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Differential grain filling in apical and basal spikelets of compact panicle rice is associated with difference in expression of miRNAs targeting gene products involved in grain filling

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ABSTRACT

Development of rice cultivars bearing numerous spikelets by breeding approach to increase production of the crop to feed the ever increasing human population accompanies poor filling of grains in their basal spikelets preventing achievement of the yield potential. To understand the reason of such poor grain filling at the level of the initial biochemical events, 5 RACE library was prepared taking RNA extracted from the spikelets of two indica rice cultivars, a compact-panicle Mahalaxmi bearing numerous spikelets per panicle (>350) and a lax-panicle Upahar bearing fewer spikelets (<250) per panicle, and the presence of the miRNA cleaved gene products involved in grain filling was identified by running PCR using gene-specific primers. Bioinformatics analysis was conducted to find if the grain filling enzymes were targeted by miRNAs. The effort led to identification of three enzymes, namely granule bound starch synthase, invertase and sucrose phosphate synthase, the transcripts of which were targeted by the miRNAs osa-miR160a-5p, osa-miR166e-3p and osa-miR156b-5p, respectively. The expressions of the three miRNAs were confirmed by Northern in the apical and basal spikelets of the two cultivars. The expression analysis of the three miRNAs further revealed that these were expressed significantly more in the basal spikelets than in the apical ones of Mahalaxmi, but not of Upahar. Real time PCR of the target genes showed an opposite result, i.e., these were expressed significantly less in the basal spikelets than in the apical ones of Mahalaxmi, but mostly not of Upahar. The finding led to the conclusion that the grain filling in rice could be regulated at the level of miRNAs, and manipulation of their expression could be proved an important tool in improving grain filling in compact-panicle rice cultivars.

Key words: *miRNAs, Oryza sativa, granule bound starch synthase, invertase, sucrose phosphate synthase*

INTRODUCTION

Rice besides being a model crop is also food crop feeding the entire human population. In the Asian countries rice meets almost 80% of the calories requirement of the human beings (Nallamilli et al., 2013; Peng et al., 2013). Because of large dependence of the human beings on rice for food, the challenges now lies in increasing its production, which should must increase to at least 800 million tons from the current figure of approximately 500 million tons (Virk et al., 2004) to meet the calories requirement of the ever increasing human population that is estimated to be 9.6 billion by 2050 (UN, 2013; FAO, 2009).

The edible part of the rice crop is the grain, which is nothing but endosperm that serves as food for the embryo during germination. The fully developed endosperm of rice grain is largely composed of starch, which is accumulated in the amyloplast of the endosperm cells during the course of grain filling that last for a little more than 25 days after anthesis or fertilization. The anthesis in rice panicle is basipetal, starting from apical spikelets towards the top of the panicle to the basal spikelets towards the base of the panicle and the process is completed in 6-7 days. Thus, the grain filling starts earlier in the apical spikelets compared with the basal spikelets. Biochemically, the grain filling is a process of unloading of sucrose into the apoplast of

caryopses, its entry into the endosperm cells and breakdown into hexoses, which are converted to glucose-1-phosphate and then into ADP-glucose, the substrate that is used in the synthesis of starch (Panda et al., 2015). The major enzymes catalyzing the entire process are sucrose synthase (SUS), ADP glucose pyrophosporylase (AGPase), starch synthase(SS), granule-bound starch synthase (GBSS), starch branching enzyme (SBE) and starch debranching enzyme (DBE) (Panda et al., 2016; Zhu et al., 2011; Odhan et al., 2005).

The number of spikelets borne on a rice panicle varies with the cultivars. In lax-panicle bearing <250 spikelets, most of them produce well filled grains. However, in the compact-panicle bearing numerous spikelets (>350), more than 1/3rd of them, mostly the basal spikelets produce poorly filled grains or do not have grain filling at all (Panda et al., 2015; Panda et al., 2016; Sekhar et al., 2015a, Sekhar et al., 2015b; Das et al., 2016; Panda et al., 2018; Yang et al., 2002; Wang et al., 2008; Fu et al., 2011), although these are fully competent to produce well filled grains (Kato, 2004). The reason of poor grain filling in the basal spikelets could be the poor activities of the starch synthesizing enzymes (Kato et al., 2007; Wang et al., 2008; Yang and Zhang, 2010; Zhang et al., 2014; Panda et al., 2015; Panda et al., 2016), but there could also be other factors not well understood, as plethora of proteins/genes are differentially expressed between the well filled apical spikelets and poorly filled basal spikelets (Sekhar et al., 2015a; Sekhar et al., 2015b; Das et al., 2016; Zhang et al., 2014).

