miR1432-OsACOT (Acyl-CoA thioesterase) module determines grain yield via enhancing grain filling rate in rice

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Summary
Rice grain filling rate contributes largely to grain productivity and accumulation of nutrients. MicroRNAs (miRNAs) are key regulators of development and physiology in plants and become a novel key target for engineering grain size and crop yield. However, there is little studies, so far, showing the miRNA regulation of grain filling and rice yield, in consequence. Here, we show that suppressed expression of rice miR1432 (STTM1432) significantly improves grain weight by enhancing grain filling rate and leads to an increase in overall grain yield up to 17.14% in a field trial. Molecular analysis identified rice Acyl-CoA thioesterase (OsACOT), which is conserved with ACOT13 in other species, as a major target of miR1432 by cleavage. Moreover, overexpression of miR1432-resistant form of OsACOT (OXmACOT) resembled the STTM1432 plants, that is, a large margin of an increase in grain weight up to 46.69% through improving the grain filling rate. Further study indicated that OsACOT was involved in biosynthesis of medium-chain fatty acids. In addition, RNA-seq based transcriptomic analyses of transgenic plants with altered expression of miR1432 demonstrated that downstream genes of miR1432-regulated network are involved in fatty acid metabolism and phytohormones biosynthesis and also overlap with the enrichment analysis of co-expressed genes of OsACOT, which is consistent with the increased levels of auxin and abscisic acid in STTM1432 and OXmACOT plants. Overall, miR1432-OsACOT module plays an important role in grain filling in rice, illustrating its capacity for engineering yield improvement in crops.

Introduction
Rice (Oryza sativa L.) is a staple food for more than half of the world’s population and yield improvement is a major goal of breeders. Rice grain yield is a complex trait multiplicatively determined by its three component traits: number of panicles, number of grains per panicle and grain weight. Grain weight, as the most important factor determining yield, is largely determined by the filling rate and duration of the filling period of rice. More importantly, rate of grain filling contributes largely to grain productivity (Zhou et al., 2013) and mainly supplies the endosperm development and accumulation of nutrients. Genetics studies have identified many grain weight-related QTLs (Si et al., 2016; Zuo and Li, 2014), however, the knowledge about regulatory networks controlling grain weight and rice yield remains limited due to the involvement of various processes and regulatory factors which are regulated post-transcriptionally or post-translationally. Therefore, new gene resources that could control the complex traits are still urgently desired for rice cultivation.

MicroRNAs (miRNAs), a class of 19–24 nt abundant small non-coding RNAs, are important regulators in both plants and animals, regulating the expression of their target genes (Achkar et al., 2016; Bartel, 2004, 2009). In the past few years, studies have demonstrated the crucial roles of miRNAs in various aspects of plant growth and development including organ morphogenesis, hormone function, signal transduction and response to environmental stimuli. More importantly, there are also reports illustrating the involvement of miRNAs in regulating agronomic traits (Tang and Chu, 2017; Zhang et al., 2017a), especially seed development (Peng et al., 2014; Xue et al., 2009; Yi et al., 2013). Recently, a list of studies in rice reported that altering the expression of a single miRNA could significantly change crop yield, including grain shape and size by regulating grain-related characteristics. Specifically, miR156 could define panicle branching by down-regulating its target OsSPL13, OsSPL14 and OsSPL16 to consequently improve grain productivity (Jiao et al., 2010; Miura et al., 2010). Blocking of miR396 resulted in different inflorescence architecture and hence increased grain size and rice yield (Gao et al., 2010; Hu et al., 2015; Li et al., 2016). Overexpressing miR397 increased the overall grain yield up to 25% by enlarging grain size and promoting panicle branching (Zhang et al., 2013). OsmR14B8 regulates OsCYP51C expression and mediates BR biosynthesis to modulate leaf angle, grain size and seed quality (Xia et al., 2015). Most recently, it has been reported that miR408 could regulate grain yield and photosynthesis via a phytocyanin protein, OsUCL8 (Zhang et al., 2017b). Manipulation of miR398 can increase panicle length, grain number and grain size (Zhang et al., 2017a) and suppressing...
expression of miR159 affects multiple traits of rice including grain shape by up-regulating its targets OsGAMYB and OsGAMYBL1 (Zhao et al., 2017). Overexpression of miR164b-resistant target, OsNAC2, could make better plant architecture, longer panicles, more grain number and yield (Jiang et al., 2018). However, so far there is little information showing the effect of miRNA on rice yield through affecting grain filling by negatively regulating its target. Therefore, identification of new regulatory networks and studies of the relevant mechanisms contributed by miRNAs on grain filling will surely help to understand the complex control of grain weight, thereby improving the grain yield and quality. Our previous studies have identified the differentially expressed miRNAs between rice superior and inferior grains during filling (Peng et al., 2011, 2014), which provides informative clue to study the role of miRNAs in filling rate regulation. Among the differentially expressed miRNAs, miR1432 shows higher expression in inferior grains than that in superior grains during seed development. Moreover, the expression levels of miR1432 during grain filling increases gradually and show a relative opposite correlation with grain filling rate, suggesting a potential role of miR1432 in grain size regulation and grain filling process. Thus, miR1432 was selected for detailed analysis to explore its possible function in seed development. In present study, we focus on studying the role of miR1432 in rice grain filling and seed size determination, and prediction and validation of its downstream target. Preliminary analysis of the target’s function and application of miR1432-target module in rice yield improvement suggest that rice miR1432 is a promising candidate gene in breeding for crop improvement.

