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Research Article

Identification of candidate genes contributing to grain filling in rice through genomic neighborhood mining

Divya P Syamaladevi*, C ReddiRoja, R K Gokulasuryan, Cheruvuri Naga Neeraja* Balachandran S M

ICAR-Indian Institute of Rice Research, Rajendranagar, Hyderabad

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****Corresponding author:***
E-mail: cnneeraja@gmail.com, dpsdevi@gmail.com

Abstract

Grain filling is an important process that contributes to grain yield in rice, especially in large panicle types. Genes associated with grain filling primarily belong to pathways in carbohydrate metabolism such as sucrose biosynthesis, sucrose transport and starch biosynthesis. The knowledge on the interplay of proteins in regulating various pathways associated with grain filling is still sparse. In this study we have surveyed the genomic neighbourhood (50Kb upstream and downstream) of six grain filling associated genes viz. Alpha-amylase, Starch synthase, Sucrose phosphate synthase, Glucose transporter, Sucrose transporter and Triose phosphate translocator. This has led to the identification of several genes coding for transcription factors, enzymes and other proteins in the neighbourhood of the foresaid grain filling associated genes. The identified neighbourhood genes constitute a prudent set of candidate genes for functional validation of their direct or indirect involvement in grain filling related pathways. A subset of the identified neighbourhood genes is expected to co-express with the known grain filling related genes. It was found that stress tolerance genes were present in the neighbourhoods of all the grain filling related enzymes selected for this study pointing to the possible stress tolerance mechanisms in action during reproductive phase.

Key words: Grain filling, Co-expression, neighbourhood genes, co-expressed genes in grain filling

1. Introduction

Rice (*Oryza sativa* L.) is the most important food crop in the developing world especially in Asia. Population growth, changing food habits and shrinking area under rice cultivation demand improvement in rice grain yield and productivity world-wide. The grain yield is an integrated function of various physiological and biochemical processes contributing to sink capacity and grain filling efficiency (Kato and Takeda, 1996). Sink capacity of a rice plant is defined by three characteristics: individual grain weight and size, total number of spikelets in a panicle and total number of panicles in a plant (Kato *et al*., 2007). The breeding efforts to increase the sink capacity has resulted in lines with extra heavy panicles which often fail to achieve their yield potential due to diminished grain-filling efficiency (Peng *et al*., 1999; Yang *et al*., 2002; Yang *et al*., 2010). Grain filling is the process by which fertilized ovaries in each of the spikelet develop into caryopses (fruit) by accumulating starch in the kernel. In a panicle, large and heavy superior grains are formed from spikelets located on apical primary branches which flower first while spikelets located on the basal secondary branches reach anthesis later and generate smaller or chaffy grains (Peng *et al*., 2016). Inferior spikelets fail to fill completely owing to a low grain filling rate in secondary branches (Wei *et al*., 2011).

Genes associated with sucrose transport and starch metabolism have considerable effect on grain filling (Chen *et al.*, 2019). An important aspect of grain filling process is the transportation of soluble sugars from source tissues to the spikelets through sugar transporters (Wang *et al.* 2019). Sucrose translocation is the major contributor of filling inferior grains (Wang *et al.*, 2015). Starch synthesis and accumulation in grains is also of great importance, as it forms almost the entire of the seeds (Wei *et al.*, 2017). Some transcription factors related to sucrose and starch metabolism have been found to affect grain filling. For instance, differential, transient and over expression analysis of NAC, GATA and WRKY genes have shown their involvement in the process of grain filling in inferior grains (Sperotto *et al.*, 2009; Wang *et al.*, 2019; Zhang *et al.*, 2011).

Segments of genetic material with specific characteristics viz. epigenetic modifications, physical interaction with the nuclear lamina, etc. have been defined as "genomic neighbourhoods" or "domains" (De and Babu, 2010). Apart from the presence of such regulatory sites, larger segments of the neighbourhoods of a gene, harbour other genes coding for various proteins or RNAs. These genes many-a-times function in related pathways or have related biological functions and may be co-expressed (Wang *et al.* 2011, Ghanbarian and Hurst, 2015). Such intra-chromosomal colocalization can strengthen co-expression, co-modification, and evolutionary conservation of neighbouring genes (Lian *et al.* 2018)

In this study, we identified many transcription factors and other genes in the 50Kb neighbourhood of known grain filling related genes.

