10^{fghijk}	25.75^{fghij}	151.85^{ghijklm}	83^{abcdef}	9.69^{defgh}	2.11^{n}	4.72^{b}	20.40^{defghi}	20.08^{bcdefgh}
27	Solumpiket	9.84^{cde}	26.00^{bcdef}	99.93^{ijkl}	84.25^{mno}	25.30^{fghij}	109.55^{mnopq}	80^{abcdefg}	9.30^{fghij}	2.50^{hijk}	3.78^{efgh}	23.99^{abcdef}	18.78^{defgh}
28	Taramati	9.53^{cde}	22.13^{hi}	100.87^{hijkl}	170.27^{bcde}	59.67^{ab}	229.93^{abc}	74^{efgh}	7.71^{pq}	2.29^{lm}	3.37^{hijk}	16.08^{ghi}	13.11^{fghi}
29	TKM6	10.13^{bcde}	25.07^{defg}	82.40^{mn}	114.87^{ghijklm}	**76.13^{a}**	191.00^{cdefgh}	**60^{i}**	9.93^{bcde}	2.22^{mn}	4.47^{bcd}	17.95^{fghi}	29.93^{ab}
30	WAB-450	9.00^{de}	25.20^{defg}	101.00^{hijkl}	104.80^{hijklmn}	56.60^{abc}	161.40^{fghijkl}	66^{hi}	8.97^{ijkl}	2.64^{fgh}	3.39^{ghijk}	20.43^{defghi}	12.11^{ghi}
	Mean	**10.64**	**25.37**	**108.49**	**125.82**	**31.12**	**156.94**	**80.00**	**9.12**	**2.57**	**3.65**	**22.58**	**18.47**
	Max.	**21.60**	**32.33**	**175.33**	**221.63**	**76.13**	**252.22**	**91.00**	**11.49**	**3.49**	**6.50**	**30.03**	**32.72**
	Min.	**6.80**	**21.13**	**81.20**	**58.58**	**7.11**	**70.18**	**60.00**	**7.36**	**1.77**	**2.22**	**15.00**	**7.90**
	SD	**3.18**	**2.54**	**21.67**	**42.03**	**16.36**	**49.31**	**0.08**	**0.99**	**0.37**	**0.85**	**3.80**	**6.70**
	Skewness	**1.56**	**0.62**	**1.31**	**0.44**	**0.87**	**0.15**	**-0.76**	**0.37**	**0.36**	**1.45**	**-0.22**	**0.35**
	Kurtosis	**3.71**	**0.47**	**2.23**	**-0.67**	**0.56**	**-0.81**	**0.06**	**-0.48**	**0.76**	**3.68**	**-0.40**	**-0.67**
	ANOVA *P*-value	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**

PT, Number of productive per plant; PL, Panicle length; PH, Plant height; FG, Number of filled grains per panicle; CG, Number of chaffy grains per panicle; GN, Total grains number per panicle; SF, Spikelet fertility; GL, Grain length(mm); GW, Grain width(mm); GL/GW, Grain length to width ratio; TGW,1000-grain weight; YP, Yield per plant and SD, Standard Deviation.

Trait maximum and minimum values under each trait are shown with bold font.Genotype ranking was assigned based on DMRT performed in SPSS version 20.Trait means, range,standard deviation, ANOVA (*P*-Value), skewness and kurtosis were done with Microsoft Excel Programme.

https://doi.org/10.37992/2024.1502.048

Table 3. Five best performed genotypes based on mean performance of 12 yield contributing traits among 30 rice genotypes

