Journal of Cereal Science 101 (2021) 103317

Contents lists available at ScienceDirect

Journal of Cereal Science

journal homepage: www.elsevier.com/locate/jcs

GUN4-mediated tetrapyrrole metabolites regulates starch biosynthesis during early seed development in rice

Ruiqing Li [a,b,1], Meng Jiang [a,1], Wenying Zheng [a], Huali Zhang [b,*]

[a] College of Agronomy, Anhui Agricultural University, Hefei, 230036, PR China
[b] State Key Laboratory of Rice Biology and Chinese National Center for Rice Improvement, China National Rice Research Institute, Hangzhou, 310029, PR China

ARTICLE INFO

Keywords:
OsGUN4
Starch biosynthesis
Rice
Heme
Grain development

ABSTRACT

Seed formation requires the supply of sucrose derived from leaves and the functioning of starch biosynthetic enzymes during seed development, of which are related to the tetrapyrrole metabolism. Here, we found that, aside from leaves, the *Genome Uncoupled 4* (*OsGUN4*) was also predominantly expressed at 7–14 days after flowering (DAF) in developing seeds, whereas its down-regulation produced aberrant grain appearances and altered storage substances in the epi-genetic mutant of $gun4^{epi}$ seeds, which was attributed to abnormal performance of metabolites and starch biosynthetic enzymes. Moreover, the *OsGUN4* mutation would greatly affect the sucrose accumulation in leaves and the sucrose transportation in phloem, which was related to the weak photosynthesis resulted from reduced chlorophylls. Conversely, in developing grains, the *OsGUN4* mutation led to the accumulation of sucrose and the tetrapyrrole metabolites, i.e., protoporphyrin IX (PPIX) and heme, but inhibited synthesis of storage starch. Additionally, feeding of the exogenous heme also greatly suppressed the expression of starch biosynthetic genes at 7 to 14 DAF in developing grains, suggesting its similar effects to the *OsGUN4* mutation during starch biosynthesis. Collectively, these findings demonstrated that *OsGUN4* was a potential regulator to mediate starch biosynthesis via tetrapyrrole metabolites early during seed development.

1. Introduction

Starch is the major storage carbohydrate in higher plants, displaying critical role of starch in plant growth and productivity and our reliance on starch as a major component of our diet (Bahaji et al., 2014). With the increasing demand of starch from food and non-food industries but the growing loss of arable land to urbanization, understanding starch biosynthesis and its regulation is vital to produce the rational design of agronomic traits and more and better polymers via biotechnological approaches in crop plants (Geigenberger et al., 2004). Hitherto, while a sound knowledge of the enzymes involved in starch metabolism has been revealed, little is known about the detailed regulatory mechanisms of starch biosynthesis (Bahaji et al., 2014), thus, to understand these mechanisms is crucial for ultimately controlling these properties, which is a goal that has significant industrial interest.

However, although highly similar, transient and storage starch syntheses occurring in plastid stroma involve in dedicated enzymatic activities, which were typically supported by several genetically independent isoforms (Bahaji et al., 2014; Geiger, 2011). The storage starch is synthesized in the amyloplasts of non-photosynthetic sink tissues, i.e., seeds endosperm, and requires the supply of carbon precursors and energy from the source organs, i.e., leaves (Bahaji et al., 2014). Supplies in this process mainly depends on sucrose transporters, which delivers sucrose and energy from source to sink organs throughout the vascular system (phloem) in the plant (Geiger, 2011). Besides, as one of the important variations, amyloplast displays a high morphology and function in plant cells, whereas its development from undifferentiated proplastid requires coordinated regulation of nucleus and plastids to achieve a specific enzymatic configuration for functional diversity (Chan et al., 2016). The vast majority of plastid proteome is encoded by nucleus, but the expression of plastid genes is essential for metabolic processes such as photosynthesis and lipid biosynthesis (Jarvis and López-Juez, 2013). Amyloplasts are essential for starch biosynthesis and storage in cereal crops, but nearly all studies on signaling pathway between the nucleus and chloroplasts have been focused in photosynthetic cells.

The Genome uncoupled 4 (GUN4) were identified to be involved in chlorophyll biosynthesis and plastid-to-nucleus signaling pathway in

* Corresponding author.
E-mail address: zhanghuali621@126.com (H. Zhang).
[1] Authors contributed equally to this work.

https://doi.org/10.1016/j.jcs.2021.103317
Received 31 March 2021; Received in revised form 10 July 2021; Accepted 29 August 2021
Available online 31 August 2021

Arabidopsis thaliana (Peter and Grimm, 2009; Adhikari et al., 2011), *Chlamydomonas reinhardtii* (Brzezowski et al., 2014), *Synechocystis* sp. (Chen et al., 2015) and rice (Li et al., 2014, 2017; Jiang et al., 2019). Furthermore, the mutation of *OsGUN4* in rice have been revealed to greatly affect tetrapyrrole intermediates, including heme, protoporphyrin IX (PPIX) and Mg-PPIX, and deregulate transcription of photosynthesis-associated nuclear genes (PhANGs) depending on disruption of singlet oxygen ($^1O_2$)-induced signaling pathway (Li et al., 2017). Recently, we also demonstrated the roles of *OsGUN4* in the regulation of ROS and peroxidases (PRX) genes (Li et al., 2021). Moreover, expression of starch biosynthesis-associated genes is partly mediated by tetrapyrrole intermediates, i.e., heme, indicating a possible retrograde signaling from nucleus to non-photosynthetic plastids—amyloplasts during starch biosynthesis in BY cells (Enami et al., 2011). But whether the similar roles are existed in rice developing grains remains largely unknown.

Here, to explore the function of *OsGUN4* in the development of grains, we further employed the rice epi-genetic mutant *gun4*^{epi} to examine the mature seed properties, its carbon metabolites and starch biosynthetic enzymes at 7–20 days after flowering (DAF) in developing grains. The transcriptional expression of genes involved in starch biosynthesis were also investigated to verify the role of OsGUN4 in starch biosynthesis. Our results demonstrated that OsGUN4 was involved in playing roles during starch biosynthesis via tetrapyrrole metabolites to regulate transcription of starch biosynthetic genes.

2. Materials and methods

2.1. Plant materials

From wildtype *indica* variety Longtepu B (LTB), we previously developed a *xantha* mutant line, Huangyu B (HYB) by using gamma ray mutagenesis (Zhou et al., 2006). The epigenetic mutation of *OsGUN4, gun4*^{epi}, underlies the *xantha* phenotype of HYB (Li et al., 2014). The unique *xantha* marker trait of HYB was resulted from elevated CHG methylation in a 374-bp region (−2497 to −2124 bp) within the CpG island of the *OsGUN4* promoter (Li et al., 2014). Further, through gamma ray irradiation, a few *OsGUN4* mutant alleles were derived from the cross between M_2 plants of a *japonica* line GS113 and HYB (Li et al., 2014). The hybrid F_1 with *GUN4/gun4*^{epi} genotype were further self-crossed and the homozygous F_2 individuals were selected. The floury seeds derived from the *GUN4/GUN4* genotype seedlings with green leaf color, as well as the non-floury seeds from *gun4*^{epi}*/gun4*^{epi} genotype seedlings with yellow leaf color were consecutively selected to F_8 generation. In the current study, the harvest F_8 seeds, as well as the F_8 seedlings, were used as the investigated materials, and thereby, the *GUN4/GUN4* genotype materials were used as wild-type (WT), whereas the *gun4*^{epi}*/gun4*^{epi} genotype materials were termed as *gun4*^{epi} (Figure S1).