Results

miR1432 is highly expressed in developing rice seeds

In order to investigate the spatial expression patterns of miR1432 during rice development, qRT-PCR and β-glucuronidase (GUS) activity assays were used to analyse miR1432 expression in various organs of wild type (WT) plants. The expression level of miR1432 varied among different tissues and developmental stages (Figure 1). It was found that miR1432 is preferentially highly expressed in root (Figures 1a,c), seedling (Figure 1a), stem (Figures 1a,e), and developing seeds (Figures 1b,g–j), whereas it is lowly expressed in other tissues including panicle (Figure 1a) and spikelets (Figures 1a,f). Specifically, the transcriptional level of miR1432 increased along with the seed development, which was negatively correlated with the grain filling rate (Figures 1b, g–i), indicating that miR1432 may have roles in controlling rice seed development.

miR1432 negatively regulates grain size in rice

To evaluate the effect of miR1432 on rice grain development, its expression in Nipponbare endosperm was either increased (OXmiR1432) or suppressed by STTM (STTM1432), driven by an endosperm-specific promoter Gt13a respectively. To detect whether expressions of miR1432 changed or not, northern blot and qRT-PCR were used to analyse its expression level in 10 DAF endosperm. As expected, expressions of miR1432 increased in OXmiR1432 transgenic plants (Figures 2a,c), whereas expression of miR1432 was suppressed effectively in STTM1432 transgenic lines compared with that of WT (Figures 2b,c).

Figure 1  Expression pattern analysis of rice miR1432 showed a negative correlation with grain filling rate. (a) Expression analysis of rice miR1432 in vegetative and reproductive organs till 5 days after fertilization (DAF) by stem-loop qPCR; (b) Expression of rice miR1432 and grain filling rate in Nipponbare plants during grain development. Experiments were repeated in three independent biological samples and error bars indicate standard deviations of three biological replicates. (c–i) Spatial expression of miR1432 by GUS staining. (c) seedling; (d) leaf; (e) stem; (f) spikelet; (g–i) developing grains at 5 DAF; (g) 15 DAF (h) and 30 DAF (i) respectively; (j) magnified view of the boxed area in (i) (30 DAF grain); analysis was repeated in three independent biological samples and representative images were shown.

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Phenotypic observation of the positive transgenic lines in 3 years of field experiments showed that altered miR1432 expression results in the changed grain size, i.e. bigger or smaller seeds under suppressed or enhanced expressions of miR1432 respectively (Figures 2e–f, S1a,b, S2). Detailed measurement of grain-related traits revealed the increased 1,000-hulled grain weight 18.88%–20.28% ($P < 0.01$) (Figures 2d, S1c), grain length 8.03%–10.54% ($P < 0.01$) (Figures 1g, S1e), width 4.76%–6.72% ($P < 0.01$) (Figures 2h, S1f), and thickness 4.76%–6.72% ($P < 0.01$) (Figures 2i, S1g) of STTM1432 seeds without any notable difference in aspects of main panicle features, such as length of main panicle, numbers of primary and secondary branches, effective grain numbers and seed setting rate per panicle (Tables 1 and S1, Figure S2a). On the contrary, increased expression of miR1432 (OXmiR1432) results in the seeds with decreased length, width and thickness, and hence reduced 1,000-hulled grain weight (Figures 2d–f, S1a–c, S2). In brief, grain length, grain width and grain thickness significantly reduced 8.03%–10.54% ($P < 0.01$) (Figures 2g, S1e), 6.10%–6.35% ($P < 0.01$) (Figure 2h, S1f), and 9.48%–10.34% ($P < 0.01$) (Figures 2i, S1g) in OXmiR1432 transgenic plants when compared with wild-type plants respectively. Consequently, 1,000-hulled grain weight decreased 23.58%–25.47% ($P < 0.01$) (Figures 2d, S1c), indicating a negative effect of miR1432 in seed development and yield. In addition, the actual yield of the transgenic plants under the altered expressions of miR1432 was measured using field experiments. Overall, the yield in STTM1432 plants increased by 13.20%–17.14% ($P < 0.01$), while in OXmiR1432 plants decreased by 17.94%–29.12% ($P < 0.01$) (Tables 1 and S1).

miR1432 controls rice grain size through altering grain filling rate

Considering the concomitant increase in the expression of miR1432 with seed maturation and importance of grain filling in contributing to seed size and yield, the seed filling rate in plants

