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Designing and Validation of Primers for High grain number for use in Molecular Breeding in rice

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Abstract Improvement of rice grain yield is an important goal in rice breeding. Yield improvement efficiency depends upon yield related components like grain number per panicle, grain weight, tillers number per plant, etc. Of these factors, grain number per panicle was shown to be highly correlated with yield. Pyramiding genes for grain number is highly indispensable in rice improvement programs. The high grain number genes, *Apo1*, *Dep1*, *Ghd7*are responsible for more seed production leading to increase in grain yield in rice. The length, sequence ID, chromosomal position and protein evidences of these genes were found from Orzabase, RAP-DB and Gramene database. The promoter, poly-A signal, DNA strand, open reading frame, initial signal/3' splice site score, 5' splice site, coding region, probability of exon and intron of high grain number genes under study were found out. These informations were used to design primers for these high grain number genes. The similarity search of designed primers showed 100% similarity with respective high grain number genes. The secondary structure prediction along with the comparative structural analysis of their respective proteins were performed. Three deigned primers for *Dep1*gene, one for *Ghd7* gene and one for *Apo1* gene were tested for amplification from high and low grain rice varieties, HGN1 and Heera, respectively. Two primers, Dep1-1 (promotor) for *Dep1*gene and Ghd7-2(Exon1) for *Ghd7* gene amplified fragments from high grain variety, HGN1 while failed to amplify from low grain variety, Heera, indicating that these primers would be useful for introgression of genes from high grain varieties into low grain varieties to improve yield potential in rice through molecular breeding approach in future.
Keywords High grain number genes; Comparative structural analysis of high grain number proteins; Primer designing; Amplification of genes

Introduction

Rice is cultivated in more than 100 countries worldwide on approximately 150 million hectares of land. Among the rice growing countries in the world, India has the largest area under rice crop and ranks second in production next to China. In India, it is grown in 44 million hectares accounting for 43% of food grain production and 46% of the total cerea production. Even though rice covers largest area under cultivation, the potential of rice production till has to be exploited. Rice productivity has more than doubled since the green revolution in the 1960s. Thus, further improving rice yield potential has posed tremendous challenges to rice breeders. Recent estimation suggests that a 40% increase in the production of rice is must by 2030 to meet the demands of the ever increasing population (Khush, 2003). The cultivated rice (*Oryza sativa* L.) is rich in genetic diversity apart from highly diverse 21 wild progenitors and the African cultivated rice, *Oryzaglaberrima*Steud. In addition to staple food, rice has extensive protective and curative properties against human ailments like epilepsy, chronic headache, rheumatism, paralysis, skin diseases, diabetes, arthritis, indigestion, blood pressure, colon cancer, internal rejuvenation of tissues and overcoming postnatal weaknesses (Kirtikar and Basu, 1935). Identification and pyramiding of favorable/superior alleles for yield and its component traits would make a significant contribution to breeding high-yielding rice varieties.Improvement of rice grain yield is an important goal in rice breeding. Unfortunately, yield improvement efficiency is not

adequate due to its complex property of related components like grain number per panicle, grain weight, tillers per plant, etc. Of these factors, grain number was shown to be highly correlated with yield (Hua et al., 2002). Three hundred sixty nine QTLs distributed across all over the 12 chromosomes of rice controlling grain number per-panicle have been identified (http://www.gramene.org) using various mapping populations derived from inter-specific, *indica-japonica*, *indica-indica*and *japonica-japonica* crosses. Some of them were fine mapped to less than 1 cM intervals and few have been cloned using QTL-based near isogenic lines (NIL). Genes controlling grain number per-panicle directly or indirectly, i.e. *Gn1a, Ghd7, Dep1, fz, Sp1, rcn2, lax1* and *Apo1* also have been isolated from rice (Li et al., 2003; Jin et al.,2008; Tan et al., 2008; Li et al.,2009; Piao et al., 2009; Terao et al.,2010; Zha et al., 2009).The recent development in molecular biology, genomics and bioinformatics has helped in the fine mapping and cloning of major genes/QTLs associated with rice yield traits at a rapid pace for the past 25 years. With the information on the cloned genes and closely linked markers associated with agronomic and yield traits in rice, accumulating beneficial alleles/genes using MAS has become a straight forward approach for improving target traits in rice (Wang et al., 2012). Pyramiding of genes in the elite genetic backgrounds has been reported to result in higher yield potential, longer grains, and a more suitable heading date. The novel genes are preserved in many related wild species, land races and cultivated varieties of rice. Therefore, pyramiding genes for grain number is highly indispensable in rice improvement programs. Here we have considered four different high grain number genes *APO1, DEP1, GHD7, GN1A*. The main objective of the work is to find out the exons and promoters of genes, to predict their restriction sites, comparative homology modeling of corresponding proteins, designing and validation of primers for high grain number for use in molecular breeding in rice.