Leaves, roots, stems at 35-days after germination (DAG, depicted as vegetative stage), 14-days after pollination (DAF, depicted as reproductive stage) and 42 DAF (depicted as ripening stage), as well as developing grains at 0, 3, 7, 10, 14, 28, 42 DAF, were collected for phenotypes, carbon metabolites, enzyme assays and RT-qPCR analysis.

For heme treatments, the hulled grains of 7, 10 and 14 DAF were collected from the developing grains and subsequently cultured in 1 × MS medium containing 40 μM heme at 30 °C for 24 h under 16 h/8 h light (100 μmol photons m^{-2} s^{-1})/dark.

2.2. Scanning electron microscopy analysis

Scanning electron microscopy analysis was performed with previous described methods (She et al., 2010). Dried seeds were cut across the short axis with a razor blade, and then the sputter-coated with gold to scan with a Hitachi S–3000N SEM according to the manufacturer's protocol (Hitachi, Tokyo, Japan).

2.3. Determination of PPIX, Mg-PPIX and heme

Extraction of PPIX, Mg-PPIX and heme were performed with the previous methods (Papenbrock and Grimm, 2001). In brief, 0.3 g hulled or unhulled grounded grains were suspended with cold alkaline acetone containing 0.1 N NH_4OH (9:1; v/v), and then the supernatants were subsequently collected with centrifugation (5 min at 16,000×*g*) for determination of PPIX and Mg-PPIX with the enzyme linked immunosorbent assay (ELISA) method by using commercial kits (Elisa kits, Jingmei Tech., China) according to the manufacturer's instructions. While the pellets were resuspended in acetone/HCl/DMSO (10:0.5:2, v/v/v) and the supernatant were collected for heme determination with the previous method (Peter and Grimm, 2009).

2.4. Analysis of metabolites

Determination of amylose, starch, and protein were performed as described previously (Han et al., 2012). Sucrose, fructose, and glucose were analyzed using the methods described by Tang et al. (2016).

2.5. Characterization of amylopectin structure

Chain length distributions of amylopectin was determined using high-performance anion-exchange chromatography with pulsed amperometry detection (She et al., 2010).

2.6. Enzyme activity assays

About 0.1 g samples were homogenized in ice-cold buffer [50 mM HEPES-NaOH, pH 7.4, 2 mM $MgCl_2$, 50 mM -mercaptoethanol, 12.5% (v/v) glycerol], and the homogenate was then centrifuged at 20,000×*g* for 10 min at 4 °C. The supernatants were used for enzyme activity assays with the plant enzyme-linked immunosorbent assay (ELISA) kits (Mlbio, Shanghai) as follows: sucrose synthase (SS, NO.ml077397), sucrose phosphate synthase (SPS, NO.ml062647), ADP-Glc pyrophosphorylase (AGPase, NO.ml076671), granule-bound starch synthase (GBSS ml076667), soluble starch synthase (SSS, NO.ml076670), starch branching enzyme (SBE, NO.ml076664) according to the manufacturer's instructions.

2.7. Western blotting

Briefly, 0.5 g developing grains of 7, 10, 14 DAF were ground, and resuspended in 1 mL extraction buffer [2% SDS, 50 mM Na_2CO_3, 12% sucrose, 50 mM DTT and 2 mM EDTA, pH8.0, with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and protein inhibitors (Sigma)] for protein extraction. Antibodies against OsGUN4 (Li et al., 2014) was generated by BPI (Beijing Protein Innovation, Beijing, China), the proteins were immunoblotted according to Li et al. (2014) with OsHSP (Os09g0482600) as a reference protein.

2.8. Quantitative real-time PCR analysis (qRT-PCR)

Quantitative real-time PCR analysis was performed following the previous method (Li et al., 2017). Relative gene expression was calculated in relation to the rice *Ubiquitin* gene using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Gene-specific primers for qRT- PCR were listed in Table S1.

2.9. Accession numbers

Genes accession number were identified through homolog search of GenBank/EMBL database (https://www.ncbi.nlm.nih.gov/).

2.10. Statistical analysis

Data were expressed as means ± standard deviations (n = 6) and analyzed using two-way ANOVA test followed by the Tukey's Multiple Comparison Test with $P < 0.05$.

3. Results

3.1. The OsGUN4 mutation produced aberrant grain appearances and abnormal storage substances in seeds

Previous studies revealed that the *OsGUN4* mutation negatively affected the photosynthesis in leaves (Zhou et al., 2006), which was generally thought to lead to lanky seeds. Thus, to determine the effects of *OsGUN4* mutation on seeds, the grain appearance and starch granules of *gun4*^{epi} were examined. Unlike the white and floury endosperm of wild-type, the *gun4*^{epi} seeds showed relatively transparent appearance in brown seeds (Fig. 1A). Additionally, seed length had no differences between WT and *gun4*^{epi}, but both of the seed width and thickness were significantly reduced in *gun4*^{epi} (Fig. 1B). Besides, the polished seeds also performed similar appearances to brown seeds (Fig. 1C). Consistent with the phenotypic traits, the transparency of *gun4*^{epi} seeds exhibited higher values as compared with wild-type (Fig. 1D). Moreover, the cross sections of *gun4*^{epi} seeds became greatly downsized both in the peripheral and central regions of endosperm in relative to that in wild-type (Fig. 1E). Accordingly, scanning electron microscopy assays indicated that the endosperm of *gun4*^{epi} grains, were filled with densely packed, large, and irregularly polyhedral starch grains, while the wild-type was packed with loosely arranged compound starch grains (Fig. 1F and G). Finally, the amylose content performed significant increase in *gun4*^{epi} grains compared with that in wild-type (Fig. 1H).

Furthermore, the structural changes of amylopectin indicated that the endosperm greatly increased the middle chains consisting of 15–40 degrees of polymerization (DP), while decreased the short chains of 6–14 DPs and long chains of more than 40 DPs (Fig. 1I). All these results indicated that the *OsGUN4* mutation not only produced lanky seeds, but also made neglect effects on the seed appearances as well as the storage substances.

3.2. Temporal and spatial expression pattern of OsGUN4

OsGUN4 was predominately expressed in leaves and made abnormal effects on the formation from proplastid to chloroplast (Li et al., 2014). Nonetheless, lanky phenotypes were derived from the insufficient photosynthetic products, which could not fully explain the accumulation of amylose and alterative storage substances. So we further investigated the expression pattern of *OsGUN4* at the temporal and spatial scales.

As shown in Fig. 2A, aside from vegetative leaves, reproductive and ripening leaves and stem, *OsGUN4* was also predominately expressed at 7, 10 and 14 DAF in developing grains, but had not obvious expression at 0, 3, 28 and 42 DAF, suggesting OsGUN4 may be involved in grain development at 7 to 14 DAF (Fig. 2A).

To ensure the expression of *OsGUN4* early during seed development, the hulled grains of 7, 10 and 14 DAF were collected to detect the OsGUN4 expression at protein levels. Consistent with the results as shown in qRT-PCR, the translated OsGUN4 also showed relatively high expression (Fig. 2B). Thus, these results indicated that OsGUN4 played potential roles early during grain development.