S.No.	Trait	Genotypes
1	Productive tillers per plant	Sharbati, HIM799, **Azucena**, Kesari, Pathariya
2	Plant height	Daddiga, **Mrunalini, SannaJajula, Burma Black,**Numali
3	Panicle length	**Burma Black, Mrunalini**, Badshabhog, **SannaJajula** Pant Sugandh 15,
4	Grain number per panicle	**SannaJajula**, Krishna, **Mrunalini**, Taramati, Numali
5	Filled grains per panicle	Krishna, Daddiga, **Mrunalini, Burma Black**, Numali
6	Chaffy grains per panicle	**Mrunalini**, Krishna, WAB450, Taramati, TKM6
7	Spikelet fertility	Solumpiket, Daddiga, Kesari, **Burma Black**, Pathariya
8	Grain length	NL3, **Azucena**, Pant Sugandh 15, **SannaJajula,**Sharbati
9	Grain width	**Azucena**, Anjali, Pathariya, Daddiga, **Burma Black**
10	Grain length to width ratio	Badshabhog, TKM6, Pant Sugandh 15, **SannaJajula**, Sharbati
11	1000-grain weight	NL16, RPBio/248, **Azucena, Burma Black**, HIM799
12	Grain yield per plant	AMO, **Burma Black**, **Azucena**, Kesari, **Mrunalini**

kurtosis,-0.67) showed near normal distribution. The distribution of these traits, as reported in many studies, proved the quantitative inheritance nature of these traits.

GENOTYPING OF THE RICE GENOTYPES: A total of 37 gene-specific markers were used for genotyping of the 30 rice genotypes, used in the current study. The primers were standardized for their annealing temperatures.

Primer Annealing Temperature (ºc) and their Product Size (Bp): The annealing temperatures **(ºC)** for the primers ranged from 50ºC to 65ºC (**Table 4**). Of 37 primers, seven primers were not amplified. The amplified primers were used for genotyping of the rice genotypes. Molecular screening of rice genotypes resulted in the generation of allele size range of 50bp (GW2SNB-2) to 1800bp (DEP1-1). Of the 30 primers amplified, two primers namely GW004 (750bp and 1050bp) and RM223 (165bp and 175bp) yielded two kinds of alleles among the genotypes (**Table 4, Fig.1**) and thus were polymorphic. Remaining 28 primers exhibited monomorphic alleles (**Table 4, Fig. 2**).

Diversity among the Rice Genotypes: Two alleles per loci was identified from genotyping of 30 accessions using yield gen-specific polymorphic markers *viz*. RM223 (*YLD* gene on chromosome 8) and GW004 (*GW2* gene on chromosome 2) (**Table 4**). The major alleles for the primers RM223 (165bp) and GW004 (1050bp) were observed with frequency of 90% and 77%, respectively among the genotypes. The polymorphic information content (PIC) value for these primers was 0.41 (RM223) and 0.32 (GW004) with an average PIC value of 0.37. This is line with several studies were the PIC values ranged from 0.30 to 0.40 (Ngangkham *et al*., 2018 , Swamy *et al*., 2017, Gull *et al*., 2019) . The study also showed that the GW004 of *GW2* gene on chromosome 2 and RM223 on chromosome 8 are potentially informative for grain width and yield genes, respectively with average PIC of 0.37, and hence, can be used to assess the genetic diversity of germplasm for the respective traits.

Cluster Analysis: Cluster analysis was done using DARWIN version 6.0.010 software (http://darwin.cirad.fr/). UPGMA dendrogram grouped the 30 genotypes into three clusters *viz*., A, B and Cwith >70% boot strapping values (**Fig.3**). Cluster A contained 20 genotypes, which