1 Material and Methods

1.1 Retrieval of nucleotide sequence

The nucleotide sequences of the high grain number genes i.e*Apo1, Dep1, Ghd7,* were retrieved from oryzabase, RAP-DB and gramene databases and from NCBI (http://www.ncbi.nlm.nih.gov/) by searching against nucleotide.

1.2 Similarity search for high grain number genes

Then the sequences were subjected for similarity search by using BlastP (http://blast.ncbi.nlm.nih.gov/Blast.cgiPROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

1.3 Gene prediction

The coding and non-coding regions were predicted using Genscan tool (http://genes.mit.edu/GENSCAN.html). From this we could find out the exon (functional region) and intron (non-functional region).

1.4 Protein structure prediction

The secondary structures were predicted by using SOPMA server (http://www.rcsb.org/pdb/home/home.do). From this prediction it was easy to find out the helices, beta and turns. Then the comparative homology modeling was done by using modeller9.12 tool. The templates were identified from BlastP after similarity search and collected from PDBin fasta format. Then the align2d.py, model-single.py and evaluate model.py files were run on modeller9.12. The best model was selected on the basis of lowest DOPE score.

1.5 Model optimization and evaluation

Then the final models were visualized by using visualization tools i.epymol, rasmol and discovery studio visualiser. Then the backbone confirmation of the proteins were predicted by using PROCHECK Saves server (http://services.mbi.ucla.edu/SAVES/Ramachandran/), which showed the allowed and disallowed regions of proteins from where it was easy to find out the suitable protein for further study.

1.6 Primer designing

Primers were designed to amplify the particular regions of the genes which can be further used for cloning purpose leading to high grain production in rice. Primer3 tool (http://primer3.ut.ee/) was used to find out the full length primers, and genomic primers, exon primer tool (ttp://ihg.gsf.de/ihg/ExonPrimer.html) was used to find the exon specific primers and multiple primer design with primer3tool

(http://flypush.imgen.bcm.tmc.edu/primer/) was used to find out the overlapping primers.

1.7 In-silico amplification

The designed primers might amplify or not in wet lab technique, hence the in-silico method was performed to check the successful amplification. The *Insilico* PCR amplification tool (http://insilico.ehu.es/PCR/) was used to find out the amplification. Both forward and reverse primers were uploaded and after submitting it showed the amplification.