Figure 2  Rice miR1432 negatively regulates grain filling (in the year 2017-Zhengzhou). Northern blot analysis of enhanced (a) or suppressed (b) expressions of rice miR1432 in OXmiR1432 or STTM1432 transgenic plants respectively; (c) Validation of increased or decreased expressions of rice miR1432 in OXmiR1432 or STTM1432 transgenic plants respectively, by stem-loop qRT-PCR; (d) Measurement of the 1,000-hulled grain weight of Nipponbare (WT), STTM1432, and OXmiR1432 transgenic plants; (e-f) Phenotypic observation of grain size of Nipponbare (WT), STTM1432 and OXmiR1432 transgenic plants. Scale bars, 5 mm; (g-i) Detailed analysis of grain traits including grain length (g), width (h), and thickness (i) of Nipponbare (WT) and miR1432 transgenic plants; (j) Measurements of grain filling rate in Nipponbare (WT), STTM1432 and OXmiR1432 transgenic plants; experiments were repeated three times and data are presented as mean ± SD ($n = 1000$ grains); statistical analysis was performed by Student’s t-test ($**P < 0.01$; *$P < 0.05$). (k) Morphologies of seeds of Nipponbare (WT), STTM1432 and OXmiR1432 transgenic plants at different stage of grain filling. Scale bar, 1 mm.
with altered miR1432 expression was firstly examined. Results showed that compared with WT, the grain filling rate of STTM1432 was significantly increased, while that of OXmiR1432 showed significant decrease along with seed development (Figures 2)-k, S1d). This is consistent with the altered miR1432 expression in endosperm and increased or decreased grain weight of STTM1432 or OXmiR1432 seeds respectively, which suggests the possible role of miR1432 in filling regulation. To further detect the filling characters, detailed parameters related to filling, such as initial filling potential, maximum and mean filling rate, day reaching maximum filling rate, days of active grain filling were investigated and fitted according to Richards equation (Benjamini and Hochberg, 1995). Among these parameters, maximum filling rate increased or decreased 0.25 mg/(grain day) (<P < 0.01) or 0.56 mg/(grain-day) (<P < 0.01) and mean filling rate increased or decreased 0.14 mg/(grain-day) (<P < 0.01) or 0.32 mg/(grain-day) (<P < 0.01) in STTM1432 and OXmiR1432 transgenic plants respectively (Tables 2 and S2). These results confirmed miR1432 held an important role in rice grain filling regulation and repression of miR1432 can increase grain weight by enhancing the grain filling rate.

**OsACOT is targeted by miR1432 through cleavage**

It is well known that plant miRNAs have near-perfect pairing with their targets and function through cleaving them directly, or in some cases, by their translational repression. Therefore, identifying miRNAs target genes is important to understand their specific contributions. To identify the molecular mechanism of miR1432 regulating grain size, potential targets of miR1432 were first computationally predicted using psRNATarget (http://plantgr. noble.org/psRNATarget/), a miRNA target prediction online tool (Dai et al., 2018). In total, 16 potential targets of miR1432 were predicted with relatively strict parameters, and all of them belong to different protein families (Table S4). In general, the expression of miRNAs shows a negatively correlation with their real targets. RNA-seq results showed only LOC_Os04g35590 to be highly expressed in STTM1432 and lowly expressed in OXmiR1432 transgenic plants (Table S5). Therefore, it was speculated as the most prospective target of miR1432.

To further validate whether LOC_Os04g35590 is the real target of miR1432, agroinfiltration of *Nicotiana benthamiana* leaves, as a transient expression method, was used to validate the interaction between miR1432 and its putative targets in vitro. As expected, transcript expression of LOC_Os04g35590 decreased sharply when fused with the precursor of miR1432 (Figure 3a). To further validate the target of miR1432, we mapped the miR1 432-directed cleavage sites in *OsACOT* using RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE). The results showed that cleavage occurred between the 16th and 17th base pair of the miR1432 target site (Figure 3b), indicating that OsACOT can be precisely cleaved in vivo by miR1432. Furthermore, we investigated the spatial expression pattern of OsACOT in various rice tissues using qRT-PCR analysis (Figure 3c). It revealed that OsACOT was mainly expressed in roots and

### Table 1 Grain yield and associated components of transgenic plants with altered expressions of miR1432 and its target in field trials

| Characters                  | WT     | L21    | L24    | L26    | L32    | L1     | L3     |
|-----------------------------|--------|--------|--------|--------|--------|--------|--------|
| Tiller numbers              | 17.67 ± 0.50 | 16.17 ± 0.57 | 16.20 ± 0.47 | 17.20 ± 0.52 | 17.27 ± 0.47 | 16.93 ± 0.57 | 16.67 ± 0.47 |
| Spikelet number             | 128.15 ± 2.02  | 126.53 ± 1.58  | 130.5 ± 2.47  | 127.93 ± 2.02  | 120.13 ± 2.14  | 135.95 ± 1.39  | 132.32 ± 1.62  |
| Seed setting rate (%)       | 92.35 ± 0.39  | 91.64 ± 0.56  | 92.22 ± 1.12  | 90.84 ± 0.46  | 93.26 ± 0.39  | 91.02 ± 0.38  | 91.73 ± 0.30  |
| Length of panicle (cm)      | 21.58 ± 0.33  | 20.03 ± 0.15  | 20.57 ± 0.16  | 22.44 ± 0.41  | 20.71 ± 0.21  | 21.22 ± 0.15  | 22.09 ± 0.20  |
| Primary branch numbers      | 12.03 ± 0.11  | 12.17 ± 0.14  | 12.82 ± 0.20  | 12.60 ± 0.16  | 12.05 ± 0.13  | 11.83 ± 0.14  | 12.42 ± 0.19  |
| Secondary branch numbers    | 20.23 ± 0.59  | 20.87 ± 0.49  | 21.87 ± 0.63  | 19.05 ± 0.55  | 19.05 ± 0.63  | 19.42 ± 0.63  | 20.77 ± 0.81  |
| 1,000-hulled grain weight (g) | 21.20 ± 0.26 | 15.80 ± 0.26  | 16.20 ± 0.39  | 25.50 ± 0.36  | 25.20 ± 0.33  | 29.45 ± 0.28  | 31.10 ± 0.33  |
| Grain yield per plant (g)   | 44.30 ± 3.20  | 32.50 ± 3.30  | 35.70 ± 4.10  | 52.10 ± 4.70  | 50.90 ± 4.50  | 57.70 ± 5.40  | 58.90 ± 2.30  |
| Yield increase (%)          | –       | –26.64    | –19.41    | 17.61    | 14.90    | 30.20    | 32.90    |
| Grain yield (kg per plot)   | 1.55 ± 0.01  | 1.10 ± 0.11** | 1.27 ± 0.06** | 1.81 ± 0.13** | 1.75 ± 0.07** | –        | –        |
| Yield increase (%)          | –       | –29.12    | –17.94    | 17.14    | 13.20    | –        | –        |