3.3. Mutation of OsGUN4 affected the grain formation at 5–35 Days after pollination

To explore effects of the mutation of *OsGUN4* on grain development, the seed appearances during 5–35 DAF were investigated (Fig. 3). Both of the seed size in wild-type and the *gun4*^{epi} mutant were enlarged with

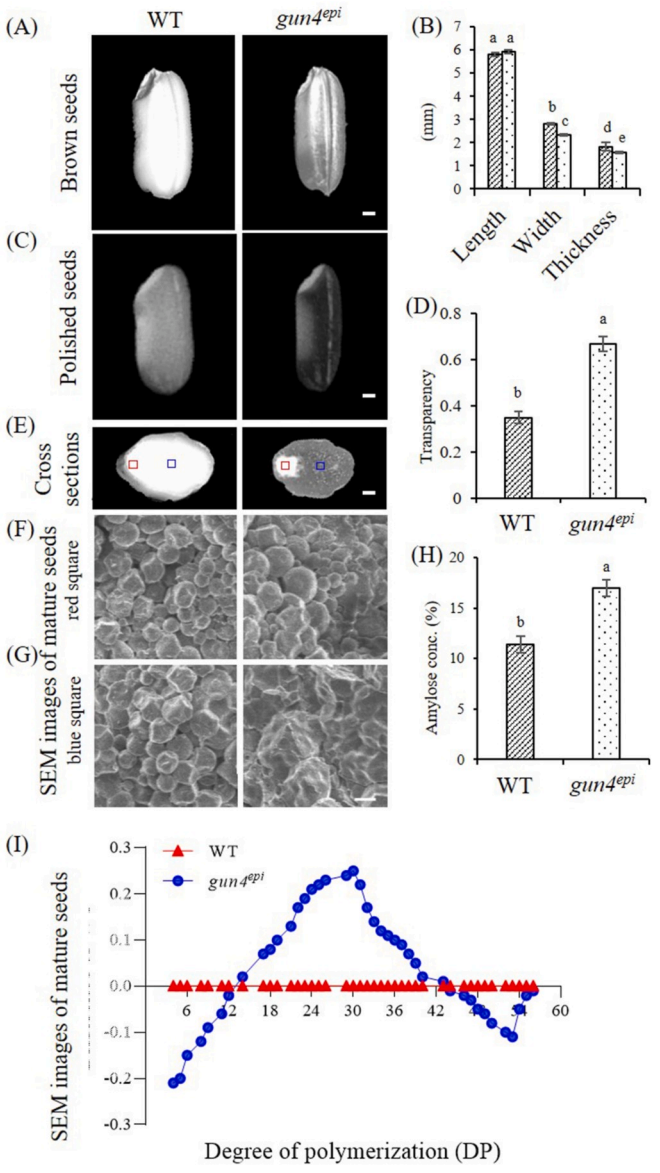

Fig. 1. Properties of mature seeds in wild type (WT) and the epi-mutant *gun4*^{epi}. (A) Representative brown seeds. Bar = 500 μm; (B) Quantification of seed length, width and thickness; (C) Representative polished seeds. Bar = 500 μm; (D) Transparency of polished seeds; (E) Seed cross sections. Bar = 300 μm; (F) Scanning electron microscopy images of the kernel peripheral regions indicated by red squares; Bar = 5 μm. (G) Scanning electron microscopy images of the seed central regions indicated by green squares; Bar = 5 μm. (H) Amylose content of mature seeds. Values are means ± standard deviations (n = 6) and analyzed for significant differences by two-way ANOVA followed by the Tukey's multiple comparison test with $P < 0.05$. (I) Comparison of amylopectin chain-length distribution. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

grain development (Fig. 3A). However, compared to WT, the *gun4*^{epi} seeds were obviously shorter, and showed significant difference at 7–20 DAF but narrowed after 25 DAF (Fig. 3A). Moreover, unlike the white and floury endosperm of wild-type, the *gun4*^{epi} seeds showed greatly downsized both in the peripheral and central regions of endosperm in relative to wild-type (Fig. 3B). All these results indicated that the *OsGUN4* mutation made neglect effects on the grain formation and storage substances during seed development.

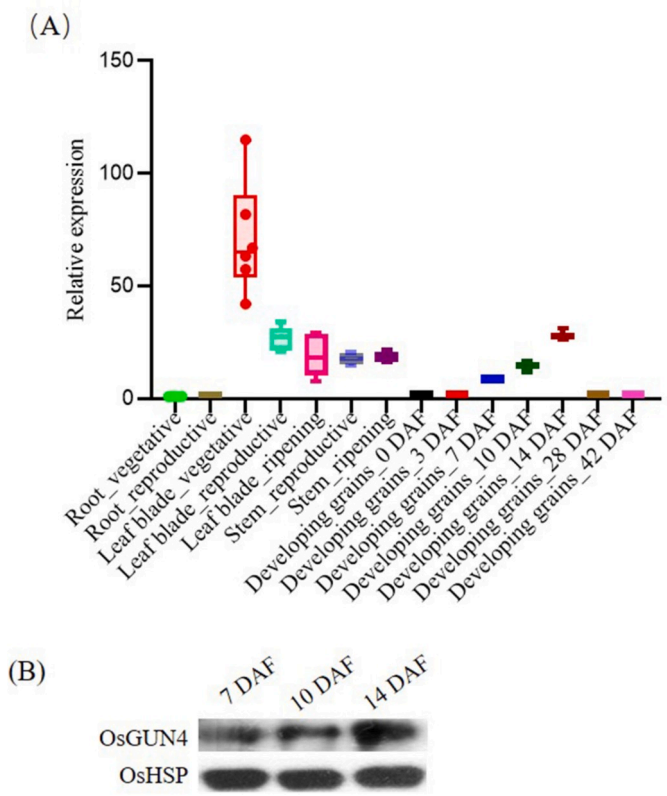

Fig. 2. Tissue-specific expression patterns of OsGUN4. (A) Expression is reported relative to the expression of *OsGUN4* in the vegetative root, which was assigned a value of 1. Values are means ± standard deviations (n = 6). (B) Western blot for the OsGUN4 expression at 35 days after pollination (DAF) in WT and *gun4*^{epi}.

3.4. Mutation of OsGUN4 performed aberrant starch metabolism in developing grains

The storage starch is synthesized in non-photosynthetic sink tissues, i.e., seed endosperm, and requires the supply of sucrose and energy from the green tissues, i.e. leaves (Bahaji et al., 2014). To determine whether the aberrant formation of seeds was resulted from lacking of sucrose from leaves, we next investigated the dynamic concentrations of sugars at 7 to 14 DAF in developing grains.

At 7 to 14 DAF, both of the sucrose contents in leaves and phloem of *gun4*^{epi} were significantly lower in relative to WT, only amount to 48.58%–86.88% and 65.78%–71.08% of that in WT, respectively (Fig. 4A and B; Table S2). In contrast to leaves and phloem, the sucrose contents of developing grains in *gun4*^{epi} were significantly higher than that in wild-type (Fig. 4C). Besides, the contents of glucose and fructose became gradually increases from 7 to 14 DAF both in wild-type and *gun4*^{epi}, while the concentrations of glucose and fructose in *gun4*^{epi} were greatly lower as compared to wild-type (Figure S2).

We next examined whether the *OsGUN4* mutation also suppressed the storage starch and proteins. The total starch and proteins displayed decreased concentrations in *gun4*^{epi}, while the amylose contents in *gun4*^{epi} were significantly higher than that in wild-type (Fig. 4D–F). However, despite the reduced total starch were detected in *gun4*^{epi}, the supply of sucrose performed enhanced accumulations in the developing grains of *gun4*^{epi}. These results indicated that the *OsGUN4* mutation seemed to suppress the sucrose transports from leaves to developing grains, but instead promoted the accumulation of sucrose in developing grains during the critical stage of seed development. So, it can be concluded that the lanky seeds of *gun4*^{epi} was largely attributed to the suppressed transformation from sucrose to starch.