1.8 Genomic DNA isolation

The genomic DNA isolation process was followed by using CTAB powder method (Murray and Thompson, 1990). The cut pieces of rice leaf variety HEENA and HGN1 were homogenized using liquid nitrogen. The fine powder leaves were then kept in -20 degree centigrade freezer for 12 hours. 10ml of extraction buffer was added and incubated at 56 degree centigrade for 20minutes at 150rpm in shaker. 10ml of chloroform: isogamy alcohol mixture was added to get an emulsion. Emulsion was transferred to 30ml Oakridge polypropylene tube and centrifuged at 8000rpm for 25minutes at room temperature. 600ul of 10% CTAB and chloroform: isogamy alcohol (24:1) was added and subjected to centrifuge for 25minutes at room temperature. 0.7 volume of cold filter sterilized iso-propanol was added, precipitated dna was spooled out and again subjected to centrifuge at 5000-6000rpm/10minutes, rinsed in 70% ethanol, air dried or vaccum dried and dissolved in 4ml of TE buffer. The sample was then treated with 4ul of DNA free rnase and incubated at 37 degree centigrade for 1 hour. The DNA was re-precipitated with 0.1 volume of 3m sodium acetate and 2 volume of cold absolute ethanol. The precipitated DNA was spoole, rinsed in 70% ethanol, dissolved in 1ml of TE and stored at -20 degree centigrade for further analysis. Agarose gel electrophoresis method was done to find out the quality of the DNA. Gradient PCR was performed by setting the annealing temperature of 53^{o}C, 54^{o}C, 56^{o}C and 57^{o}C. The PCR amplification was performed in a 20 µl reaction mixture volume containing 2 µl (30 ng) of genomic DNA, 1× PCR buffer, 200 µM dNTP mix, 4 pM of each of forward and reverse primers, 2 mM of magnesium chloride, 1 U of *Taq* (*Thermos aquaticus*) DNA polymerase.

The reaction mixture (master mix) was prepared as follows:

Reagent	Final concentration	Vol. in µl
Sterile de-ionized water	-	12.04 ×12 = 144.48
10 X PCR buffer	1×	2.0 ×12 =24.00
10mM dNTP mix	200 µM of each	0.16 ×12 =1.92
25 mM magnesium chloride	2m M	0.8 ×12 =6.0
Primer (F)	4pM	1 ×12 =12.0
Primer(R)	4pM	1 ×12 =12.0
Taq DNA polymerase	1U	1 ×12 =12.0
		18.0 ×12 = 216.0

The reaction mixture was mixed well, 18 µl was distributed to each of 12 tubes. Two µl of DNA sample each genotype was added to corresponding tube, mixed well, briefly centrifuged to collect drops from wall of tube.

1.9 Gel electrophoresis and detection of amplified products

After gradient PCR, the products were separated on 2.5- 3% agarose gel. Twelve micro liters of amplified products of each sample was loaded on 2.5% agarose gel in 1× TBE buffer to separate the amplified fragments. The electrophoresis was done for about 3 hours at 70 volts. The molecular weight marker (50 bp ladder) was used to compare the molecular weights of amplified products. 14 µl of Ethidium bromide was added to the molten gel and mixed well before pouring to the mold. The gel was visualized under UV and photographed using Chemi-Doc system (Alpha Innotech).

1.10 Data analysis

DNA fragment sizing and matching was done by scoring photographs directly. Individual bands within lanes were assigned to a particular molecular weight comparing with the DNA molecular weight marker. The amplified bands/alleles were scored as present (+) or absent (-) for each genotype and primer combination.

2 Results and Discussion

2.1 Similarity search analysis

The *Apo1* gene showed 100% similarity with *oryza sativa* group *Apo1* for panicle organization related protein and genomic DNA of *oryza sativa* on chromosome no.6. *Dep1* gene showed 100% similarity with genomic DNA of *japonica* group on chromosome no.9 and with *Dep1* complete cds. *Ghd7* showed 100% similarity with *indica* group cultivator *minghui63Ghd7* complete cds. The gene prediction which was performed is given in Table 1.