Values shown are the mean ± SD (n = 60 panicles, n = 10 plants, n = 3 plots). Significant differences were identified using Student’s t-test. **P < 0.01.

### Table 2 The grain filling parameters for wild-type and different transgenic plants of miR1432 and OXmACOT

| Parameters      | WT     | L21    | L24    | L26    | L32    | L1     | L3     |
|-----------------|--------|--------|--------|--------|--------|--------|--------|
| GR0             | 3.48 ± 0.34 | 2.09 ± 0.31 | 2.65 ± 0.30 | 4.69 ± 0.53 | 3.93 ± 0.17 | 2.46 ± 0.56 | 1.89 ± 0.34 |
| Vamax (mg/grain-day) | 1.40 ± 0.01 | 1.09 ± 0.05** | 0.84 ± 0.04** | 1.65 ± 0.02** | 1.55 ± 0.05** | 1.72 ± 0.02** | 1.73 ± 0.03** |
| Va (mg/grain-day) | 0.91 ± 0.01 | 0.76 ± 0.03** | 0.59 ± 0.03** | 1.02 ± 0.02** | 1.05 ± 0.03** | 1.17 ± 0.01** | 1.18 ± 0.04** |
| tmax (day)      | 10.54 ± 0.41 | 11.21 ± 0.19 | 12.03 ± 0.32 | 9.46 ± 0.56 | 9.88 ± 0.54 | 10.37 ± 0.38 | 10.39 ± 0.36 |
| Active period (day) | 22.23 ± 0.33 | 21.94 ± 0.35 | 21.57 ± 0.49 | 22.63 ± 0.61 | 22.20 ± 0.21 | 24.13 ± 0.34 | 24.00 ± 0.67 |

GR0: Initial filling potential; Vam: Maximum filling rate; Va: Mean filling rate; tmax: Date when reaches maximum filling rate; Active period: Days of active grain filling. Values shown are the mean ± SD (n = 60). Significant differences were identified using Student’s t-test. **P < 0.01.
developing seeds, which was similar to the spatial expression pattern of miR1432. The expression levels of OsACOT were negatively correlated with those of miR1432 in STTM1432 or OXmiR1432 transgenic lines (Figure 3d). All these results indicate that OsACOT is an authentic target gene of miR1432 and its expression is negatively regulated by miR1432-directed cleavage.

Overexpression of miR1432-resistant OsACOT phenocopies STTM1432 plants

To further check whether miR1432-mediated negative regulation of OsACOT affects grain size and filling rate, we hypothesized that higher expression of OsACOT in the endosperm of rice would lead to an increase in grain productivity. Therefore, we generated transgenic plants (OXmACOT) expressing an miR1432-cleavage resistant OsACOT gene (mACOT) that contained seven mismatches to the site targeted by miR1432 without any amino acid changes with Gt13a endosperm-specific expression promoter (Figure 3b). qRT-PCR analysis of OsACOT mRNA expression in these transgenic lines indicated significantly increased level of OsACOT mRNA in the OXmACOT transgenic lines (Figure 4a). To further explore the relationship between the dosage of the OsACOT mRNA and the grain weight, expression of OsACOT in OXmACOT plants with varying grain weights was examined. As expected, a significant positive correlation was observed between the OsACOT expression level and the 1000-hulled grain weight (Figure S5a–c). Moreover, consistent with that of STTM1432, grain size of OXmACOT was significantly increased compared to WT in the two sites’ field experiments (Figures 4b,c, S3a,b, S4) and the grain length, grain width, and grain thickness increased 15.87%–19.24% (P < 0.01), 6.89%–7.30% (P < 0.01) and 9.55%–12.18% (P < 0.01) respectively. Overall, 1,000-hulled grain weight significantly increased 38.92%–46.70% (P < 0.01) (Figures 4d–g, S3c, e–g). Further detailed analysis of grain filling parameters indicated both mean and maximum filling rate of OXmACOT transgenic plants to be significantly higher than those of WT (Figures 4h–i, S3d, Tables 2 and S3). Specifically, maximum filling rate and mean filling rate increased 0.33 mg/(grain/day) (P < 0.01) and 0.27 mg/(grain/day) (P < 0.01) respectively. All these evidences further support the hypothesis that STTM1432 increased grain filling through down-regulation of miR1432, which resulted into decreased miR1432-guided cleavage of the OsACOT mRNA. In addition, we tried to generate the