The differential expression of genes between the apical and basal spikelets may be a result of differential expression of transcription factors, such as RITA, bZip, Myb, RISBZ, RPBF and C3H family protein (Venu et al., 2011; Zhu et al., 2003; Sekhar et al., 2015a;Sekhar et al., 2015b). However, no transcription factors have been reported to be expressed differentially in the spikelets based on their spatial location that regulate the expression of starch biosynthesizing enzymes (Das et al., 2016; Zhu et al., 2003; Venu et al., 2011; Sekhar et al., 2015b; Liu et al., 2010; Zhang et al., 2014; Chen et al., 2016), despite the fact that transcriptional control of the gene involved in grain filling is very important for the proper development of endosperm (Zhu et al., 2003). In recent decades, however, post transcription regulation of gene expression by miRNAs have been reported that could greatly influence the function of a gene (Bartel, 2004; Jones-Rhodes et al., 2006). They could also regulate the expression of the effector proteins by targeting their transcription factors.

Temporal expression of miRNAs in spikelets during grain development has been reported by a few workers (Lan et al., 2012; Zhu et al., 2008; Peng et al., 2013; Xue et al., 2009; Yi et al., 2013) by deep sequencing to get an insight into their role in grain development. In addition, at least two studies have been conducted on spatial difference in expression of miRNAs in spikelets in *Japonica* cultivar by high throughput sequencing in order to understand their role in grain development (Peng et al., 2011; Peng et al., 2014). However, none of the above studies have been able to put forth a clear understanding of the influence of any miRNA in grain filling, particularly with regard to the miRNA target identification. The present study has taken a reverse approach in identifying the miRNAs likely to be involved in regulation of grain filling in rice; firstly, the miRNA cleaved fragments of a few enzymes known for their role in grain filling were identified by 5 RACE PCR, and then the miRNAs targeting these enzymes was identified bioinformatically. The paper additionally reports spatial difference in expression of the identified miRNAs by Northern and spatial variation in expression of their targetsin rice cultivars contrast for panicle compactness and grain filling to show the possible regulatory role of the miRNAs in grain filling in rice.

MATERIALS AND METHODS

Two cultivars of *Oryza sativa* ssp. *Indica*, the lax-panicle Upahar with little difference in grain filling in the apical and basal spikelets, and the compact-panicle Mahalaxmi exhibiting significant difference in grain filling between the apical and basal spikelets, were selected for the study (Sekhar et al., 2015a; Panda et al., 2015; Sekhar et al., 2015b). The seeds of the two cultivars were obtained from ICAR-National Rice Research Institute, Cuttack, India and the plants were raised at the field facility of the Institute following standard agriculture practices (Panda et al., 2015). The primary tillers of the plants were tagged in the morning on the day the spikelets on the apical region of the

panicle, the apicalspikelets, reached anthesis, and this day was referred to as 0 DAA (days after anthesis). The spikelets located on the basal region of the panicle, the basal spikelets, reached anthesis after four days and corresponded to 0 DAA for the inferior spikelets. The spikelets were sampled from the apical and basal regions of the panicle on the 6^{th}, 9^{th},12^{th} and 15^{th} day after anthesis/fertilization, and referred as 6, 9, 12, 15 DAA samples (Panda et al., 2015; Sekhar et al., 2015a). These were placed in 15 mL falcon tubes separately, frozen immediately in liquid nitrogen and stored at -86 C until analysis.