Table 1 gene prediction of *Apo1, Dep1, Ghd7* high grain number genes

Gene	Gene exon type	Begin	End	Length	Fr	Ph	I/ac	Do/t	Tscr
APO1	1.01INIT+	166	869	704	0	2	88	115	94.56
(genomic)	1.0 TERM+	952	1537	586	1	1	98	49	75.75
DEP1	1.01INIT+	117	245	129	2	0	85	105	17.76
(genomic)	1.0 TERM+	288	356	69	2	0	79	38	-5.66
	1.03PIYA+	374	379	6					-0.45
	2.03 PIYA+	589	484	6					-1.75
	2.02TERM-	640	503	138	1	0	39	42	-4.74
	2.01INIT-	824	681	144	2	0	85	82	16.53
	2.00PROM-	917	878	40					-5.56
	3.00 PROM-	1175	1170	6					1.06
	3.01 PROM-	4183	3296	888	1	0	_1	83	43.66
GHD7 (genomic)	1.01 TERM+	110	8181558	509	2	2	35	43	81.37
	1.02 PIYA+	1553	2317	6					1.06
	2.00PROM+	2278	2722	40					-4.76
	2.01INIT+	2491		232	0	1	122	18	28.37

2.2 Secondary structure prediction analysis

The secondary structure prediction of the aberrant panicle organisation1 protein showed that it has 429 amino acids, 23.54% alpha helix, 20.28% extended strand, 9.09% beta turn, 47.09% random coils, whereas dense erect panicle1 protein has 426 amino acids, 12.68% alpha helix, 9.62% extended strand, 3.76% beta turn, 73.94% random coils and grain heading date7 protein has 257 amino acids, 17.90% alpha helix, 13.62% extended strand, 5.45% beta turn, 63.04% random coils.

Figure 1 3D structure of aberrant panicle organisation1 protein

2.3 Comparative homology analysis

The final models were visualized using pymol and discovery studio visualizer. The backbone confirmation of all the proteins were analyzed which showed that*Apo1* protein lies in 84.9% of allowed region and 0.9% of disallowed region, *Dep1* protein lies in 80.6% of allowed region and 2.6% of disallowed region, *Ghd7* lies in 85.4% of allowed region and of disallowed region. The homology models are given in Figure 1-Figure 3 and the backbone confirmations of all the tertiary structures are given in Figure 4-Figure 6.