Given the repressed effects of the *OsGUN4* mutation on starch synthesis, we subsequently analyzed the activities of key enzyme participating in starch biosynthesis, including SS, SPS, GBSS, AGPase, SSS and SBE (Fig. 4; Table S3). Consistent with the contents of metabolites, the activities of SS (Fig. 4G) and SPS (Fig. 4H) for sucrose utilizations were significantly higher in *gun4*^{epi} than that in WT, whereas AGPase (Fig. 4I), SSS (Fig. 4K) and SBE (Fig. 4L) involved in starch synthase showed reduced activities in *gun4*^{epi} as compared to WT. Besides, activities of

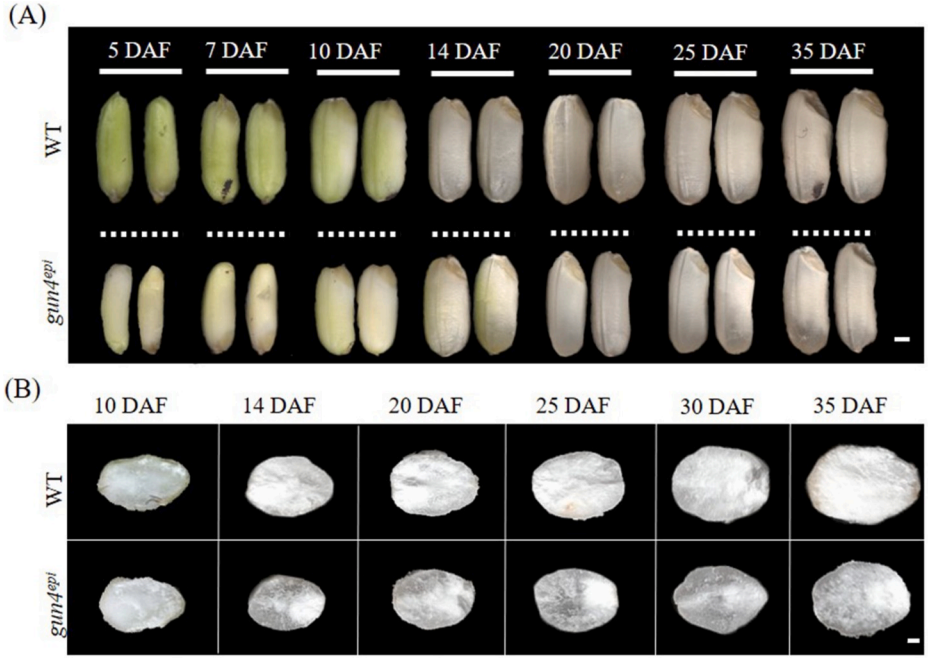

Fig. 3. Effects of *OsGUN4* mutation on the seed development. (A) Representative brown seeds. Bar = 700 μm; (B) Seed cross sections. Bar = 300 μm. DAF, days after pollination. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

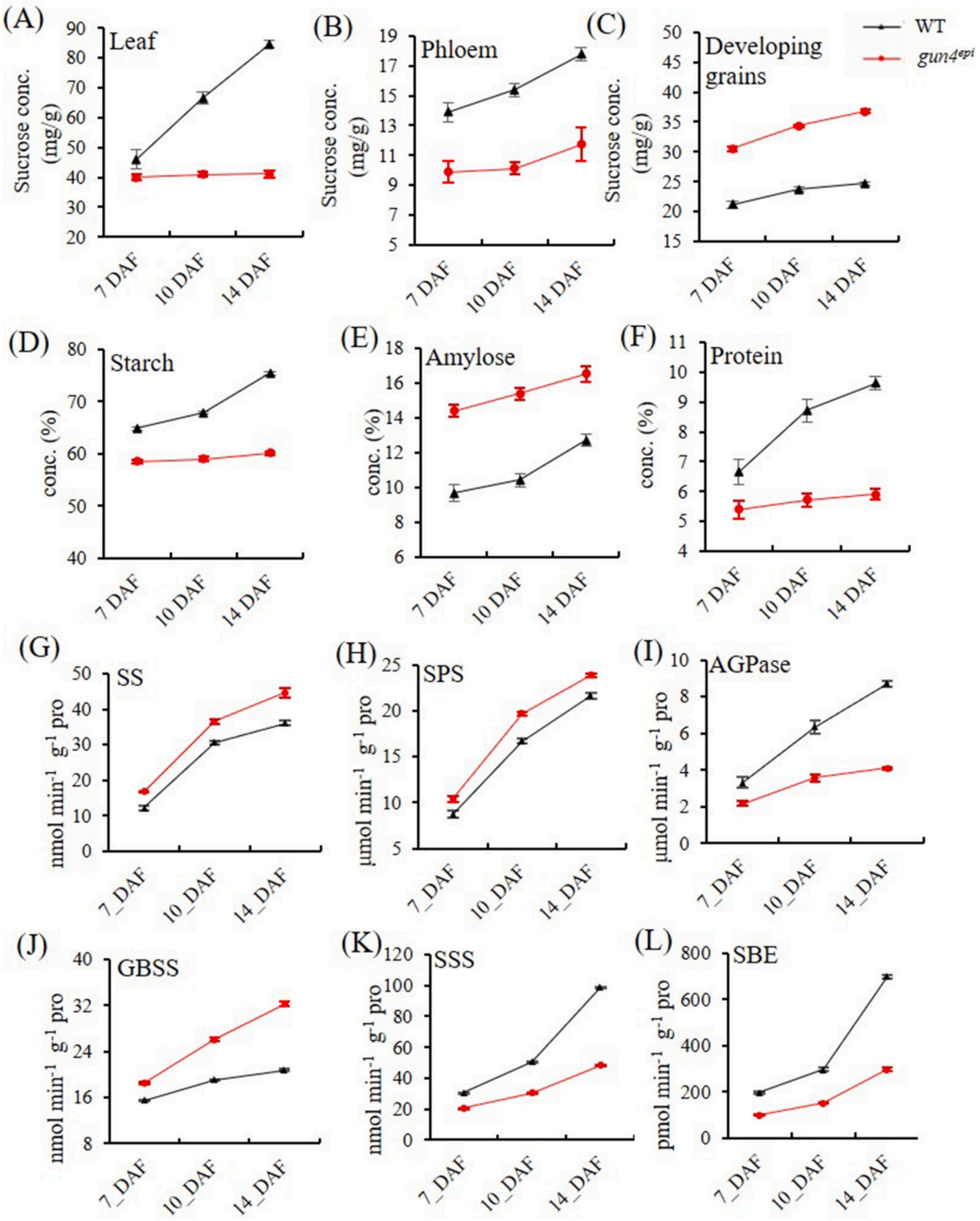

Fig. 4. Detection of quality traits during grain development. (A) Sucrose content in leaves, (B) Sucrose content in phloem, (C) Sucrose content in developing grains, (D) Total starch content, (E) Amylose content, (F) Protein content, (G) Activities of sucrose synthase (SS), (H) Activities of sucrose phosphate synthase (SPS), (I) Activities of ADP-Glc pyrophosphorylase (AGPase), (J) Activities of granule-bound starch synthase (GBSS), (K) Activities of soluble starch synthase (SSS), (L) Activities of starch branching enzyme (SBE) at 7, 10 and 14 DAF in wild type (WT) and the epi-mutant *gun4*^{epi}. Values are means ± standard deviations (n = 6) and analyzed for significant differences by two-way ANOVA followed by the Tukey's multiple comparison test with $P < 0.05$. DAF, days after pollination.

GBSS in *gun4*^{epi} were higher than that in WT (Fig. 4J). Thus, the suppressed flow of sucrose-to-starch in *gun4*^{epi} grains was derived from the suppressed activities of the key starch biosynthetic enzymes.