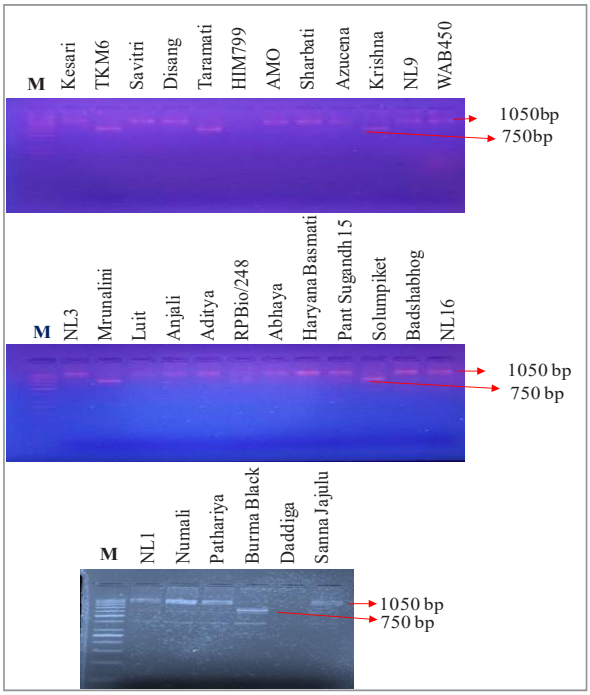

Fig.1. Image showing agarose gel electrophoresis of PCR products amplified among 30 rice genotypes using the markers GW004

Table 4. Annealing temperature (°C) and allele sizes observed (bp) for the yield gene specific markers used to screen among the genotype panel under study

S.No.	Trait	Gene	Chr.	Primer	AT (°C)	EPS (bp)	OPS (bp)
1	Plant height	*sd1*	1	sd1-h	55	800, 843	850
2	Grain number	*GN1A*	2	RM3535	58	185	190
3	Grain width (weight)	*GW2*	2	GW2 SNP-2	58	50	NA
4				GW2SNB-2	58	50	50
5				**GW004**	57	750, 1050	**750, 1050**
6	Grain size (length)	*GS2*	2	RM 3212	58	181	NA
7	Plant architecture	*IPA1*	2	S5- 803	59	243	NA
8				S5- 1	59	457/ 321	450
9	Grain size (length)	*GS3*	3	RGS1	55	180, 200	180
10				SR17	58	1000,1400	1400
11	Grain filling	*GIF1*	4	EX GIF1	58	80	80
12				RM 16942	58	181	NA
13	Spikelet number	*SPIKE*	4	Spike indel 3	62	150,170	170
14	Grain size (length)	*GS5*	5	RM574	58	500, 520	500
15				RM593	58	300	300
16				GS5 INDEL1	57	67	65
17	Grain width (weight)	*GW5*	5	RM513	59	275	280
18	Seed width (weight)	*SW5*	5	RM3328	57	119	120
19				N1212	59	60	60
20	Tillering (Monoculm)	*MOC1*	6	MOC1	60	-	NA
21	Strong culm	*SCM2*	6	SCM2 INDEL1	57	117	120
22	Heading date	*HD 1*	6	HD 1	50	-	NA
23		*Hd1*	6	Hd1agc	65	441	440
24		*Hd 3*	6	Hd3a	62	144	150
25	Grain width (weight)	*GW7*	7	RM 22015	58	176	180
26	Grain length and width	*GS7*	7	GLW7	52	140	140
27	Grain filling	*GLW7*	7	RM 505	55	160, 180	180
28	Grain and cooking quality	*GLW7*	7	RM 21945	55	292	290
29	Grain size, shape and quality	*OsSPL 14*	8	OSSPL14	50	500	500
30	Grain yield	*YLD*	8	PAY1SP6	53	200	200
31				**RM 223**	58	165	**165**
32	Heading date	*DTH-8*	8	DTH-8 INDEL	57	350	350
33	Grain width (weight)	*GW8*	8	GW8PRO2	58	-	NA
34				GW8-InDel	57	290-300	300
35	Dense and erect panicle	*DEP 1*	9	DEP1S7	59	110	110
36				DEP1 INDEL1	58	1031	1030
37				DEP1-1	58	1860,1235	1800

Chr, chromosome; AT, annealing temperature; EPS, expected product size; OPS, observed product size; NA, not amplified. Polymorphic markers were indicated in bold font

included four Nerica Lines, *indica*, aromatic *indica* and aromatic japonica sub-groups. Cluster B contained three genotypes one Nerica Line (NL1) and two indica genotypes (AMO and Numali). Clusters C included seven genotypes belongs to *indica* (Daddiga, Burma Black, Solumpiket, Krishna, Mrunalini and TKM6) and aromatic *indica* (Taramati) sub-groups.