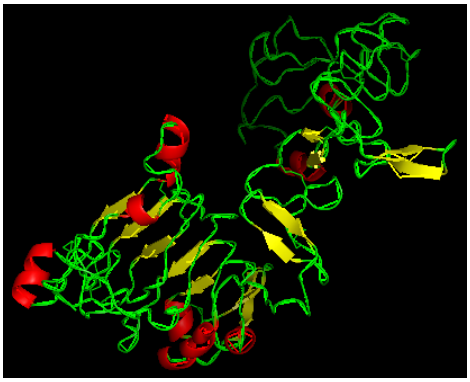

Figure 2 3D structure of dense erect panicle1 protein

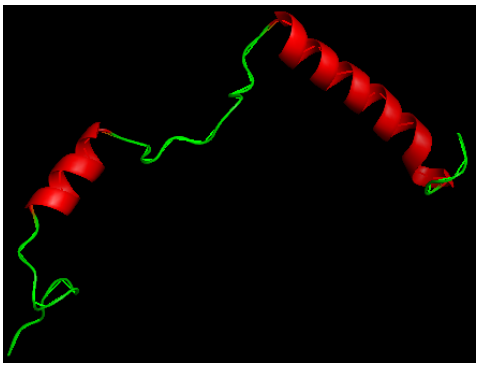

Figure 3 3D structure of grain heading date7 protein

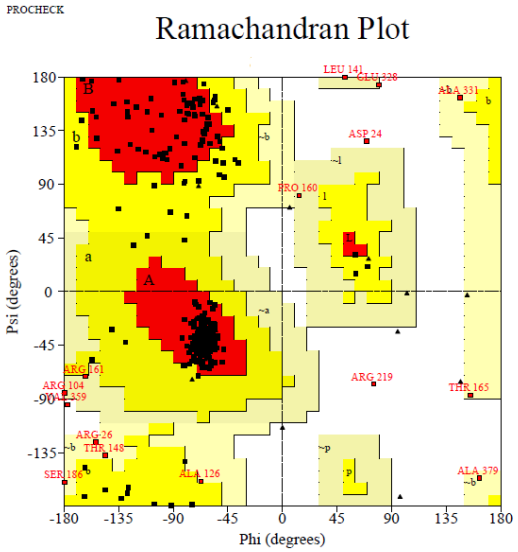

Figure 4 Ramachandran plot ofaberrant panicle organisation1 protein

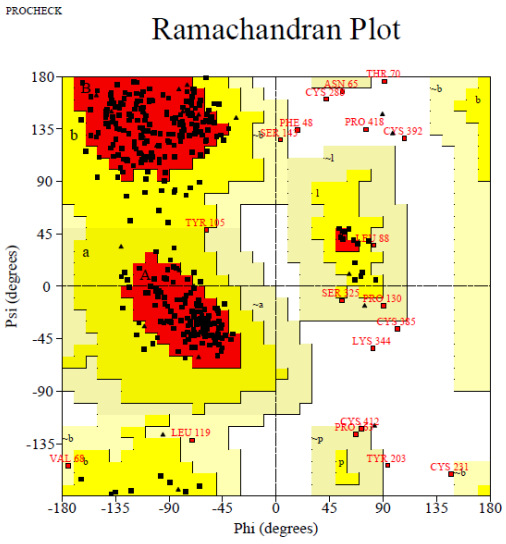

Figure 5 Ramachandran plot of dense erect panicle1 protein

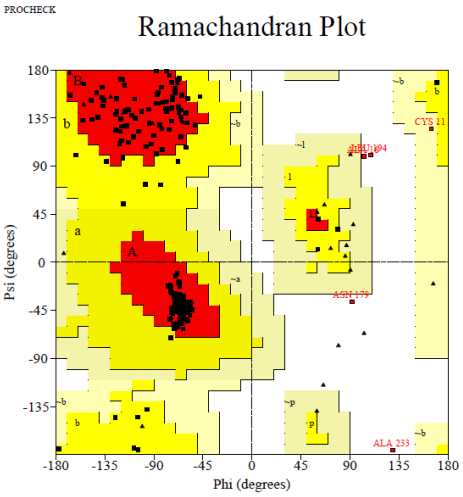

Figure 6 Ramachandran plot of grain heading date7 protein

2.4 Final primers

The final primers were selected on the basis of gc%, tm, product size and hairpin loop sequence. The gc content must be 40%-80%, tm must be 55°C-65°C, hairpin loop sequence must be 0.00 and the product size must be more depending upon the sequence length.

These primers were ordered for checking amplification and following primers amplified successfully whereas *Apo*1-1 promoter primer showed negative amplification.

Four primer pairs(three for *Dep1* gene, one for *Ghd7* gene and one for *Apo1* gene were used to amplify from high grain, HGN1 and low grain, Heera using six different temperature regime each with six different temperatures.

The primer pair Dep1-1 (Promoter) for *Dep1* gene amplified band of 600bp in all the six temperatures from high grain variety, HGN1 while fail to amplify from Heera (Figure 7).

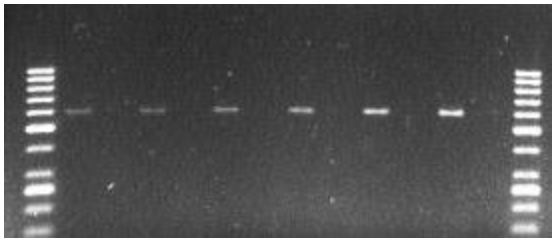

Figure 7 Gradient PCR with *Dep1* gene specific primer, Dep1-1 (Promo 1 - HGN1 (high grain), 2- Heera(low grain)
Note: Annealing Temperatures are given on the top of gel

Optimum temperature of amplification was found to be 57^0C and 58^0C as amplified band found to be deeper as compared to other annealing temperature. This indicated that the primer pair Dep1-1 (Promoter) for *Dep1* gene would be useful for introgressing genes from high grain varieties into low grain varieties in order to improve yield potential through molecular breeding approach.