The spikelets samples of 6, 9, 12 and 15 DAA of Mahalaxmi and Upaharwere mixed, and total RNA was extracted using PureLink™ Plant RNA Reagent (Thermo Fisher Scientific). A known amount of RNA was incubated with 5' adapter and other reagents supplied along the Clontech RLM-RACE kit, and incubated at 37 ^{o}C for 1 h following the kit protocol to facilitate ligation of the adapter at the 5' end of the miRNA cleaved mRNA that contains a free phosphate group (Awasthi et al., 2019). Further, the 5' adapter ligated RNA was reverse transcribed using random decamers and M-MLV reverse transcriptase and other reagents supplied along with the kit to make the 5' RACE library (Awasthi et al., 2019). The reverse transcription required incubation of the mixture at 42 ^{o}C for 1 h. Gene specific primers of several enzymes/ proteins reportedly involved in grain filling were used to amplify their cleaved fragments. Each such amplification required running PCR taking 1 μL of the RACE library, PCR reagents including gene-specific and adapter-specific outer primers, followed by a 2^{nd} PCR taking the product of the 1^{st} PCR as template and PCR reagents including gene-specific and adapter-specific inner primers. The PCR product was visualised on 1.5% agarose gel, the band was excised and eluted using Qiagen get extraction kit, the amplicons were cloned into pGEMT vector and transformed into DH5 competent bacteria. The transformed bacteria were grown in LB medium on a shaker and plated on ampicillin containing LB plates for the colonies to develop. The white colonies were subjected to colony PCR using SP6 and T7 primers and the amplicons were sequenced on automated sequencer. The sequences were BLAST searched for identification. The number of colonies sequenced and those containing the gene sequence of interest were recorded.

The CDS of the individual gene identified was loaded on the online psRNATarget software and queried for the know *O. sativa* miRNA that would cleave the gene product. The miRNA cleaving a gene product was recorded.

Total RNA was isolated from the 6 DAA apical and basal spikelets of Mahalaxmi and Upahar separately, as stated above. The concentration of the RNA in each extract was adjusted to 1 g μL^{-1}, 20 L of each extract was mixed with equal volume of 2X loading buffer and run on 7 x 10 cm 15% denaturing polyacrylamide gel. After completion of the run, the gel was removed and the RNA was transferred onto Hybond-N+ nylon membrane using semi-dry electrophoretic transfer unit (Bio-Rad). The membrane was air-dried and the RNA was cross-linked on a UV cross-linker (HoeferTM). Each membrane was then hybridized separately with the complimentary miRNA probe end-labelled with [γ^{-32}P]dATP. The membrane was washed in non-stringent and stringent SSC buffer, air-dried, pressed against X-ray film, housed in a cassette and kept at -80 ^{o}C for 72 h (Gharat et al., 2015). The film was developed after exposure and imaged on a gel-doc. The blot was then stripped off the probe and used again to probe the U6 RNA using [γ^{-32}P]dATP labelled U6 for a small stretch of the sequence (Gharat et al., 2015). The band intensities from the miRNA and U6 were subjected to densitometric analysis, and the expression of the induvial miRNA was presented as relative value.

The CDS of the target mRNAs of interest was retrieved from NCBI database and primers were designed using Primer Blast software for the study of spatio-temporal expression of the individual target mRNAs. Total RNA was extracted from the apical and basal spikelets of 6, 9, 12 and 15 DAA samples, as stated above. After quality check, the RNA in the individual extract was reverse transcribed using QuantiTect Reverse Transcription kit (Qiagen). The cDNA obtained served as template for qPCR. QuantiTect SYBR Green PCR kit was used to carry out qPCR of the gene of interest on LightCycler 480II (Roche). Each reaction was run in triplicate and two cDNA preparationswere used for qPCR of each gene (n = 6). The relative levels of the templates of the

individual gene in the apical and basal spikelets of each cultivar were quantified as fold change (i.e., $2^{-\Delta\Delta C}T$) following Pfaffl (2001) considering actin template as the reference level. The result was presented as the fold change in expression of the gene in the basal spikelets over the apical ones of a particular sampling day. Student's t-test was carried out to know the significance of difference in expression of a gene in the basal spikelets over the apical ones (Gharat et al., 2015).

RESULTS AND DISCUSSION

The 5 RACE library revealed the presence of at least three cleaved gene products of the enzymes/proteins involved in grain filling (Fig. 1). These were granule-bound starch synthase 1 (GBSS1), invertase 1-like and sucrose phosphate synthase 4 (SPS4). The cleavage of the gene products of all these enzymes were, however, off the miRNA-mRNA pairing site, towards the 3 mRNA end of the miRNA-mRNA pairing. The miRNAs targeting the gene product GBSS1, invertase and SPS4 were bioinformatically identified to be osa-miR160a-5p, osa-miR166e-3p and osa-miR156b-5p.