![Figure 3](image-url) Rice miR1432 negatively regulates the expression of OsACOT. (a) OsACOT was shown to be cleaved by miR1432 using Nicotiana benthamiana-based in vitro analysis; (b) Rice miR1432 cleavage site in OsACOT mRNA was confirmed by RNA ligase-mediated 5'-RACE; (c) Expression pattern of OsACOT at vegetative and reproductive stages was analysed by quantitative real-time PCR (qPCR); (d) Suppressed or enhanced expressions of OsACOT in rice endosperms at 10 DAF (days after fertilization) of Nipponbare (WT), STTM1432 and OXmiR1432 transgenic plants; experiments were repeated three times and data are presented as mean ± SD (n = 3); statistical analysis was performed by Student’s t-test (**P < 0.01).
mutant of OsACOT by means of CRISPR/Cas9 technology. However, the resistant calli could not differentiate into seedlings in two independent transformations. Lethality under OsACOT deficiency also indicates the role of OsACOT in regulating key plant functions.

**OsACOT regulates fatty acid metabolism**

To further clarify the function of OsACOT in rice seed development, we developed a coexpression network of OsACOT from rice endosperm development chip data. Gene-clustering analysis indicated that co-expressed genes of OsACOT are notably enriched in the pathways related to lipid biosynthesis and endomembrane system organization, hormone signalling and so on (Figures 5a, S6), suggesting a possible role of OsACOT in regulating these pathways. In addition, the OsACOT co-expressed genes were up-regulated in STTM1432/OXmACOT transgenic plants but down-regulated in OXmiR1432 plants (Figure S7). Therefore, fatty acids in brown rice of wild type and OXmACOT transgenic plants were identified by GC-MS. In total, eleven fatty acids and their distribution was detected (Figure 5b). Significant differences were observed for 16:0 (palmitic acid), 18:1 (oleic acid) and 18:2 (linoleic acid) fatty acid contents between OXmACOT transgenic plants and wild type at \( P < 0.05 \) level. Interestingly, contents of 16:0 and 18:0 fatty acids were lower and those of 18:1 and 18:2 fatty acids were higher in OXmACOT transgenic plants compared to wild-type grains. All these results strongly suggested that OsACOT might be a key enzyme in fatty acid desaturation and elongation pathway, especially from fatty acid 16:0 to 18:2.

**Changed ABA and IAA levels in STTMmiR1432 and OXmACOT transgenic plants**

To further explore the role of miR1432 in rice seed development, differentially expressed genes (DEGs) were studied between STTM1432/OXmiR1432 and wild type developing grains by high throughput RNA-sequencing. The genes which were differentially expressed at least 1.5-fold were identified and subjected to further analysis. Enrichment analysis showed that DEGs are mainly involved in endomembrane organization, fatty acid metabolism, lipid biosynthesis, sugar and starch metabolism. Most importantly, biological processes involved in hormone signaling such as abscisic acid (ABA) and auxin (IAA)
were also clustered (Figure S8a). The results were further confirmed by qRT-PCR in miR1432 and OXmACOT transgenic plants (Figure S8b,c).

Interestingly, consistent with the idea that IAA and ABA are the positive regulators in grain filling and yield (Tan et al., 2009; Yang et al., 2006), there were nine DEGs, such as OsTAA1 and OsABA4 involved in IAA and ABA biosynthesis, and several DEGs, including OsARF14, OsPYL/RCAR4 and series of DEGs related to IAA and ABA response among DEGs of STTM1432 transgenic plants and OXmiR1432 transgenic plants (Figure 6a). In addition, expressions of auxin efflux carriers, such as OsPIN2, OsPIN9 and starch synthesis genes OsBT1-2, OsSSIIc were found among the DEGs as well (Figure S8a) which were further confirmed by qRT-PCR (Figure S8b,c). Furthermore, genes related to ABA synthesis and signaling were up-regulated in STTM1432 and OXmACOT plants (Figure 6f,g). Specifically, expressions of genes related to IAA and ABA synthesis and signalling increased in STTM1432 and OXmACOT transgenic plants and decreased in OXmiR1432 transgenic plants. Therefore, we speculated that rice miR1432-OsACOT module increases grain filling through activating the biosynthesis and responsiveness of IAA and ABA. In fact, endogenous IAA and ABA in endosperm at 10 DAF of WT and different transgenic plants were measured. Compared with WT, the contents of IAA and ABA were higher in the STTM1432 and OXmACOT transgenic plants, and lower in OXmiR1432 plants (Figure 6b–e). These results strongly suggested that miR1432-OsACOT module regulates grain filling through modulating IAA and ABA biosynthesis and signalling.