2. Materials and methods

Six important grain filling related genes including three enzymes (α-amylase, starch synthase, SPS) and three transporters (Glucose transporter (GLUT), Sucrose transporter (SUT) and triose phosphate translocator (TPT)) were selected for this analysis. Paralogues of the selected grain filling related genes were identified using key word searches in *Oryza sativa* genome in NCBI genome data viewer (www.ncbi.nlm.nih.gov/genome/gdv/browser/genome/?id=GCF000001405.39) with locus search option. The identified locus id was used for navigating the neighbourhoods of each paralogous loci using the INSDC annotation provided by RAPDB. Genes within 50Kb upstream and downstream of each of the loci were collected. Neighbourhood gene functions were obtained from NCBI annotations using Batch Entrez (www.ncbi.nlm.nih.gov/sites/batchentrez) option and Uniprot (www.uniprot.org/) database. The obtained neighbourhood genes were classified into enzymes, transcription factors, other proteins and hypothetical proteins. The protocol is described in the Flowchart (Figure 1). The count of neighbourhood loci in each of the category was taken.

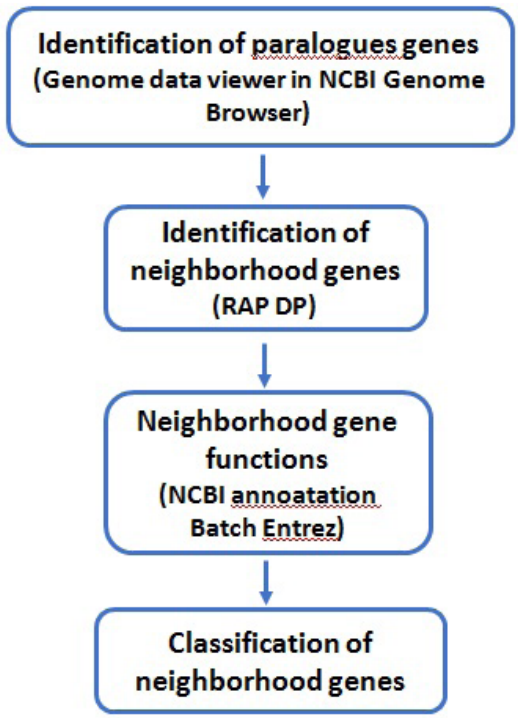

Figure 1: Protocol for identification of genes and their neighbourhood loci related to grain filling

3. Results and Discussion

3.1 Orthologues of grain filling related genes

Alpha-amylase and Triose phosphate translocator have the highest number of orthologous loci with 19 in each (Supplementary File 1). SUT hit the lowest with 6 loci. There were 11 loci in starch synthase, 8 loci in SPS and 9 in GLUT (Supplementary File 1). Chromosome 1 has the maximum grain filling related genes under investigation, followed by chromosome 2. Chromosome 11 and 12 were found to be sparsely populated with respect to the selected genes (Table 1, Supplementary File 1).

Table 1: Chromosome wise loci of selected grain filling related genes

Chromosome	Os AF	(OsSS)	(OsSPS)	OsGLUT	OsSUT	OsTPT	Total
1	2	1	2	1	0	4	10
2	2	2	1	1	2	0	8
3	1	0	0	1	1	3	6
4	2	1	1	0	1	0	5
5	0	1	0	1	0	4	6
6	1	3	2	2	0	1	9
7	6	1	0	0	0	1	8
8	2	1	1	0	0	3	7
9	2	0	0	3	0	1	6
10	0	1	0	0	1	0	2
11	0	0	1	0	0	1	2
12	1	0	0	0	1	1	3
Total	19	11	8	9	6	19	

3.2 Distribution of neighbourhood genes

Genomic regions near the grain filling related genes may harbor other genes involved in grain filling for which the grain filling function is not known hitherto. Therefore, we analyzed the genes present in 50Kb distance both upstream and downstream of six grain filling related genes. The number of neighbourhood genes in α amylase and starch synthase, were 100. SPS has slightly less populated neighbourhood with 67 loci (Figure 2). Compared to these enzymes, GLUT and SUT have most poorly populated neighbourhood with 55 genes. However, Triose phosphate translocator has an exceptionally highly populated neighbourhood with 152 loci (Figure 2).