The expressions of osa-miR160a-5p, osa-miR166e-3p and osa-miR156b-5p were studied by Northern in 6 DAA apical and basal spikelets of the two indica rice cultivars Mahalaxmi and Upahar contrast for grain filling. The expression of all the miRNAs was much greater in the basal spikelets compared with the apical spikelets in Mahalaxmi that showed poor grain filling in the basal spikelets compared with the apical spikelets (Fig. 2). On the other hand the expression of all the miRNAs was more or less similar in both apical and basal spikelets of Upahar that did not show any spatial difference in grain filling; the expression of osa-miR156b-5p and osa-miR166e-3p was a little less in the basal spikelets than in the apical spikelets and that of osa-miR160a-5p was only slightly more in the basal spikelets than in the apical spikelets.

Real-time PCR (qPCR) of the cleaved gene products identified in the 5 RACE library in time and space reveled that all these, including GBSS1, invertase and SPS4, were having a significantly lower abundance in the basal spikelets compared with the apical spikelets on all the days after anthesis in Mahalaxmi; the expression in the basal spikelets mostly less than half than that in the apical spikelets (Fig. 3). The expression of the three genes in Upahar on the other hand was more or less similar in both apical and basal spikelets on all the days after anthesis, except of GBSS1, which showed a little less, but significantly less, expression in the basal spikelets compared with the apical ones on all the days after anthesis. On an inter-cultivar comparative note, the relative expression of all the target genes in the basal spikelets over the apical ones was much lesser in Mahalaxmi compared with that in

Fig. 1. Cleavage points of the target mRNAs by the known miRNAs of *Oryza sativa* as obtained by 5 RACE PCR and cloning and sequencing of the PCR products. The arrows indicate the cleavage points. The number of clones having the ligated gene product out of the total number of clones sequenced are indicated besides the arrows indicating the cleavage points.

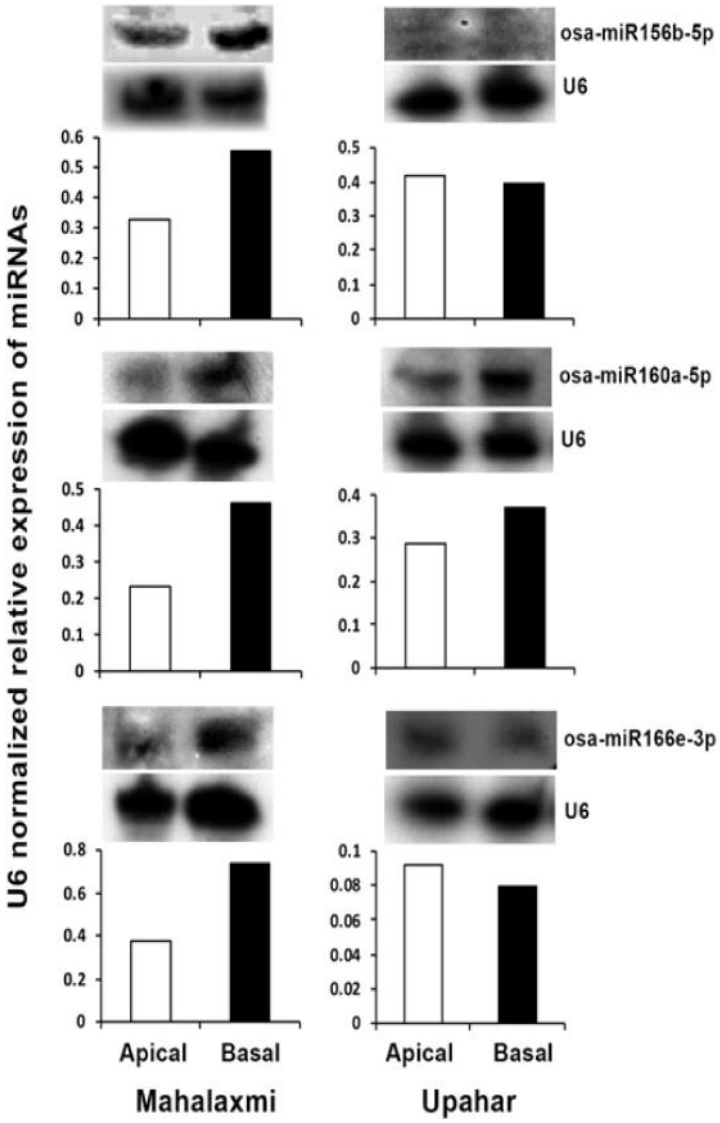

Fig. 2. Expression studies of the three known miRNAs in the apicaland basalspikelets of the compact-panicle Mahalaxmi and the lax-panicle Upahar by Northern blotting. The apicaland basalspikelets of both the cultivars were harvested on the 6th days after fertilization (6 DAF). RNA was isolated from them and subjected to Northern blot analysis for the individual miRNAs using their complimentary sequences. The expression of the individual miRNAs in the apical and basal spikelets was represented as the normalized relative value obtained by densitometric analysis of the individual miRNA bands with respect to that of the U6 bands.