Discussion

Characterization of the roles that miRNAs play in the grain size and yield is an active field of research. Previous study reported that miR1432 was one of the miRNAs that was differentially expressed during grain filling between superior and inferior grains (Peng et al., 2014). In present study, we demonstrated a novel approach to enhance grain filling rate by manipulating miRNA expression. We found that altered expression of miR1432 in rice endosperm can significantly change grain filling, which in turn affect grain size and yield. Notably, a newly identified gene, OsACOT, which plays an important role in biosynthesis of fatty acid, is validated as the direct target gene of miR1432. Increased expression of miR1432-resistant version of OsACOT (OXmACOT) results in the increased grain size and filling rate compared to those of WT plants. More importantly, field performance indicates that STTM1432 and OXmACOT transgenic plants can significantly increase plot yield by 17.14% and per plant yield by 32.90% respectively. These results suggest that miR1432-OsACOT module play important roles in regulating rice yield by enhancing grain filling in rice.
miR1432 regulates grain filling by promoting transport ability of endomembrane system through OsACOT

In present study, we found that an over-expression of OsACOT, the direct target of miR1432, generated faster grain filling rate and enlarged grains. This finding suggested a positive role of OsACOT in grain size regulation. However, there are no published reports on the relationship of function between OsACOT and grain filling in rice. Furthermore, we searched for the orthologous genes of OsACOT among different species including model plant Arabidopsis (Figure S9). The orthologous genes in other species and KEGG (Kyoto Encyclopedia of Gene and Genomes) definition indicated that OsACOT might be the 13th member of acyl-CoA thioesterase super family and is responsible for fatty acid and lipid biosynthesis (Figure S9). The altered compositions of fatty acids, especially C:16 to C:18, in brown rice of OXmACOT transgenic plants (Figure 5b), suggested the role of OsACOT in controlling lipid and fatty acid metabolism. Together with the idea that ACOT1/2/4 and ACOT7 in acyl-CoA thioesterase super family play a certain role in long chain fatty acid elongation (Ellis et al., 2013), it is not difficult to speculate that OsACOT probably took part in the lipid metabolism of fatty acids, C:16 to C:18.

Lipid biosynthesis and metabolism is essential for endomembrane system organization. Numerous studies have demonstrated that plant endomembrane system is of vital importance for intracellular protein processing, lipid modification and transport, crucial for plant development and signal transduction (Boutte and Moreau, 2014; Debono et al., 2009; Ding et al., 2014; Morita and Shimada, 2014; Surpin and Raikhel, 2004). Especially, endomembrane system such as Golgi in developing endosperm cells is responsible for transportation of nutrients including storage protein to protein storage vacuole (Liu et al., 2013). Moreover, the defects of endomembrane would bring out abnormal starch structure and decreased grain filling (Wang et al., 2010). It is also reported that auxin transporters of the PIN-FORMED (PIN) family localize asymmetrically at the plasma membrane (PM) and mediate directional intercellular auxin transport (Tanaka et al., 2006; Vanneste and Friml, 2009). It has been reported that PM-located PIN proteins play important role in regulating cytosolic auxin concentration (Scherer, 2011).

Figure 6 Rice miR1432-OsACOT module is involved in IAA and ABA homeostasis. (a) Differentially expressed genes involved in IAA and ABA biosynthesis (I), IAA and ABA signal transduction (II), and their responsiveness (III), in Nipponbare (WT), STTM1432 and OXmiR1432 transgenic plants, identified by high throughput RNA-sequencing analysis; (b-c) Contents of IAA (b) and ABA (c) in Nipponbare (WT), STTM1432 and OXmiR1432 transgenic plants assayed by ESI-HPLC-MS/MS; (d-e) Contents of IAA (d) and ABA (e) in Nipponbare (WT) and OXmACOT plants measured by ESI-HPLC-MS/MS; (f-g) qRT-PCR expression analysis of genes involved in biosynthesis and signaling of IAA and ABA in STTM1432 plants and OXmiR1432 plants (f), and were validated in OXmACOT transgenic lines by qRT-PCR (g); Rice endosperms at 10 DAF (days after fertilization) were used for analysis; experiments were repeated three times and data are shown as means ± SD; statistical analysis was performed by Student’s t-test (**P < 0.01; *P < 0.05).
Combined with the results of co-expressed genes of OsACOT and their overlap with the highly expressed genes involved in endomembrane system organization, auxin transport and starch synthesis in OXmACOT transgenic plants, it is very likely to accept that increased expression of OsACOT favours nutrients and auxin transport through the organization of endomembrane system, which in turn improves grain filling to enlarge grain size.

miR1432-OsACOT module mediates lipid and auxin signal transduction

Lipids are essential structural constituents of membranes and some also have important cell signaling roles, such as those involved in ascorbic acid, salicylic acid and auxin pathways (Janda et al., 2013). OsACOT, which encodes a thioesterase protein, is a member of Acyl-CoA thioesterase family and can hydrolyze Acyl-CoA into free fatty acids and CoA to regulate lipid metabolism, signal transduction events and gene transcription (Ying et al., 2012). At the same time, it has been reported that free fatty acid could make membranes more fluid to exert effects on membrane vesicle transport-related function, which are the basis for auxin transport (Janda et al., 2013). Therefore, free fatty acid could be regarded as potential second messengers in auxin action (Holk and Scherer, 2001). It is believed that, maybe like the function of OsCOLE1 (CONTINUOUS VASCULAR RING-LIKE 1), one of the co-expressed gene of OsACOT, OsACOT might promote the transport of auxin to enhance the grain filling rate and grain size in OXmACOT transgenic plants (Liu et al., 2016). In addition, RNA-seq data showed the expression level of OsCOLE1 to be up-regulated in STTM1432 plants and down-regulated in OXmiR1432 plants. Very-long-chain fatty acid are required for auxin transport and tissue patterning during seed development (Baud et al., 2003; Roudier et al., 2010). Together with the co-expressed genes of OsACOT during endosperm development and the role of OsACOT in fatty acid composition, we speculate that miR1432-OsACOT module may regulate the grain filling rate by mediating auxin transport. Therefore, medium chain fatty acid in regulating seed development may also function as the very-long-chain fatty acid.