The primer pair Dep1-2 (Exon1) for *Dep1* gene amplified band of 600bp in all the six temperatures from both high and grain varieties, HGN1 and Heera, respectively, though variation was found in strength of amplified bands. Optimum temperature of amplification was found to be 57^0C and 58^0C as amplified band found to be deeper as compared to other annealing temperature (Figure 8).

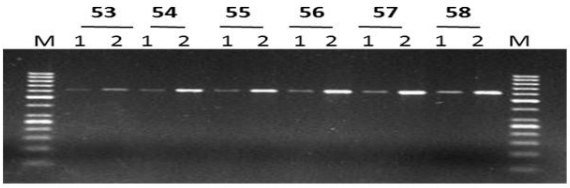

Figure 8 Gradient PCR with *Dep1* gene specific primer, Dep1-2 (Exon1) 1 - HGN1 (high grain), 2- Heera(low grain)
Note: Annealing Temperatures are given on the top of gel

This indicated that the primer pair Dep1-2 (Promoter) for *Dep1* gene would be not be useful for introgressing genes from high grain varieties into low grain rice varieties as there is no difference in length polymorphism. However, further investigation is necessary to find out whether there is difference in restriction site resulting in fragment size variation.

The primer pair Dep1-3 (Exon2) for *Dep1* gene amplified band of 600bp in all the six temperatures from both high and grain varieties, HGN1 and Heera, respectively, though variation was found in strength of amplified bands in HGN1 and Heera. Optimum temperature of amplification was found to be 56^0C, 57^0C and 58^0C as amplified band found to be deeper as compared to other annealing temperature (Figure 9).

This indicated that the primer pair Dep1-3 (Promoter) for *Dep1* gene would be not be useful for introgressing genes from high grain varieties into low

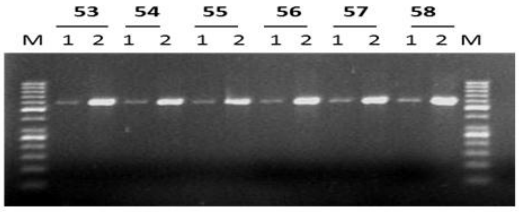

Figure 9 Gradient PCR with *Dep1* gene specific primer, Dep1-3 (Exon2) 1-HGN1 (high grain), 2- Heera(low grain) Annealing Temperatures are given on the top of gel

grain rice varieties as there is no difference in length polymorphism. However, further investigation is necessary to find out whether there is difference in restriction site resulting in fragment size variation.

The primer pair *Ghd-7* (Exon1) for *Ghd1* gene amplified band of 650bp in all the six temperatures from high grain variety, HGN1 while fail to amplify from Heera (Figure 10).

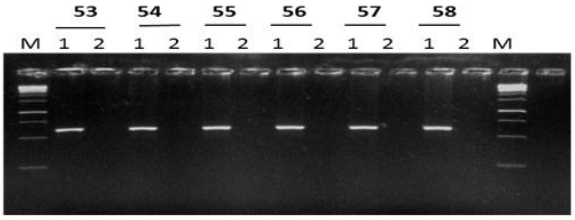

Figure 10 Gradient PCR with *Ghd7* gene specific primer, Ghd7-2 (Exon1) 1 - HGN1 (high grain), 2- Heera(low grain) Annealing Temperatures are given on the top of gel

All the six temperature of amplification were found to be optimum as amplified band found to be medium to deeper at all six annealing temperature. This indicated that the primer pair *Ghd-7* (Exon1) for *Ghd1* gene would be not be useful for introgressing genes from high grain varieties into low grain rice varieties as there is no difference in length polymorphism.

The primer pair Apo1-1 (promoter) for *Apo1* gene did not amplify any band at the six temperatures from either high or low grain varieties, HGN1 and Heera, respectively indicating that the primer pair Apo1-1 (promoter) for *Apo1* gene would be not be useful for introgressing genes from high grain varieties into low grain rice varieties (Figure 11) (Table 2 and 3).