Upahar.

The lower expression of the target proteins in the basal spikelets of Mahalaxmi compared with the apical spikelets accompanied a greater expression of the miRNAs, particularly miR156-5p and miR166-3p, targeting them (Fig. 4). The schematic representation (Fig. 4) also shows that the panicle compactness was greatly responsible for a greater expression of miR156-5p and miR166-3p in the basal spikelets of Mahalaxmi accompanied by production of poor grain quality.

It is now well established that yield potential of the breeding lines bearing compact panicle with numerous spikelets remained unachieved because of poor filling of the basal spikelets (Panda et al., 2015; Das et al., 2016; Yang and Zhang, 2010; Panda et al., 2018; Wang et al., 2008). The reason of such differential

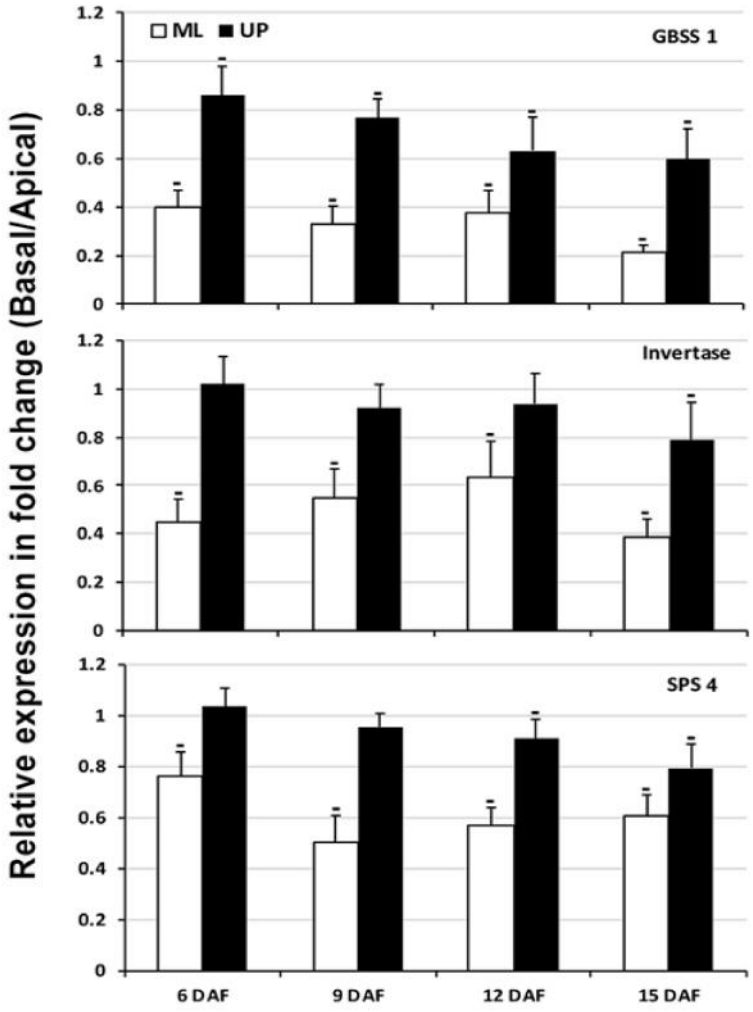

Fig. 3. Changes in expression of the target genes of the miRNAs in the apical and basal spikelets of the compact-panicle Mahalaxmi and the lax-panicle Upahar. The expression of the individual target genes was quantified by RT-qPCR and expressed as relative value (fold change) in the basal spikelets over the apical spikelets. Expression of actin served as internal control for normalization of expression of the individual gene. The data are the mean ±SD of six independent estimations considering two biological samples. The individual bars marked with (-)/(+) represent significant down regulation/upregulation of expression in the basalspikelets over the apicalspikelets at least at p 0.05.

grain filling in the apical and the basal spikelets is, however, far from understood, although it is quite known with certainty that the basal spikelets of the compact panicle rice is competent enough to produce well filled quality grain (Kato, 2004; You et al., 2016). The reason of poor filling of the basal spikelets has been attributed to several factors, including poor activities of the starch synthesizing enzymes (Panda et al., 2015; Das et al., 2016; Yang and Zhang, 2010) and differential expression of many genes in them compared to that in the apical spikelets (Sekhar et al., 2015a; Sekhar et al., 2015b; Das et al., 2016). The possible role of miRNAs in differential grain filling in the spikelets based on their spatial location has also been indicated (Peng et al., 2014; Yi et al., 2013; Peng et al., 2011). However, no work carried out so far has demonstrated that the expression of the enzymes involved in grain filling is regulated by miRNAs.