Higher contents of IAA and ABA may contribute to miR1432-OsACOT module mediated rice grain filling improvement

Grain filling is a complex process and regulated by various factors. Plant hormones are crucial regulators of growth and development, especially the processes of cell proliferation and differentiation. Studies have shown that ABA and IAA contributed to rice grain filling through regulating the activities of key enzymes involved in sucrose-to-starch conversion in cereal sink organs. Poor grain filling of inferior grains was resulted from low level of ABA and IAA (Zhang et al., 2009). ABA influences sugar metabolism and transport through regulation of the expression of some genes encoding components of the sugar response pathway (Gibson, 2004; Rook et al., 2006; Zhu et al., 2011). Importantly, it is possible that modulating auxin transport could improve grain yield (Kant et al., 2009; Liu et al., 2015; Qi et al., 2008; Takai et al., 2013). In present study, we found that expression of genes involved in both ABA signalling, and biosynthesis and auxin and starch metabolism got strengthened in STTM1432 and OXmACOT transgenic plants. The best-characterized proteins controlling auxin efflux genes, PIN-FORMED (PINs), were expressed differentially between wild type and miR1432 and its target OsACOT transgenic plants, which were consistent with the higher contents of ABA and IAA, suggesting that miR1432 may regulate grain filling through modulating ABA signalling and auxin transport to enhance sucrose to starch metabolism under the altered expression of OsACOT.

In summary, miR1432 was found to be a new regulator of grain size determination via controlling rice grain filling through negatively regulating its target, OsACOT. Therefore, manipulating the expression of miR1432 or OsACOT might be exploited in high-yield rice breeding. Further investigation is necessary to establish the molecular mechanism of miR1432 and distinct function of OsACOT in grain filling.

Experimental procedures

Rice cultivation, sampling and dry weights measurement

All experiments were performed using rice (Oryza sativa spp. japonica) cultivar Nipponbare. Wild type and transgenic lines were transplanted in the field under non-stressed conditions at a research farm of Henan Agricultural University (Henan Province), Songjiang (Shanghai), and Sanya (Hainan Province), during the rice-growing season. Hulled grain of wild type and transgenic lines were separated from the panicle at 5 DAF (days after fertilization), 10 DAF, 15 DAF, 21 DAF, 27 DAF and 35 DAF. To determine the dry weight, the samples were fast dried at 105 °C for 30 min, and then were kept at 80 °C until constant weight was reached, which indicates a complete dryness of grains.

Grain trait measurement and grain-filling rate determination

Phenotypic data were collected at the maturing stage. The hulled grain length, width was measured by an automatic seed-size-analysing system (SC-G, Wanshen, Hangzhou, China) and grain thickness was measured by an electronic digital caliper (SF2000, Guanglu, Guilin, China). Fully filled grains were used for measuring 1,000-hulled grain weight. The grain filling rates were determined by the followed equations:

\[
W = A/(1 + Be^{-kt})^N
\]

(1)

The grain filling rate (G) was calculated by the derivative of Equation 1:

\[
G = KW/N[1 - (W/A)^N]
\]

(2)

where \(W\) is the average weight of per grain (mg), \(t\) is the number of days after fertilization, \(k\), \(A\), \(B\), \(N\) are the coefficients determined from regression.

Plasmid construction and rice transformation

To overexpress miR1432, sequence of pre-miR1432 from the NCBI (http://www.ncbi.nlm.nih.gov/) with restriction enzyme cutting site KpnI and BamHI was cloned from DNA of Nipponbare and was inserted downstream of the Gt13a promoter (He et al., 2011) in pCAMBIA1301. The Short Tandem Target Mimic (STTM) (Tang et al., 2012; Yan et al., 2012) was used to suppress the expression of Osmir1432. The STTM fragment with restriction sites KpnI and BamHI (GGTACCGGTGGTGCTTTCACTTCTCCT GATTTGTGTTGTATGCTTTAATTATATAGTGCTGCTAAAGAAG AAGAATGTCGTTGTCATCAACTCCGTCGTATGGAATTC) was synthesized by Sangon Biotech (Shanghai, China) and inserted downstream of the Gt13a promoter in pCAMBIA1301. The
miR1432 suppresses rice grain filling by OsACOT

default parameters. The sequence of miR1432 was used as a custom sequence. The O. sativa TIGR genome cDNA OSA1 Release 5 (OSA1R5) was used as the genomic library for the search target.

Verification of interaction between miR1432 and its target

Agroinfiltration of Nicotiana benthamiana leaves as a transient expression method (English, 1997) was used to validate the interaction between miRNA and its putative targets in vivo. In brief, predictive targets and precursor of miR1432 were constructed in the same vector respectively. And non-sequence was used as control. Then A. tumefaciens, with the 35S promoter driving the expression of precursor of miR1432 and its target, was infiltrated into the leaves of Nicotiana benthamiana. If the interaction between miR1432 and its putative targets existed, the transcripts level of target would decrease when compared with putative targets fused with empty vector.