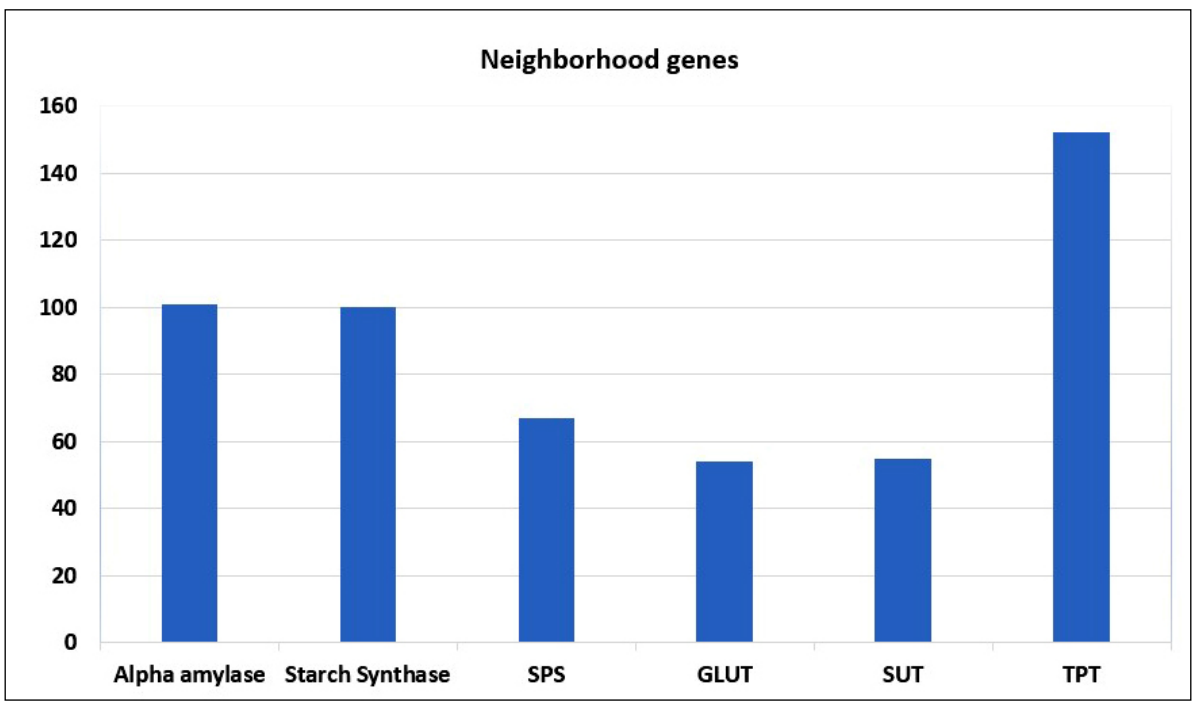

Figure 2: Graph showing distribution of total number of neighbourhood loci in genes Alpha-amylase, Starch Synthase, Sucrose Phosphate Phosphatase (SPS)Glucose transporter (GLUT), Sucrose transporter (SUT), Triose phosphate translocator (TPT)

A functional categorization of these neighbourhood genes into enzymes, transcription factors, other proteins and un-characterized proteins revealed that the gene density of these categories in the 50Kb neighbourhood of select grain filling related genes differ considerably. In TPT neighbourhood around 40 genes were coding for various enzymes. Number of enzyme coding neighbourhood genes in Alpha-amylase, Starch synthase and SPS genes were slightly less than that of TPT. GLUT and SUT gene neighbourhoods showed a much lower number of enzyme coding genes; 24 and 10 respectively.

Transcription factor genes in the neighbourhood were the lowest among all the grain filling genes surveyed. Neighbourhoods of Starch synthase, SPS and GLUT genes harbored single transcription factor locus only. Sucrose transporter has two transcription factors in its 50Kb flanking region. Alpha-amylase neighbourhood harbored four transcription factors and that of TPT housed nine (Figure 3).