In present study, based on genetic dissimilarity results, 30 genotypes were not divided into clear cut trait-based grouping *i.e.* distribution of these genotypes was not based on their phenotypic score of the respective traits governed by the genes (yield and grain width). For instance, cluster C, a small group, contained a broad yield range of 13.11 to 32.71g and grain width range of 2.16

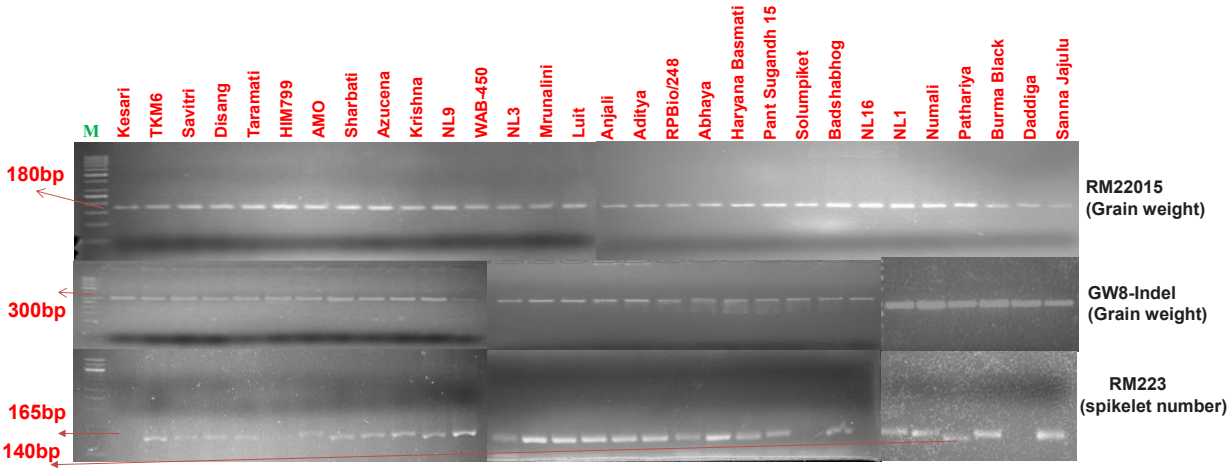

Fig. 2. Image showing agarose gel electrophoresis of PCR products amplified among 30 rice genotypes using the markers RM 22015, GW8-Indel and RM223 M:50bpladder