5’ RNA ligase-mediated rapid amplification (RACE)

Total RNA of wild-type endosperm at 10 DAF was directly ligated to an RNA adaptor (CCTCTAGAGATTCGCGGATCCACAGCCTACTGATGATCAGTCGATG), which has a 5’-hydroxyl group at both ends and, thus, can only ligate to 5’-phosphorylated RNA, including the truncated products of miRNA-guided mRNA cleavage. The ligated product was directly reverse-transcribed with an oligo (dt) primer. The cDNA was amplified by nested PCR, and the 3’AF was directly ligated to an RNA adaptor (CCTCA GAGATTCCCGGATCCACAGCCTACTGATGATCAGTCGATG G), which has a 5’-hydroxyl group at both OsACOT (LOC_Os04g35590) are shown in Table S6.

Analysis of Gene Ontology of different expressed genes

Endosperms of wild-type, STTM1432 and OXmiR1432 at 10 DAF were collected for total RNA extraction. Library construction was conducted with a NEB Next Multiplex Library Prep Set for Illumina (NEB) and then sequenced on an Illumina HiSeq2000 Platform. Number of fragments per kilobase exon per million fragments (FPKM) was calculated before gene expression analysis. Genes which were down-regulated in OXmiR1432 seeds and up-regulated in STTM1432 seeds, compared to WT, by at least 1.5-fold were subjected to further GO enrichment analysis. BIINGO was used to analyse item classification of differentially expressed genes (Maere et al., 2005). P-value of each GO item classification was calculated by hypergeometric test and was further checked by means of Benjamin & Hochberg. GO item classification involved at least five genes and checked P-value, < 0.05, was considered enriched, significantly.

Measurements of fatty acid

Identification and quantification of fatty acid in brown rice was conducted by GC-MS by assaying fatty acid methyl esters (FAMES). Mature grain of wild type and OXmACOT transgenic plants were collected and ground into powder. 50 mg of each sample was weighted to determine the fatty acid content. Thirty-seven kinds of FAMES were prepared as previously described with a few modifications. First, 1 mL chloroform was added into the power and ultrasonounded for 30 min, the methyl esterified with 1% sulphuric acid-methanol for half an hour. 25 μL internal quantitative standard with 1 mL N-hexane was added in every sample. Subsequently, the shaken sample
was prepared for GC-MS analysis. The Agilent 7890A/5975c 
CG/MS system (Agilent, Santa Clara, CA) was used in the 
GC-MS analysis. The concentrations of individual fatty acid 
were expressed as percentages of the sum of all identified fatty 
acid contents. Three biology repeats of grain samples were 
analysed.

Phytohormone measurements

Contents of auxin (IAA) and abscisic acid (ABA) were quantified 
by high-performance liquid chromatography-tandem mass spec-
trometry (ESI-HPLC-MS/MS) as previously reported (Xu et al., 
2016). Briefly, for accuracy quantitative results, 0.3 g sample 
from endosperms was collected and grounded into powder using 
liquid nitrogen. Then 3 mL isopropanol/hydrochloric acid extraction 
buffer was added into the powder, followed by shaking at 
4 °C for 30 min. After that 5 mL dichloromethane was added 
and the samples were shaken at 4 °C for 30 min. IAA and ABA 
were dissolved in the lower organic phase after centrifuging at 
4 °C for 5 min. Organic phase was dried by nitrogen protected 
from light and then dissolved by 400 μl methanol with 0.1% 
fomric acid. HPLC-MS/MS analysis was conducted after filtering 
with 0.2 μm filter membrane. HPLC-MS/MS system consists of an 
Agilent1290 HPLC system (Agilent), a SCIEX-6500Qtrap (MSMS, 
AB) with an ESI source.

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Conflict of interest

The authors declare no conflict of interests.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Study of transgenic rice with altered expression of miR1432 (in the year 2016-Shanghai).

Figure S2 Morphologies of Nipponbare (WT), STTM1432 and OXmACOT transgenic plants.

Figure S3 Study of transgenic plant expressing OsmACOT (in the year 2017-Hainan).

Figure S4 Morphologies of Nipponbare (WT) and OXmACOT transgenic plants.

Figure S5 Correlation analysis of expression level of OsACOT and grain weight.

Figure S6 Enrichment analysis of OsACOT co-expressed genes.

Figure S7 Validation of OsACOT co-expressed genes.

Figure S8 GO enrichment analysis of differentially expressed genes (DEGs) in endosperm of Nipponbare (WT) and miR1432 transgenic plants.

Figure S9 Orthologs of OsACOT in different species.

Table S1 Grain yield and associated components of main panicle in miR1432 transgenic plants in field trials in the year 2015 (Zhengzhou) and 2016 (Shanghai).

Table S2 Grain filling parameters for wild-type and different transgenic plants of miR1432 in year the 2015 (Zhengzhou) and 2016 (Shanghai).

Table S3 Grain filling parameters for wild-type and different transgenic of OsACOT in the year 2017 (Hainan).

Table S4 Potential targets of rice miR1432 predicted by psRNATarget.

Table S5 Expression analysis of potential targets of rice miR1432 in STTM1432 and OXmACOT transgenic plants by RNA-seq.

Table S6 Primers used in the study.