3.5. Mutation of OsGUN4 affected gene expression responsible for starch metabolism in developing grains

To investigate the detailed roles of *OsGUN4* on starch biosynthesis,

expression of starch biosynthetic genes were detected in the developing grains. Genes for sucrose synthesis (*OsSUS1*, *2*, *3*), SPS (*OsSPS2*, *OsSPS11*) were significantly increased in *gun4*^{epi} (Figure S3), whereas genes encoding fructokinase (Frk, *OsFrK1*) and hexokinase (HxK, *OsHXK1*, *4*, *7*) showed reduced expression at 7, 10 and 14 DAF as compared to WT (Figure S3). Besides, expression of other genes for sucrose synthesis, including *phosphoglucoseisomerase* (*OsPGI)*, *phosphoglucomutase* (*OsPGM*), and *sucrose phosphate phosphatase* (*OsSPPL1* and *OsSPPL2*) were also greatly increased in *gun4*^{epi} (Figure S3; Table S4). However, for AGPase, genes for large subunit (*OsAGPL1*, *2*, *3*, *4*) in *gun4*^{epi} were significantly increased, while genes encoding small subunit, such as *OsAGPS1*, *2a*, *2b*, show lower expression as compared to WT (Figure S3), suggesting the abnormal functioning of AGPase. All in all, up-regulation of the genes for sucrose synthesis would promote the synthesis of storage starch from the transported sucrose, but the repressed activity of AGPase, which was used for catalyzing the formation of AGP, consequently restricted the starch synthesis. This can also be concluded from the expression of genes involved in starch synthesis. Compared with WT, gene expression for soluble synthase I (SSI, *OsSSI*), SSIII (*OsSSIIIb*) and branching enzymes (BEII; *OsBEIIa*, *OsBEIIb*) was significantly reduced in *gun4*^{epi}, while genes encoding SSII (*OsSSIIa* ; *OsSSIIb*, *OsSSIIc*), SSIII (*OsSSIIIa*), SSIV (*OsSSIVa*, *OsSSIVb*) and BEI (*OsBEI*) showed increased expression in *gun4*^{epi} (Figure S3).

Besides, gene expression for isoamylases (*OsISA1*, *OsISA2*, *OsISA3*), pollulanase (*OsPUL*) and α-amylase (Amy3A, 3B, 3C) was increased as compared to wild-type (Figure S3). Gene expression for storage proteins, including protein disulfate isomerase (PDI), prolamin (CysR10), PPDKB, glutelins (GluA1, GluA2, GluA3) was significantly reduced in *gun4*^{epi} as compared to that in the wild-type, while gene expression for alanine aminotransferase (*AlaAT1*, *AlaAT4*), major allergenic protein (RA16, RA17, RA5B, RAG2 and RG21), globulins (globulin1, globulin2, 11s-globulin, 10kD-, 13kD-, 17kD-, 19kD-globulin) showed increased expression in *gun4*^{epi} (Figure S3). These results suggested that *OsGUN4* mutation down-regulated the expression of many genes participating in storage starch in developing grains, especially for *OsAGPS1*.

3.6. Mutation of OsGUN4 affected the tetrapyrrole metabolism in developing grains

Although the suppressed transport of sucrose from leaves to developing grains largely depended on less accumulation of photosynthetic products caused by the *OsGUN4* mutation, it still seemed not fully to explain why more sucrose were accumulated at 7 to 14 DAF in *gun4*^{epi}. Besides, tetrapyrrole intermediates, e.g., heme, were assumed to regulate starch biosynthesis during differentiation of plastids into amyloplasts (Enami et al., 2012). Thus, to investigate whether the similar GUN4-involved regulatory route existed during early seed development, the developing seeds of 7, 10, 14 DAF were used for determination of the tetrapyrrole intermediates. Compared with the unhulled grains, all the investigated intermediates showed significant decreases in hulled grains of WT and *gun4*^{epi}, and the similar change trends were also found between hulled and unhulled grains from 7 to 14 DAF (Fig. 5; Table S5). Moreover, no significant changes of PPIX were detected from 7 to 14 DAF in WT, while PPIX was gradually accumulated with increased DAF in *gun4*^{epi} (Fig. 5A and B). Also, more PPIX were detected in *gun4*^{epi} than that in WT (Fig. 5A and B). On the contrary, the contents of Mg-PPIX were increased with DAF in both of WT and *gun4*^{epi}, but less Mg-PPIX were accumulated in *gun4*^{epi} as compared to WT (Fig. 5C and D). Besides, the accumulations of heme were higher in *gun4*^{epi} compared with WT (Fig. 5C and D). All these results indicated that the *OsGUN4* mutation affected the tetrapyrrole metabolism early during seed development, especially for the accumulations of heme.

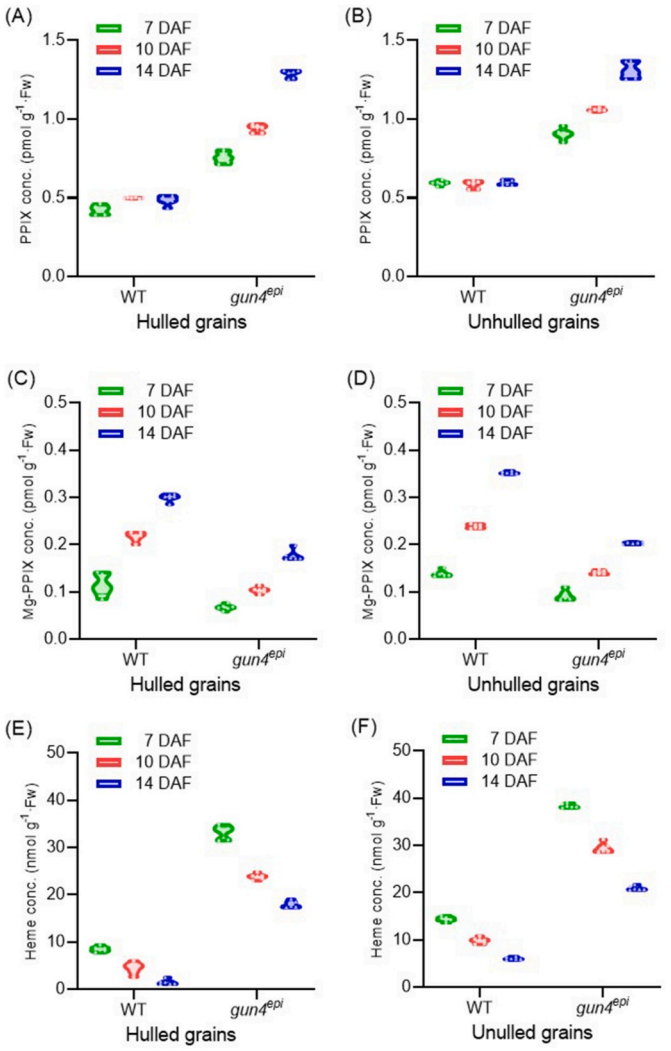

Fig. 5. Tetrapyrrole metabolisms during grain development. (A) PPIX contents in the hulled grains; (B) PPIX contents in the unhulled grains; (C) Mg-PPIX contents in the hulled grains; (D) Mg-PPIX contents in the unhulled grains; (E) Heme contents in the hulled grains; (F) Heme contents in the unhulled grains at 7, 10 and 14 days after pollination **(DAF).** Values are means ± standard deviations (n = 6) and analyzed for significant differences by two-way ANOVA followed by the Tukey's multiple comparison test with $P < 0.05$.