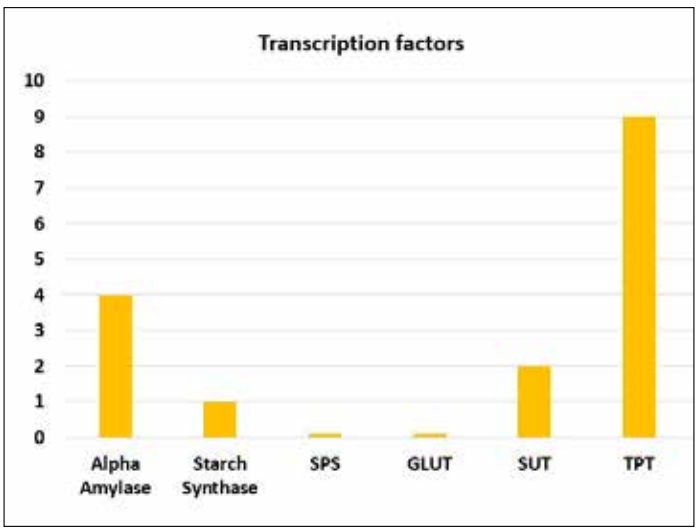

Figure 3: Transcription factors in the 50Kb upstream and downstream neighbourhood of select genes

Maximum number of 'other proteins' (Figure 4) and 'uncharacterized proteins' (Figure 5) categories were also observed in the neighbourhood of TPT (60 and 43 respectively). TPT is a triose transporter on chloroplast membrane. It is responsible for the export of the carbohydrates produced through photosynthesis which is one of the primary steps in the numerous downstream processes of grain filling (Fabre *et al.*, 2019). The crowded neighbourhood of TPT is suggestive of its co-expression with different proteins in many biological pathways owing to its regulatory role in numerous metabolic processes (Walters *et al.*, 2004).

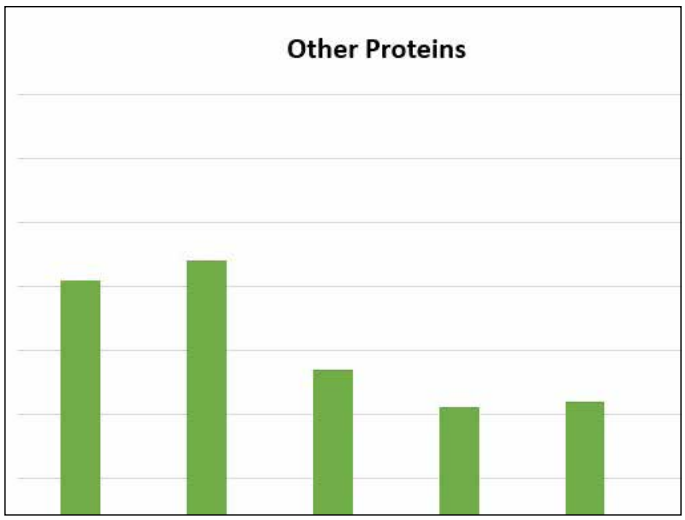

Figure 4: Other proteins in the 50Kb upstream and downstream neighbourhood of select genes

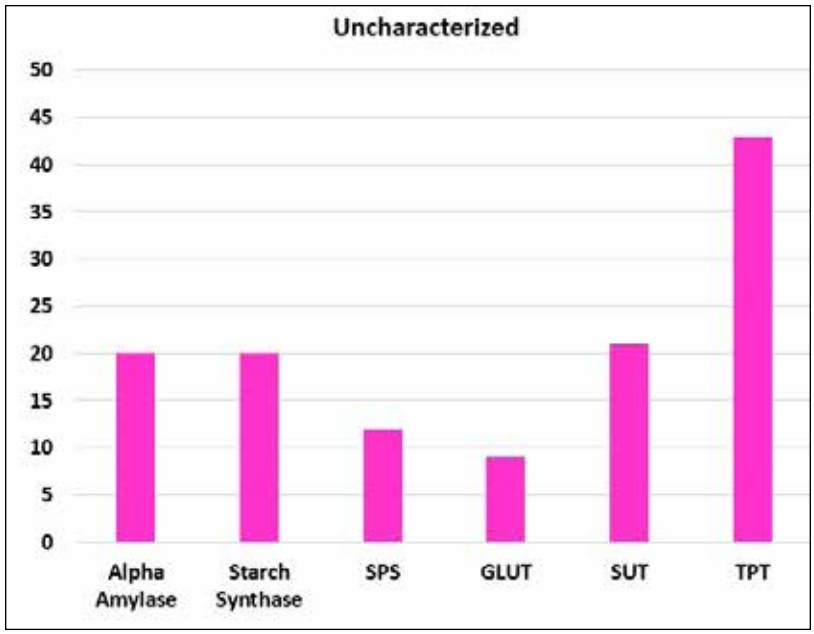

Figure 5: Uncharacterized proteins in the 50Kb upstream and downstream neighbourhood of select genes