The present results clearly show that the expression of at least a few enzymes involved in grain filling, including GBSS1, invertase and SPS4, could be

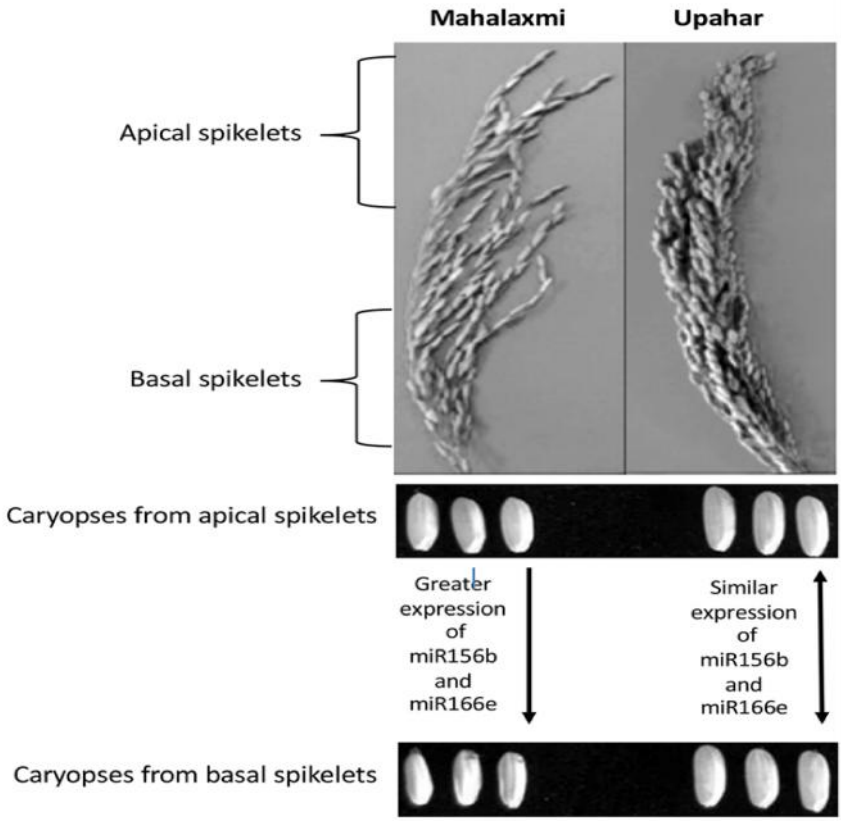

Fig. 4. Schematic representation of panicle architecture of the two rice cultivars, Mahalaxmi and Upahar and the quality of the grains (caryopses) produced by the respective apical and basal spikelets.The caryopses were removed from the spikelets 21 days after anthesis. The arrows indicate a greater expression of miR156b-5p and miR166e-3p in the basal spikelets compared with the apical spikelets in Mahalaxmi but similar expression of these miRNAs in the apical and basal spikelets of Upahar. The grain quality is accordingly affected.

regulated by miRNAs, as evidenced by identification of their miRNA cleaved fragment in 5 RACE library (Fig. 1), and hence miRNAs could have significant role in regulating the grain filling in rice. The cleavage of the target mRNAs at the places other than the ideally recognized canonical cleavage site, *i.e.*, 10/11th position from the 5' end of the miRNA-mRNA pairing site (Park and Shin, 2014; Palatnik et al., 2003), is although deviation, but not exception. For example, Wan et al. (2012) reported cleavage at the downstream of the miRNA*/mRNA pairing region by pde-miR171a, at the downstream of the miRNA*/mRNA pairing region by pda-miR162a, and at the upstream of the miRNA*/mRNA pairing region by pda-miR482a in *Pinus densata*. Similarly, De Paola et al. (2012) found cleavage at the 21-nt downstream of the canonical 10/11th position by cca-miR160 in *Cynara cardunculus*. Furthermore, Feng et al. (2015) working on *Salicornia europaea* observed cleavage of laccase by seu-miR397 at the upstream of the miRNA/mRNA pairing site, and cleavage of F-Box family protein, SAC3/GANP family protein and NADPH cytochrome P-450 reductase by seu-miR156, seu-miR171 and seu-miR15, respectively at the downstream of miRNA/mRNA pairing site. The reason of such uncanonical cleavage is unclear.