3.7. Exogenous heme repressed the gene expression involved in starch biosynthesis in developing grains

To further revealed the effects of heme on the starch biosynthesis in developing grains, the hulled grains of 7–14 DAF were co-cultured with 40 μM heme. After feeding of exogenous heme, the expression of *OsGUN4*, *OsAGPS1* and *OsGBSSI* were significant decreased both in WT and *gun4*^{epi} (Fig. 6; Table S6). However, the expression of *OsGUN4* and *OsAGPS1* showed gradual increases with DAF in WT, but displayed no significant change from 7 to 14 DAF in *gun4*^{epi} (Fig. 6A–D). Moreover, the enhanced expression of *OsGBSSI* were detected in both of WT and *gun4*^{epi}, and the expression of *OsGBSSI* were higher in *gun4*^{epi} as compared to WT (Fig. 6E and F). These results suggested that the accumulation of heme repressed the expression of starch biosynthetic genes, e.g., *OsAGPS1* and *OsGBSSI*.

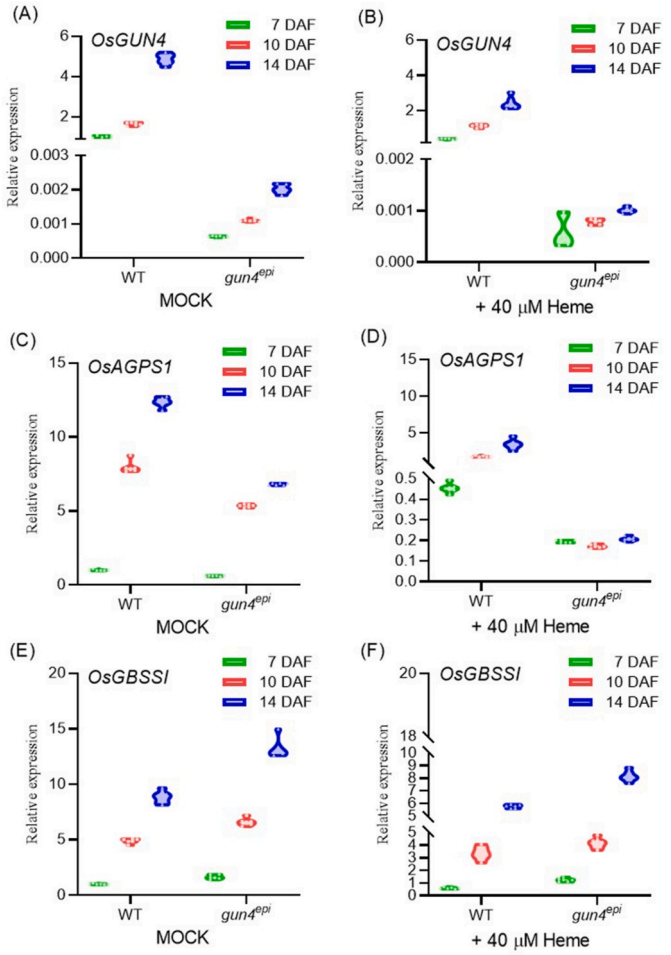

Fig. 6. Effects of exogenous heme on the developing grains. (A) Expression levels of *OsGUN4*; (B) Expression levels of *OsGUN4* after 40 μM heme treatment; (C) Expression levels of *OsAGPS1*; (D) Expression levels of *OsAGPS1* after 40 μM heme treatment; (E) Expression levels of *OsGBSSI*; (F) Expression levels of *OsGBSSI* after 40 μM heme treatment at 7, 10 and 14 days after pollination (DAF). Expression levels are reported relative to that of the WT grown at 7 DAF, which was assigned a value of 1. Values are means ± standard deviations (n = 6) and analyzed for significant differences by two-way ANOVA followed by the Tukey's multiple comparison test with $P < 0.05$.

4. Discussion

4.1. Effects of OsGUN4 on starch biosynthesis in developing grains

OsGUN4 was previously identified to be involved in chlorophyll biosynthesis and retrograde signaling in to mediate the expression of photosynthesis-associated nuclear genes (Li et al., 2014). Indeed, we here found that the *OsGUN4* mutation not only led to smaller seeds (Fig. 1A–C and Fig. 3A) but also made neglect effects on seed substances (Fig. 1I) and appearances (Fig. 1D–H and Fig. 3B). However, lanky phenotypes in *gun4*^{epi} might be derived from the insufficient photosynthetic products, but this could not fully explain the accumulation of amylose (Fig. 1H) and alterative storage substances (Fig. 1I) in the scarce expression of *OsGUN4*. Moreover, the supply of sucrose from the green tissues is essential for the biosynthesis of storage starch (Bahaji et al., 2014), but, despite of accumulation of sucrose in grains (Fig. 4C), its key downstream intermediates used for biosynthesis of storage starch, performed decreased contents in *gun4*^{epi} (Fig. 4D–F). So, it can be concluded that the lanky seeds of *gun4*^{epi} was largely attributed to the suppressed transformation from sucrose to starch during seed formation.

Correspondingly, activities of SS (Fig. 4G) and SPS (Fig. 4H) for sucrose utilizations were significantly higher in *gun4*^{epi} than that in WT, whereas AGPase (Fig. 4I), SSS (Fig. 4K) and SBE (Fig. 4L) involved in starch synthesis showed reduced activities in *gun4*^{epi} as compared to WT. These results suggested that the suppressed transformation from sucrose to starch in *gun4*^{epi} was derived from the inhibited activities of the key starch biosynthetic enzymes, and consequently caused the downsized seeds. In other words, *OsGUN4* plays vital roles in the functioning of starch biosynthetic enzymes, e.g., AGPase, SSS and SBE, which further have positive effects on seed development.

However, the phenotypes of floury endosperm in WT seemed to be paradox to that of other rice wild-type varieties in the normal presence of *OsGUN4*. Although GUN4 is not related to the phenotypes serves as one possible explanation, the restoration of transparent phenotype in the mutant of *OsGUN4* vetoed it (Figs. 1 and 3). Therefore, another possibility is that the difference largely depends on the repressed expression of *OsGBSSI* in WT as compared to other rice varieties. In fact, both expression of *OsGBSSI* and the GBSS activity in WT was even lower than that in *gun4*^{epi} (Figs. 4 and 6). GBSS/Wx is responsible for elongating the amylose polymers released from the starch granule (Ortiz-Marchena et al., 2014), while the formation of starch greatly depends on the amylose/amylopectin ratio (Sattari et al., 2015). Collectively, the repressed expression of *OsGUN4* could reasonably explain why the phenotypes of floury endosperm exists in WT as compared to the other rice wild-type varieties in the present of *OsGUN4*. Nevertheless, the restoration of transparent appearance in *gun4*^{epi} also seemed to rely on the increased expression of *OsGBSSI* (Figure S3) and the enhanced activity of GBSS at 7 to 14 DAF (Fig. 4). Interestingly, the expression of *OsGBSSI* maybe also relevant to the tetrapyrrole metabolism, e.g., PPIX. The simultaneous accumulation of heme and PPIX (Fig. 5) was beneficial to the expression of *OsGBSSI*, and eventually promoted the restricted carbon precursors for preferential synthesis of amylose. To sum up, *OsGUN4* was involved in starch biosynthesis and remodeling starch granule structure during early seed development.