3.3 Transcription factors in neighbourhood

Further to understand on the functional implications of the neighbourhood genes, we examined the tanscription factor genes. It was found that the genes coding for transcription factors viz., WRKY 24, ERF 109, bZIP 23, TCP21, were located in the neighbourhood of alpha-Amylase gene (Table 2). WRKY has been previously shown to affect grain filling (Zhang *et al.*, 2011). Some ethylene-responsive transcription factors (ERFs) have also been known to be involved in grain filling (Schmidt *et al.*, 2014). Starch Synthase harboured ERF11, which is also a growth associated factor (Dubois *et al.*, 2015). Glucose transporter neighbourhoods harboured no transcription factor gene instead it has a transcription co-activator – multi protein bridging factor 1c, which was recently reported to be associated with salinity tolerance (Zhao *et al.*, 2019). TPT harboured nine in its vicinity which included stress responsive transcription factors apart from the general transcription factors (Table 2). Implications of the presence of the transcription factors near the grain filling genes are yet to be elucidated. We propose that the transcription factor genes may be expressed together with the known grain filling related genes and may have a role in regulating their expression.

Table 2: Transcription factors in the vicinity of select grain filling related genes

Sl No	Gene	Transcription factors in neighbourhood
1	Alpha-amylase	WRKY transcription factor WRKY24
		ethylene-responsive transcription factor ERF109
		bZIP transcription factor 23
		transcription factor TCP21
2	Starch synthase	ethylene-responsive transcription factor 11
3	Sucrose phosphate synthase	Nil
4	Glucose transporter	Nil
5	Sucrose transporter	transcription termination factor MTEF1
		transcription factor ILI

6	Triose phosphate translocator	transcription factor IIIA
		transcription elongation factor SPT6
		AP2-like ethylene-responsive transcription factor TOE3
		NAC domain-containing protein 41
		probable WRKY transcription factor 65
		heat stress transcription factor A-4b
		basic leucine zipper 19
		probable transcription factor GLK2
		general transcription factor IIE subunit 1

3.4 Common genes in the neighbourhoods

There were no neighbourhood gene common between all the 6 genes selected. One gene PCMP-E42 (Pentatricopeptide repeat proteins) was found to be common among the 50Kb neighbourhood of 5 genes – Alpha-amylase, Starch synthase, SPS, SUT and TPT (Figure 7a, 7b). Pentatricopeptide repeat proteins regulate gene expression at RNA level (Manna *et al.*, 2015). Presence of PCMP gene implicates a possible common post transcriptional gene regulation pathway for these genes.

Another gene Ubiquitin ligase which controls several aspects of eukaryotic biology by promoting protein ubiquitination and degradation is present in the neighbourhood of 4 genes –Alpha-amylase, Starch synthase, SPS (Figure 6a) and TPT (Figure 6b). However, these ubiquitin ligase genes belong to different families. Alpha-amylase and SPS have BOI RNF4 family Ubiquitin ligases which are associated with biotic and abiotic stress (Sun *et al.*, 2007), whereas Starch synthase has WAV3 Ubiquitin ligase which is functionally related to root growth (Sakai *et al.*, 2012). Ubiquitin ligases RHC1A, SIRP1, RGLG4, UPL4 and EL5 were present in TPT. The role of RING-Finger type Ubiquitin ligases in grain filling has been elucidated previously (Matsuoka and Ashikari, 2007).

Splicing factor 1 (SF1) was found to be common among four genes viz., Starch synthase (Figure 6a), GLUT, SUT and TPT (Figure 6b). The splicing factor is involved in the ATP-dependent formation of the spliceosome complex and its presence in the neighbourhood of these genes suggests involvement of their splice variants in grain filling (see Chen and Cheng, 2012 for a review).