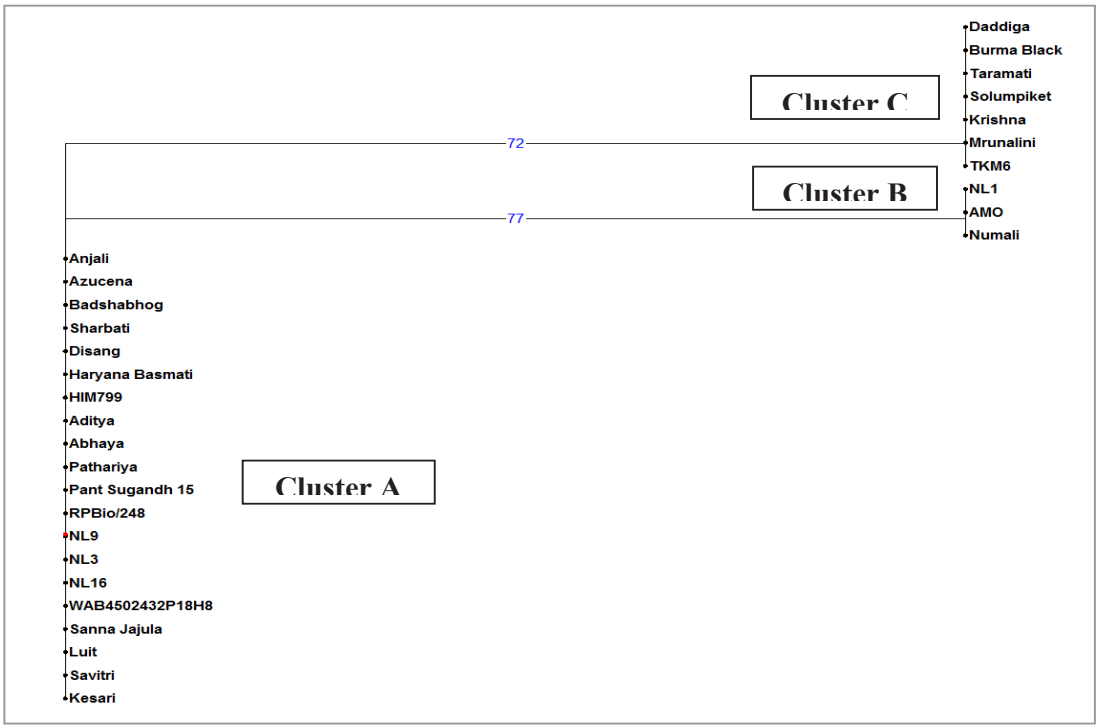

Fig. 3. Dendrogram of 30 rice genotypes generated from UPGMA analysis of RM223 and GW004 markers.
Boot strapping was done with 1000 permutations

to 3.49 mm. Similarly, cluster B was observed to have yield range from 13.59 to 25.49 g and 2.45 to 2.81 mm for grain width. Further, the results indicated that the genetic distances within sub-groups *(indica* or aromatic etc.) for these loci also do not correspond well and as a result the genetic relationships among the respective genotypes are not clear. Similar kind of ungrouping was observed by Anh *et al*. (2015) using genic SSR markers. One possible explanation for this might be the non-functional nature (association) of the identified alleles at respective traits and as well it showed conservation of the genomic regions (alleles/ genes) across sub-groups (indica/japonica/ aromatic). Therefore, the grouping of genotypes does not reflect at functional genic variation of the traits governed. Furthermore, using SNP markers Ma *et al*. (2019) in their study reported that there was no clear differentiation of 270 rice germplasm sub-groups, which includes four *aus* accessions, 111 *japonica* accessions, and 155 *indica* accessions.

Marker-Trait Association Analysis : Marker-trait association analysis was done using TASSEL Version 2.1 software (http://www.maizegenetics.net/). Both GLM (General linear model) and MLM (Multiple linear model) were carried out to identify the significant associations among markers and yield related traits (**Table 4**). Of the two polymorphic markers *viz*. GW004 and RM223 used in marker-trait association analysis in the present study, only one marker GW004 of *GW2* locus (*LOC_Os02g14720*) found to be significantly associated with three yield governing traits *viz*. Panicle length (PL, *P*= 0.0198), Plant height (PH, *P*= 0.0274) and Fertile grains (FG, *P* =0.0052), through GLM approach (**Table 5**). However, GW004 was also significantly associated with PH (*P*= 0.0274), when analysed the data through MLM approach (**Table 5**). This kind of common association of a marker/ gene with multitude of traits indicates that there is clear pleiotropy nature of gene at molecular level and supports the assumption of selection for one trait obviously influences other. Pearson correlation study (**Table 6**) also supported the molecular level association through phenotypic associations for trait pairs with significant correlations between PH and FG (r^2= 0.546**) and as well as PL and plant PH (r^2= 0.589**).

However, the marker GW004, which was previously reported to be associated with the gene controlling grain width and weight (GW2), did not show any significant association with grain size traits such as grain width (GW, P = 0.3433), grain length (GL, P = 0.6131), and grain width-

Table 5. Marker-Trait associations identified by GLM and MLM models (TASSEL v.5) for the yield related traits measured in 30 rice genotypes