4.2. OsGUN4 was involved in mediating starch biosynthesis through tetrapyrrole intermediates during seed development

Rice seeds kept size enlarging and weight increasing during 6–20 DAF of grain filling periods (Xu et al., 2008). Interestingly, *OsGUN4* was also displayed high expression at 7 to 14 DAF in developing grains (Fig. 2), which was consistent with the reported results of Cy3 signal intensity from database of RiceXPro (https://ricexpro.dna.affrc.go.jp/GGEP/gene-search.php?keyword = AK061014; Figure S4). However, due to the limitation of inherent lethal defects of *gun4*-null mutant plants during seedling stages (Li et al., 2014), nearly scarce studies have been directly related to OsGUN4 during seed development in rice, not to mention its possible roles in seed development. Nonetheless, in BY cells, tetrapyrrole intermediates, i.e., heme, have been reported to partly mediate a possible retrograde signaling from nucleus to non-photosynthetic plastids—amyloplasts during starch biosynthesis (Enami et al., 2011).

Here, we demonstrated that the *OsGUN4* mutation greatly suppressed the synthesis of sucrose in leaves and the transport of sucrose in phloem during seed formation (Fig. 4). However, by contrast, more sucrose was accumulated in the developing grains of *gun4*^{epi}, while less storage starch and proteins were detected as compared to WT (Fig. 4). This provided a possible explanation why the seeds of *gun4*^{epi} became smaller. In other words, the repressed flow of sucrose to starch in the developing grains were responsible for the smaller seeds of *gun4*^{epi}, which was also highly correlated with activities of the key starch biosynthetic enzymes, i.e., AGPase (Fig. 4). But, how OsGUN4 may function during starch biosynthesis is still unknown.

Leaves is thought to be the tissue-specific parts of *OsGUN4* (Li et al., 2014). However, we here revealed that *OsGUN4* was also showed highly expression at 7–14 DAF in developing grains (Fig. 2), which was consistent with the results of Cy3 signal intensity assays (https://ricexp

ro.dna.affrc.go.jp/GGEP/gene-search.php?keyword = AK061014; Figure S4). Thus, it could be concluded that effects of *OsGUN4* on the seed development not only depends on the retarded sucrose from leaves, but also greatly relevant to the starch biosynthesis during early seed development. Actually, the *OsGUN4* mutation made negative effects on the seed size, and also was prone to remodel the starch composition (Fig. 1).

As depicted above, tetrapyrrole intermediates, e.g., heme, were assumed to regulate starch biosynthesis during differentiation of plastids into amyloplasts (Enami et al., 2012). In this study, the *OsGUN4* mutation led to more accumulations of heme (Fig. 5), which would greatly down-regulate the expression of genes involved in starch biosynthesis, i.e. *OsAPGS1* (Figure S3), and eventually reduced the activity of AGPase (Fig. 4) to suppress the transformation from sucrose to starch (Fig. 4) during seed development (Fig. 3). Additionally, this can be confirmed from the down-regulated expression of *OsAGPS1* after feeding with exogenous heme (Fig. 6). Besides, the expression of *OsGBSSI*, *OsAGPS1* and *OsGUN4* performed decreased trends upon on the exogenous heme treatments in WT, while *OsGUN4* was insensitive to the heme treatments in *gun4*^{epi} (Fig. 6), suggesting PPIX was more prone for the synthesis of heme in the conditions of *OsGUN4* mutation and eventually to suppress the expression of starch biosynthetic genes.

Nonetheless, accumulations of heme seem to not fully explain the increased expression of *OsGBSSI* (Figure S3) and the enhanced activity of GBSS (Fig. 4) at 7 to 14 DAF in the *OsGUN4* mutant. Although the exogenous heme treatments would lead to the decreased expression of *OsGBSSI* (Fig. 6), the contents of heme displayed inconsistent changes with the *OsGBSSI* expression in the *OsGUN4* mutant (Figure S3 and Fig. 5). Therefore, the expression of *OsGBSSI* maybe also relevant to other tetrapyrrole metabolism, e.g., PPIX. In fact, the *OsGUN4* mutation led to the accumulations of heme and PPIX (Fig. 5), which also served as one of the retrograde signals (Tabrizi et al., 2016). Actually, activation of signaling resulted from PPIX accumulations in the *OsGUN4* mutant would promote the expression of *OsGBSSI* to enhance the activity of GBSS, which eventually increased the amylose contents for the grain elongation during early seed development.

Besides, the exogenous heme treatments produced similar effects with the *OsGUN4* mutation on starch biosynthesis (Fig. 6 and Figure S3), which furthermore lead to the accumulation of heme (Fig. 5). Simultaneously, the *OsGUN4* mutation also led to the accumulation of PPIX, which was beneficial to the expression of *OsGBSSI*, and eventually promoted the restricted carbon precursors for preferential synthesis of amylose.

Collectively, under normal conditions, GUN4 would promote the Mg-PPIX synthesis from PPIX but suppress the formation of heme, which would promote expression of the starch biosynthetic genes and activate the associated enzymes, i.e., GBSS and AGPase, and eventually promote the transformation from sucrose to starch. However, in the conditions of heme accumulation, e.g., the *OsGUN4* mutation and the feeding of exogenous heme, PPIX would also be simultaneously accumulated. Moreover, the accumulated heme would suppress the expression of starch biosynthetic genes, leading to the reduced carbon flow of sucrose to starch, while the accumulation of PPIX seems to activate the expression of *OsGBSSI* to promote the amylose synthesis, which was helpful to the maintenance of cell morphology.

4.3. Potential economic values on starch utilization of *gun4*^{epi}

Rice quality, especially eating and cooking qualities (ECQs), determines its economic value and consumer recognition in the market. However, the formation of ECQs depends on many factors, e.g., amylose content (AC), flavor substances, fatty acid content, gel consistency (GC) and protein content, and in addition, the amylose/amylopectin ratio, while the amylopectin fine structure determines the physicochemical properties, nutritional quality and final yield of rice grains addition (Bao et al., 2006). Here, interestingly, although possibly not suitable for the general public, more than 17% of amylose in the mature seeds of *gun4*^{epi} (Fig. 1H) showed potential values in the formation of resistant starch (RS) for some special populations, due to its improvement of seed quality. The high contents of amylose and the changed structure of amylopectin determined the unique rice qualities of *gun4*^{epi} and its potential market values. RS is the sum of starch and products derived from starch degradation which does not undergo digestion and absorption by the small intestine (Reddy et al., 2013). Both of SSIIIa and GBSSI are involved in the synthesis of resistant starch. The regulation of SSIIIa on RS depends on the high expression of *GBSSI* gene, while mutation of *SSIIIa* would reduce the expression of *GBSSI*, and consequently lead to the decrease of RS content and amylose-lipid complex (Zhou et al., 2016). This is consistent with the results as shown in Fig. 4 and Figure S4, which showed the higher expression of genes for SSIIIa and GBSSI.

Collectively, we here demonstrated that OsGUN4 play regulatory roles in storage starch biosynthesis, which helps to provide the foundation for utilizations of resistant starch in rice.

Ethics approval and consent to participate

The authors declare that the experiments were performed in compliance with the current laws of China.

Consent for publication

All authors are consent for publication.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary files.

Funding

This work is funded by a grant from the Natural Foundation of Anhui Province (1908085QC103), the Open Project Program of State Key Laboratory of Rice Biology (20200104), Funding of Introduction & Stability for Talents supported by Anhui Agricultural University (yj 2018-39).

Authors' contributions

RL and HZ conceived the study. RL, MJ and WZ carried out the experimental analysis. RL and HZ finished the first draft, and RL finished the final version.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgements

We thank Prof. Q Shu for providing the seeds of HYB.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcs.2021.103317.

References

Adhikari, N.D., Froehlich, J.E., Strand, D.D., Buck, S.M., Kramer, D.M., Larkin, R.M., 2011. GUN4-porphyrin complexes bind the ChlH/GUN5 subunit of Mg-chelatase and promote chlorophyll biosynthesis in Arabidopsis. Plant Cell 23 (23), 1449–1467.