Table 2 The primers which used for checking amplification

SL NO.	PRIMER NAME	SEQUENCE (5-3)*	SPECIFIC	START-END
1	Dep1-1F	CGAGGGGTGGTTCTGAGTT	Overlapping	22
2	Dep1-1R	CGCTCTTCTTTTCTCCAACG	Overlapping	611
3	Dep1-2F	GCGTTGTTAATTGCGGTAGC	Exon1_2 (420-1080bp)	426
4	Dep1-2R	AGATTCCTGCTTTACTGTGTAAGTG	Exon1_2 (420-1080bp)	1029
5	Dep1-3F	AATCAAATGCGCTTTAGACATC	Exon1_3 (900-1560)	912
6	Dep1-3R	CAAAATATCTGTAATGCATGAAGGG	Exon1_3 (900-1560)	1502
17	APO-1F	CACTCCACTTCACCCTTGAAA	OVERLAPPING	6
18	APO-1R	GAAGGGGGACACGAGGTC	OVERLAPPING	644
27	GHD7-2F	TGATTGATTCATCGATCCTCC	Exon1_3 (900-1560bp)	818
28	GHD7-2R	TCAACAAACTGTAAGTAAATAGACCG	Exon1_3 (900-1560bp)	1549

Table 3 Amplification pattern of genomes of high and low grain rice varieties with designed primers

Gene	Primer	Promoter/Exon	Annealing Temperature (^{0}C)	Size of product (bp)	Amplification*		Comment**	
					HGN1	Heera	HGN1	Heera
Dep1	Dep1-1	Promoter	53	600	+	-	F	-
			54	600	+	-	F	-
			55	600	+	-	F	-
			56	600	+	-	M	-
			57	600	+	-	M	-
			58	600	+	-	D	-
	Dep1-2	Exon 1	53	600	+	+	F	F
			54	600	+	+	F	M
			55	600	+	+	F	M
			56	600	+	+	F	D
			57	600	+	+	F	D
			58	600	+	+	M	M
	Dep1-3	Exon 2	53	600	+	+	F	VD
			54	600	+	+	F	VD
			55	600	+	+	F	VD
			56	600	+	+	F	VD
			57	600	+	+	M	VD
			58	600	+	+	M	VD
Ghd7	Ghd7-2	Promoter	53		+	-	M	-
			54		+	-	M	-
			55		+	-	D	-
			56		+	-	D	-
			57		+	-	D	-
			58		+	-	D	-
Apo1	Apo1-1	Promoter	53	-	-	-	-	-
			54	-	-	-	-	-
			55	-	-	-	-	-
			56	-	-	-	-	-
			57	-	-	-	-	-
			58	-	-	-	-	-

Note: * + = Presence of band, - = Absence of band, ** Strength(concentration of amplified band; F= Faint band, M= Medium band, D= Deep band, VD= Very deep band

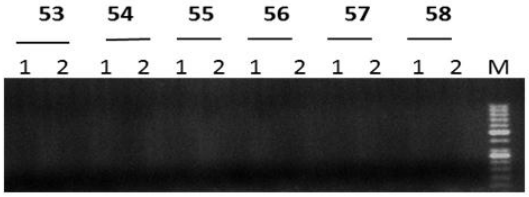

Figure 11 Gradient PCR with *Apo1* gene specific primer, Apo1-1 (promoter) 1 - HGN1 (high grain), 2- Heera(low grain) Annealing Temperatures are given on the top of gel

3 Conclusions

As mentioned above the main objective was to find out the functional, non-functional regions of high grain number genes along with the comparative structural analysis of their respective proteins. Three deigned primers for *Dep1*gene, one for *Ghd1* gene and one for *Apo1* gene were tested for amplification from high and low grain varieties, HGN1 and Heera, respectively. Two primers, Dep1-2 (promotor) for *Dep1*gene and Ghd7-2(Exon1) for *Ghd7* gene amplified fragments from high grain variety, HGN1 while failed to amplify from low grain variety, Heera, indicating that these primers would be useful for introgressing genes from high grain varieties into low grain varieties to improve yield potential through molecular breeding approach in future.

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