		Panicle length(PL)		**Plant height(PH)**		**Filled grains(FG)**		
Gene	Marker	*P*-Value	R^2_marker	*P*-Value	R^2_marker	*P*-Value	R^2_marker	**Model**
GW2(LOC_ Os02g14720)	GW004	**0.0198**	0.1790	**0.0274**	0.1621	**0.0052**	0.2474	GLM
		0.2922	-1.42E+02	**0.0274**	-1.15E+02	0.2096	-1.44E+02	MLM
		Grain length(GL)		**Grain width(GW)**		**Grain ratio(GR)**		
Gene	Marker	*P*-Value	R^2_marker	*P*-Value	R^2_marker	*P*-Value	R^2_marker	
GW2(LOC_ Os02g14720)	GW004	0.6131	0.0093	0.3433	0.0321	0.0903	0.099	GLM
		0.6131	-3.40E+01	0.3433	-7.69E+01	0.0904	-9.24E+01	MLM
		Significant at *P* value <0.05						

Table 6. Correlation coefficients for yield and its related traits on 30 rice genotypes

Trait	**PT**	**PL(cm)**	**PH(cm)**	**FG**	**CG**	**GN**	**SF(%)**	**GL(mm)**	**GW(mm)**	**GR**	**TGW(g)**	**YP(g)**
PT	1.000											
PL(cm)	-0.028	1.000										
PH(cm)	-0.129	0.589**	1.000									
FG	-0.331*	0.121	0.546**	1.000								
CG	-0.224	0.076	-0.223	0.288	1.000							
GN	-0.356*	0.128	0.391*	0.948**	0.578**	1.000						
SF(%)	0.103	-0.044	0.519**	0.236	-0.832**	-0.075	1.000					
GL(mm)	0.099	0.643**	0.039	-0.164	0.216	-0.068	-0.382*	1.000				
GW(mm)	-0.011	-0.183	0.298	-0.027	-0.481**	-0.182	0.455**	-0.423**	1.000			
GR	0.047	0.527**	-0.031	0.001	0.378*	0.126	-0.407**	0.799**	-0.847**	1.000		
TGW(g)	0.170	0.197	0.192	-0.325*	-0.461**	-0.430**	0.213	0.060	0.670**	-0.424**	1.000	
YP(g)	0.204	0.273	0.485**	0.671**	0.020	0.578**	0.316*	0.086	0.058	0.031	0.080	1.000

PT, Number of productive per plant; PL, Panicle length; PH, Plant height; FG, Number of filled grains per panicle; CG, Number of chaffy grains per panicle; GN, Total grains number per panicle, SF: Spikelet fertility, GL, Grain length; GW, Grain width; GL/GW, Grain length to width ratio; TGW,1000-grain weight and YP, Yield per plant.
Significance level: *Significant at *P* <0.05, 0.317; ** Highly significant at*P*<0.01, 0.479.

to-length ratio (GR, P = 0.0903), as determined by both the General Linear Model (GLM) and the Mixed Linear Model (MLM) approaches (**Table 5**). These findings are consistent with the results reported by Ngangkham *et al.* (2018), who also found no significant association between the GW004 marker and grain width or other grain traits. Therefore, it is evident that the GW004 marker (alleles) is not suitable for selection of grain width controlled by the GW2 gene. It may be worthwhile to explore the development and screening of other functional markers, such as Sequence Tagged Sites (STS), insertion/deletion (indel), or single nucleotide polymorphism (SNP) markers, for donor selection in order to facilitate directional selection of grain size traits in general and grain width in particular through the GW2 locus. On the other hand, significant associations were reported by Gull *et al*. (2019) for other genes governing grain size. Five markers, namely GW8-InDel, GW8-InDel1A, GW8-InDel2B, GS2-InDel1A, and GS2-InDel2B, corresponding to genes GW8 and GS2, respectively, were found to be significantly associated with grain width. Similarly, the marker GW5-InDel, corresponding to gene GW5, was also found to be significantly associated with grain width. Additionally, for total grain weight (TGW), one marker, namely GW8-InDel2B, corresponding to gene GW8, showed significant association with grain weight. Similarly, three markers, namely GW5-InDel, GS5-InDel1A, and GS5-InDel2B, corresponding to two genes, GW8 and GS5, were also significantly associated with grain weight at a significance level of $P \leq 0.05$.