Bao, J.S., Sun, M., Corke, H., 2006. Microsatellites, single nucleotide polymorphisms and a sequence tagged site in starch-synthesizing genes in relation to starch

physicochemical properties in nonwaxy rice (*Oryza sativa* L.). Theor. Appl. Genet. 113, 1185–1196.
Bahaji, A., Li, J., Sánchez-López, Á.M., Baroja-Fernández, E., Muñoz, F.J., Ovecka, M., Almagro, G., Montero, M., Ezquer, I., Etxeberria, E., Pozueta-Romero, J., 2014. Starch biosynthesis, its regulation and biotechnological approaches to improve crop yields. Biotechnol. Adv. 32 (1), 87–106.
Brzezowski, P., Schlicke, H., Richter, A., Dent, R.M., Niyogi, K.K., Grimm, B., 2014. The GUN4 protein plays a regulatory role in tetrapyrrole biosynthesis and chloroplast-to-nucleus signaling in *Chlamydomonas reinhardtii*. Plant J. 79, 285–298.
Chan, K.X., Su, Y.P., Crisp, P., Mcquinn, R., Pogson, B.J., 2016. Learning the languages of the chloroplast: retrograde signaling and beyond. Annu. Rev. Plant Biol. 67 (1), 25.
Chen, X., Hua, P., Xiao, W., Long, W., Lin, R., Lin, L., 2015. Crystal structures of GUN4 in complex with porphyrins. Mol. Plant 8, 1125–1127.
Enami, K., Ozawa, T., Motohashi, N., Nakamura, M., Tanaka, K., Hanaoka, M., 2011. Plastid-to-nucleus retrograde signals are essential for the expression of nuclear starch biosynthesis genes during amyloplast differentiation in tobacco BY-2 cultured cells. Plant Physiol. 157, 518–530.
Enami, K., Tanaka, K., Hanaoka, M., 2012. Retrograde signals arise from reciprocal crosstalk within plastids. Plant Signal. Behav. 7 (1), 142–144.
Geigenberger, P., Stitt, M., Fernie, A.R., 2004. Metabolic control analysis and regulation of the conversion of sucrose to starch in growing potato tubers. Plant Cell Environ. 27, 655–673.
Geiger, D., 2011. Plant sucrose transporters from a biophysical point of view. Mol. Plant 4, 395–406.
Han, X.H., Wang, Y.H., Liu, X., 2012. The failure to express a protein disulphide isomerase-like protein results in a floury endosperm and an endoplasmic reticulum stress response in rice. J. Exp. Bot. 63, 121–130.
Jarvis, P., López-Juez, E., 2013. Biogenesis and homeostasis of chloroplasts and other plastids. Nat. Rev. Mol. Cell Biol. 14 (12), 787–802.
Jiang, M., Liu, Y.H., Li, R.Q., Zheng, Y.C., Fu, H.W., Tan, Y.Y., Moller, I.M., Fan, L.J., Shu, Q.Y., Huang, J.Z., 2019. A suppressor mutation partially reverts the *xantha* trait via lowered methylation in the promoter of *genomes uncoupled 4* in rice. Front. Plant Sci. 10, 1003.
Li, R.Q., Huang, J.Z., Zhao, H.J., Fu, H.W., Li, Y.F., Liu, G.Z., Shu, Q.Y., 2014. A down-regulated epi-allele of the genomes uncoupled 4 gene generates a *xantha* marker trait in rice. Theor. Appl. Genet. 127 (11), 491–501.
Li, R.Q., Jiang, M., Huang, J.Z., Møller, I.M., Shu, Q.Y., 2021. Mutations of the *Genomes Uncoupled 4* gene cause ROS accumulation and repress expression of peroxidase genes in rice. Front. Plant Sci. https://doi.org/10.3389/fpls.2021.682453.
Li, R.Q., Jiang, M., Liu, Y.H., Zheng, Y.C., Huang, J.Z., Wu, J.M., Shu, Q.Y., 2017. The *xantha* marker trait is associated with altered tetrapyrrole biosynthesis and deregulated transcription of PhANGs in rice. Front. Plant Sci. 8, 901.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta c(t)) method. Methods 25, 402–408.
Ortiz-Marchena, M.I., Albi, T., Lucas-Reina, E., Said, F.E., Romero-Campero, F.J., 2014. Photoperiodic control of carbon distribution during the floral transition in Arabidopsis. Plant Cell 26, 565–584.
Papenbrock, J., Grimm, B., 2001. Regulatory network of tetrapyrrole biosynthesis studies of intracellular signalling involved in metabolic and developmental control of plastids. Planta 213 (5), 667–681.
Peter, E., Grimm, B., 2009. GUN4 is required for posttranslational control of plant tetrapyrrole biosynthesis. Mol. Plant 2, 1198–1210.
Reddy, C.K., Suriya, M., Haripriya, S., 2013. Physico-chemical and functional properties of resistant starch prepared from red kidney beans (*Phaseolus vulgaris* L.) starch by enzymatic method. Carbohydr. Polym. 95 (1), 220–226.
Sattari, A., Mahdinezhad, N., Fakheri, B., Noroozi, M., Beheshtizadeh, H., 2015. Improvement of the eating and cooking qualities of rice: a review. Int. J. Farming Allied Sci. 4 (2), 153–160.
She, K.C., Kusano, H., Koizumi, K., Yamakawa, H., Hakata, M., Imamura, T., Fukuda, M., Naito, N., Tsurumaki, Y., Yaeshima, M., Tsuge, T., Matsumoto, K., Kudoh, M., Itoh, E., Kikuchi, S., Kishimoto, N., Yazaki, J., Ando, T., Yano, M., Aoyama, T., Sasaki, T., Satoh, H., Shimada, H., 2010. A novel factor floury endosperm2 is involved in regulation of rice grain size and starch quality. Plant Cell 22 (10), 3280–3294.
Tabrizi, S.T., Sawicki, A., Zhou, S., Luo, M., Willows, R.D., 2016. GUN4-protoporphyrin IX is a singlet oxygen generator with consequences for plastid retrograde signaling. J. Biol. Chem. 291, 8978–8984.
Tang, X.J., Peng, C., Zhang, J., Cai, Y., You, X.M., Kong, F., Yan, H.G., Wang, G.X., Wang, L., Jin, J., Chen, W.W., Chen, X.G., Ma, J., Wang, P., Jiang, L., Zhang, W.W., Wan, J.M., 2016. ADP-glucose pyrophosphorylase large subunit 2 is essential for storage substance accumulation and subunit interactions in rice endosperm. Plant Sci. 249, 70–83.
Xu, S.B., Li, T., Deng, Z.Y., Chong, K., Xue, Y., Wang, T., 2008. Dynamic proteomic analysis reveals a switch between central carbon metabolism and alcoholic fermentation in rice filling grains. Plant Physiol. 148 (2), 908–925.
Zhou, H., Wang, L., Liu, G., Meng, X., Jing, Y., Shu, X., Kong, X., Sun, J., Yu, H., Smith, S. M., Wu, D., Li, J., 2016. From the cover: critical roles of soluble starch synthase SSIIIa and granule-bound starch synthase waxy in synthesizing resistant starch in rice. Proc. Natl. Acad. Sci. Unit. States Am. 113 (45), 12844–12849.
Zhou, X.S., Shen, S.Q., Wu, D.X., Sun, J.W., Shu, Q.Y., 2006. Introduction of a *xantha* mutation for testing and increasing varietal purity in hybrid rice. Field Crop. Res. 96, 71–79.