Alpha-amylase, GLUT and TPT have UDP-glycosyltransferase (UGT) as common (Figure 7a, 7b). UGT catalyzes the addition of the glycosyl group from a UTP-sugar to a small hydrophobic molecule (Meech *et al.*, 2019).

Alpha-amylase, SPS and TPT have probable auxin efflux carrier component (PIN) as common neighbourhood gene. Alpha-amylase and SPS were known to enhance the levels of sucrose in plants (Yang *et al.*, 2001) The Sucrose molecule in turn is involved in auxin transport through PIN (stokes *et al.*, 2013). Therefore, the nearness of these genes with PIN can be suggestive of their co-expression.

There were some common genes between the genes of antagonistic function in grain filling For instance, starch degrading Alpha-amylase and Starch synthase have some common neighbourhood genes viz. RGA3, NAC, LRR and ER which are biotic stress tolerance related. This indicates possible co-expression or co-inheritance of these genes with both Alpha-amylase and Starch synthase. (Figure 6a). AA and TPT neighbourhood harbour NAC41 and Starch synthase harbor NAC94. NAC domain containing protein may play important roles in the regulation of the transcriptional reprogramming associated with plant stress responses (Sun *et al.*, 2018; Murozuka *et al.*, 2018)

The presence of WAK (associated with growth) in the vicinity of SPS and starch synthase suggests their co-functioning or co-inheritance. WAK, which is involved in cell signalling has been implicated in seed development as well, explaining its localization in the neighbourhood of both SS and SPS (Wang *et al.*, 2012).

Universal stress protein gene (USP) was found to be common for starch synthase and TPT (Figure 6a, 6b). USP

genes protect the organism from environmental stress and also protect the DNA and more generally the cell from further damage. Presence of USP gene suggests importance of stress responsiveness of the plant during grain filling.

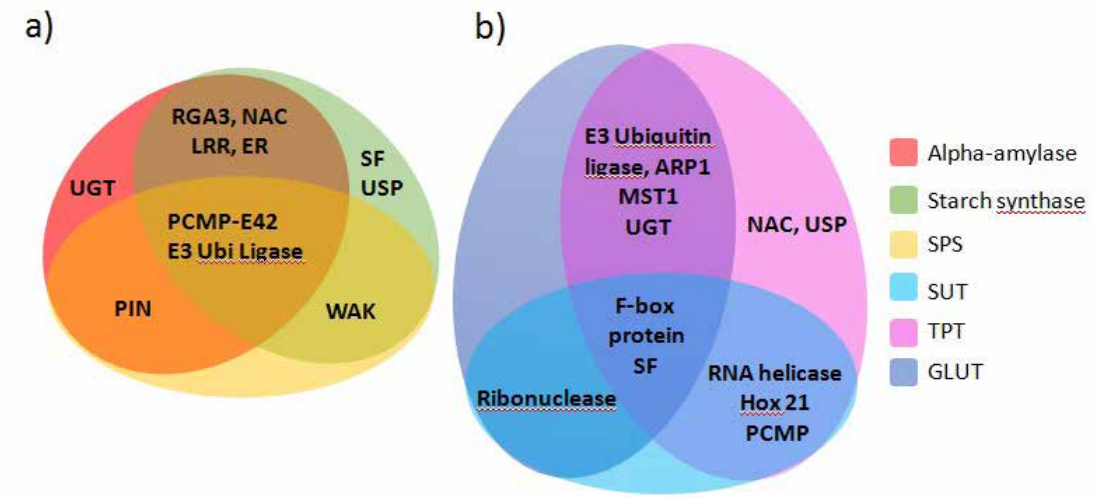

Figure 6: Common neighbourhood genes in 50kb both upstream and downstream neighbourhood of a) Alpha-amylase, Starch Synthase and SPS b) Glucose transporter (GLUT), Sucrose transporter (SUT), Triose phosphate translocator (TPT). The details of functions are given in Table 3.

E3 Ubiquitin lagase, ARP1, MST1, SF, UGT are common for glucose transporter and triose phosphate translocator. F-box protein and SF was found to be common for Glucose transporter, sucrose transporter and triose phosphate translocator.RNA helicase, Hox21 and PCMPwas found to be common for Sucrose transporter and triose phosphate translocator.