In the current study, the phenotypic variance of the marker that associated with PH (GLM: 0.1790, MLM: -1.42E+02), PL (GLM: 0.1621 MLM: -1.15E+02) and FG (GLM: 0.2474 MLM: -1.44E+02) are >15% (**Table 5**), suggesting the major role of this locus/gene in controlling the respective traits. Thus, GW004 can be employed as foreground marker in marker assisted breeding, in the simultaneous improvement of plant height (PH), panicle length (PL) and filled grain number/panicle (FG) and could be of great value in multiple trait enhancement breeding and thereby the grain yield in rice.

GW2 gene controls grain width: GW2 (Os02g024410) is a gene that was initially identified as the causative gene for a grain width quantitative trait locus (QTL) called qgw2, which is associated with large grain size in the Japonica rice variety WY3 (Song *et al*., 2007). The GW2 gene encodes a new RING protein with E3 ubiquitin ligase activity, which is involved in the breakdown of proteins in the ubiquitin proteasome pathway (Stone *et al*., 2005). RING-type E3 ubiquitin ligases control seed development by catalyzing the ubiquitination of expansin-like 1 (EXPLA1), a cell wall loosening protein that increases cell growth and size (Choi *et al*., 2018). In WY3, the GW2 gene has a loss of function mutation due to a frame shift caused by a single nucleotide deletion in exon -4, resulting in a truncated protein.

Studies have shown that the GW2 gene plays a significant role in determining grain size and shape. In a near-isogenic line of the indica rice variety Fengaizhan-1 (FAZ1) with the GW2 gene from the Japonica rice variety WY3, the width of the awn (a structure covering the grain) was increased by 26.2% due to increased cell number (Song *et al*., 2007). GAO Xuan *et al*. (2016) identified a donor with potent alleles of GW2 that control desirable grain width and thickness. They found that the grain size and shape QTL occupied the same chromosomal locations for milled grains and brown grains, indicating that the size and shape of brown grains are controlled by the palea and lemma, which are protective structures of the rice grain. Furthermore, they showed that the GW2 mutant allele from the BL129 line increased the width, thickness, and weight of grains in the elite indica variety Huazhan, where it is used as one of the parental lines in hybrid rice breeding.

Tomita *et al.* (2019) revealed that integration of GW2 into the isogenic Koshihikari rice variety resulted in a 34% increase in thousand kernel weight compared to Koshihikari, while maintaining a taste score of 80. They further developed a large grain/semi-dwarf isogenic Koshihikari rice variety by integrating GW2 with the semi-dwarfing gene d60, which is localized on chromosome 2. This combined genotype showed high yield potential and robustness to withstand climate change, which could contribute to the development of a New Green Revolution in rice breeding

GW2 is highly expressed in various plant organs of rice: Furthermore, GW2 has been shown to interact with other genes that play a role in grain size regulation. For example, GW8, another gene that regulates grain width, has been found to interact with GW2 in a complex regulatory network that controls grain size in rice. Additionally, GW2 has been found to interact with OsMADS1, a gene that regulates flower development, and OsMADS34, a gene that regulates seed development, suggesting that GW2 may play a role in coordinating seed and flower development (Duan *et al*., 2017). These findings suggest that GW2 plays a central role in the regulation of grain size and that it interacts with multiple genes and pathways to coordinate seed development.

Additionally, *GW2* participates in vegetative growth, and protects the seed from pathogen attack. Histochemical *β-glucuronidase* staining showed strong expression of *GW2* in leaf and root tissues but weak expression in leaf sheaths and internodes (Lee *et al*., 2018). Zhang *et al.* (2014) employing RNA-Seq approach, reported that spatio-temporal expression of *GW2* in shoots of rice with FPKM (Fragments Per Kilobase of transcript per Million mapped reads) value of 8.0 (cut off 0.5) (Li *et al*., 2015). Shim *et al*. (2020) mapped a major QTL- qCC2, for rice leaf chlorophyll content employing F_2 population derived from an interspecific cross between *O. sativa*

(cv. Hwaseong) x *O. grandiglumis*. The *O. grandiglumis* allele at qCC2 increased chlorophyll content and delayed senescence. Through transcriptomic dynamics they revealed the positive regulation of *GW2* gene in the qCC2 region towards control of leaf senescence in rice.