Among the enzymes identified to be cleaved, the role of GBSS in grain filling is well established. It catalyzes addition of ADP-Glucose (ADP-Glc) to the nonreducing end of growing chain of the amylose polyglucan by α-1,4 linkage (Odhan et al., 2005; James et al., 2003). Thus, any inhibition in the activity of GBSS is likely to decrease the starch synthesis and deposition process, and the poor filling of grain in the basal spikelets

of Mahalaxmi could be a result of low expression of GBSS in the basal spikelets compared with the apical spikelets (Fig. 3), which in turn could be a result of a higher expression of the miRNA osa-miR160a-5p in the former than in the latter (Fig. 2). The role of invertase is no less than GBSS in starch synthesis, as sucrose, the substrate of starch, is synthesized in leaves from where it is transported to the endosperm cells, and before its assimilation to starch in the endosperm, it has to be broken down to hexoses to initiate the process of starch synthesis. The cleavage of sucrose to hexoses is facilitated by two enzymes, sucrose synthase and invertase; while invertase breaks down sucrose into glucose and fructose, sucrose synthase converts sucrose to UDP-glucose and fructose utilizing UDP (Kumar and Asthir, 2016). It has been clearly demonstrated by radiotracer studies that sucrose is hydrolyzed in the cell wall free space to hexose, which is taken up by the endosperm (Shannon and Dougherty, 1972). It has also been seen that starch synthesis in kernel is positively correlated with both sucrose synthase and invertase activities (Ou-Lee and Setter, 1985; Kumari and Asthir, 2016) and thus significantly low expression of invertase in the basal spikelets compared with apical ones in Mahalaxmi but not in Upahar (Fig. 3) could be one of the possible reasons of the poor grain filling in the former than in the latter. Furthermore, a higher expression of osa-miR166e-3p in the basal spikelets compared with the apical ones in Mahalaxmi but not in Upahar (Fig. 2) eloquently indicate the same to be the reason of significantly low expression of invertase in the basal spikelets compared with the apical ones in Mahalaxmi but not in Upahar and hence, the miRNA could be an important regulator of the grain filling process in rice. In contrast to osa-miR160a-5p and osa-miR166e-3p, a higher expression of osa-miR156b-5p in the basal spikelets compared with the apical spikelets of Mahalaxmi but not of Upahar (Fig. 2) also suggested that the regulation of starch synthesis not only occurs at the level of its synthesis, but also at the level of synthesis of the substrate sucrose itself, as SPS, the enzyme involved in the synthesis of sucrose is targeted by osa-miR156b-5p, which is reflected from significantly low expression of SPS in the basal spikelets compared with the apical ones of Mahalaxmi but not of Upahar (Fig. 3). The role of SPS in grain filling is well established, as its activity positively correlates with accumulation of sucrose (Yang et al., 2001) and pick-upat the active grain filling stage (Kumari and Asthir, 2016).

The study thus identified three important enzymes, namely GBSS, invertase and SPS, involved in grain filling that are targeted by the known miRNAs like osa-miR160a-5p, osa-miR166e-3p and osa-miR156b-5p, respectively. The study also showed that the expression of these enzymes were negatively correlated with the expression of the respective miRNAs targeting them in the basal spikelets of the compact panicle Mahalaxmi. Among the three miRNAs, only miR156-5p and miR166e-3p showed a greater expression in the basal spikelets compared with the apical spikelets in Mahalaxmi, but not in Upahar (Fig. 2). Hence, the role of these two miRNAs in poor grain filling in the basal spikelets of Mahalaxmi might be of much significance (Fig. 4). However, it is yet to be known with certainty if the greater expression of miR156-5p and miR166e-3p in basal spikelets compared with the apical ones in Mahalaxmi is associated with panicle compactness of the cultivar (Fig. 4). Overall, the study indicated important regulatory role of miRNAs in the grain filling process in rice, and manipulation of their expression may be proved to be important biotechnological intervention in improving grain filling in compact-panicle rice cultivars.

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