AA and TPTgenes have WRKY transcription factor WRKY24 (WRKY24), ras-related protein (RRP), THO complex subunit THOC, probable calcium-binding protein CML in their 50Kb neighbourhood(Figure 7). Ras related protein mainly involved Intracellular vesicle trafficking and protein transport. THO complex is a nuclear structure with important role in biogenesis of mRNPs involved in the process between transcription elongation and mRNA maturation / export. The function of Calcium binding protein CML is to regulate the amount of cytosolic free Ca^{2+}. Elucidating the functional implications of these genes in the neighbourhood of grain filling genes need further experimentations.

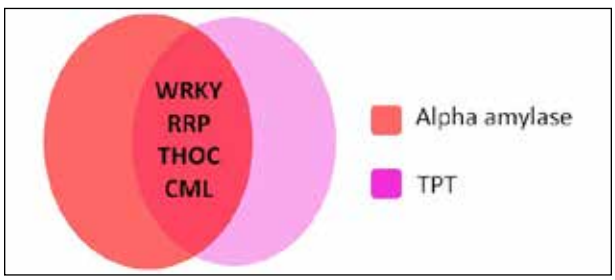

Figure 7: Common neighbourhood genes between Alpha-amylase and TPT

Table 3: List of Common genes in the neighbourhood of grain filling related genes

Sl No	Common Gene	Function	Reference
1	PCMP-E42	Regulation of Gene expression, grain filling	Manna 2015, Li *et al.*, 2014
2	E3 Ubi Ligase	Drought tolerance, immune response, grain filling	Zheng *et al.*, 2017; Bae *et al.*, 2011; Kachewar *et al.*, 2019 Capron *et al.*, 2012; Song *et al.*, 2007; Matsuoka & Ashikari, 2007
3	UGT	Stress responsive regulation	Meech *etal.*, 2019 Rehman *et al.*, 2018
4	PIN	Auxin carriers Growth and grain yield	Zhou *et al.*, 2018, Sharma *et al.*, 2018

5	WAK	cell signalling, Seed development	Anderson *et al.*, 2001, Wang *et al*., 2012
6	SF	Developmental &morphological regulation	Kalyna *et al.* 2003
7	USP	abiotic stress tolerance	Wang *et al.*,2017
8	RGA3	Putative disease resistance gene	Vossen *et al.*, 2003
9	NAC	transcription factor involved in multiple biological processes.	Sun *et al.*, 2018 & Murozuka *et al.*, 2018
10	LRR	Pathogen responsive	Moffett *et al.*, 2002
11	ER	stress signalling, wound repair.	Heyman *et al.,* 2018
12	F-box protein	control many important biological functions.	Xu *et al.,* 2009 & Chen *et al.*, 2013
13	SF	RNA Splicing	Kumar *et al.,* 2012
14	Ribonuclease	RNA degradation.	Schein *et al.,* 2008
16	ARP1	mRNA localization	Hong *et al.,* 2019
17	MST1	Monosacharide transporter	Vikram *et al.,* 2019
18	RNA helicase	chloroplast gene expression regulation	Nawaz *et al.,* 2019
19	Hox21	seed maturation, floral induction, stress and hormone signaling	Nijhawan *et al.,* 2008
20	WRKY	seed development	Zhang *et al.*,2011
21	RRP	Cell signalling, heat tolerance and grain yield	El-Esawi & Alayafi, 2019
22	THO Complex Subunit	Transcriptional elongation, mRNA export	Jimeno *et al.*, 2002
23	CML	Calcium binding protein	Perochon *et al.*, 2011

Conclusion

This study presents a set of gene loci that are in the 50Kb genomic neighbourhood regions of known grain filling pathway genes in *Oryza sativa* genome. The identified neighbourhood genes include transcription factors and other gene expression regulators. The physical proximity of these genes with the known grain filling related genes implicates the involvement of these puta tive candidate genes in related pathways through co-expression and co-inheritance. Over all, the neighbourhood genes suggested in this study could have direct or indirect effects on grain filling and therefore can be considered as putative candidate genes.

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Author Contributions

DPS, CR and RKG prepared the manuscript and DPS, CNN and BSM helped in preparing the final version of the manuscript and correspond to the journal.

Ethical Approval

This article does not contain any studies involving human or animal participants performed by any of the authors.

Conflicts of Interest:

The authors declare no conflict of interest.

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