As per the Rice Annotation Project Data Base (RAP-DB-https://rapdb.dna.affrc.go.jp/) around 88 RING E3 ligase genes were annotated in rice genome. These are found to be involved in various functions as represented in **Table 7** which further supports the diverse roles of RING E3 ligase gene family, apart from controlling grain size traits (reproductive stage).

To brief from the results of current study as well in the light of previous reports and annotation project's gene functional description (**Table 7**), the RING E3 gene family has broad range of roles in rice plant development *viz*. vegetative and reproductive stage control, nutrient uptake, defense responses etc. Therefore, screening of diverse genotypes at molecular level can result in identification of novel donors along with the tagged alleles that might result in association with real spectrum of traits they control.

Association analysis is a widely used technique in identification of novel alleles that accumulated over the evolutionary process across the crop genetic resources. These genic variations once identified can be characterized at functional level to know the plethora of functions governed by the respective genes and to characterize their interactions at molecular level

Table 7. Brief list of RINGE3 ligase genes involved in various functions apart from controlling grain size

S.No.	Gene functional description	Gene ID
	Regulation of vegetative and reproductive stages	
1	Plant architecture	Os01g0350900
2	Root development, Maintenance of cell viability after the initiation of root primordial formation, Defense response	Os02g0559800 Os02g0560200 Os02g0560600
3	Regulation of flowering under LD conditions	Os08g0539300
4	Anther development, regulaton of flowering time by affecting histone H2B mono-ubiquitination	Os10g0565600
5	Modulation of heading date by physically interacting with *Hd1*	Os04g0648800
6	Control of pollen tube growth, regulation of seed setting rate	Os05g0145000
	Regulation of nutrient up-take	
7	Pi homeostasis	Os01g0954400 Os07g0673200
8	Phosphate and nitrate signaling	Os01g0755700
	Regulation in biotic stress tolerance	
9	Innate immunity and broad-spectrum disease resistance	Os05g0279400 Os06g0125800
10	PAMP-triggered immunity	Os02g0182900
	Regulation in abiotic stress tolerance	
11	Salt and osmotic stress tolerance	Os03g0798200 Os04g0571200 Os06g0695600
12	Salt/drought stress response	Os01g0369000
13	Salt/drought stress response, UV-B stress response	Os05g0149600
14	Negative regulator of drought and salt stress responses, Positive factor of cold stress response	Os06g0687200
15	Salinity tolerance	Os04g0571200 Os11g0175500
16	Drought stress response	Os02g0682300
17	Abiotic stress tolerance	Os02g0150700 Os03g0348900 Os05g0497600
18	Root-specific ethylene response	Os04g0101800
19	Heat tolerance, Modulation of hydrogen peroxide-induced stomatal closure	Os09g0323100

(through knowing the diverse transcripts and their interactive elements). Further, the allelic variants could be developed into functional markers by further confirming their trait-association with large number of genotypes and introgression populations to use in precise phenotyping through Marker Assisted Breeding programmes.

From the current study it can be concluded that *GW2* regulates rice growth and development during vegetative growth through plant height and plant architecture while, reproductive stage through panicle length and filled grains per panicle. Thus, GW004 marker is helpful in selection of these three traits and to use in breeding programmes to improve the performance of respective traits.Further, it can be concluded that each gene controls multitude of traits within the plant system, wherein functional characterization of genes needs to be done in-depth (using both forward and reverse genetic approaches, genome editing tools) to understand completely the diverse roles of a gene at molecular level and for utilization of the knowledge in plant breeding.

ACKNOWLEDGEMENT

The authors are grateful to ANGRAU for providing the financial support to conduct the work.

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