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Effects of OsCDPK1 on the Structure and Physicochemical Properties of Starch in Developing Rice Seeds

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Abstract

Overexpression of a constitutively active truncated form of *OsCDPK1* (*OEtr*) in rice produced smaller seeds, but a double-stranded RNA gene-silenced form of *OsCDPK1* (*Ri*) yielded large seeds, suggesting that *OsCDPK1* plays a functional role in rice seed development. In the study presented here, we propose a model in which *OsCDPK1* plays key roles in negatively controlling the grain size, amylose content, and endosperm appearance, and also affects the physicochemical properties of the starch. The dehulled transgenic *OEtr* grains were smaller than the dehulled wild-type grains, and the *OEtr* endosperm was opaque and had a low amylose content and numerous small loosely packed polyhedral starch granules. However, the *OEtr* grain sizes and endosperm appearances were not affected by the temperature being either optimal (25 °C) or low (22 °C) or high (31 °C) during the grain-filling phase. In contrast, the transgenic *Ri* grains were larger, had higher amylose contents, and had more transparent endosperms filled with tightly packed polyhedral starch granules. This demonstrates that *OsCDPK1* plays a novel functional role in starch biosynthesis during seed development and affects the transparent appearance of the endosperm. These results improve our understanding of the molecular mechanisms through which the grain filling process occurs in rice.

Keywords: rice; OsCDPK1; grain size; amylose content; endosperm appearance

1. Introduction

The quality of rice (*Oryza sativa* L.) grain is defined in terms of several main factors, including (i) the eating and cooking qualities and (ii) the milling qualities and appearance [1]. The eating and cooking qualities are determined by the amylose content, amylopectin structure, gelatinization temperature, and pasting viscosity [2], and the milling qualities and appearance correlate strongly with the transparency, flouriness, and chalkiness of the endosperm [3]. The filling and accumulation of starch granules in developing rice endosperm can accelerate at high temperatures, causing the starch in the endosperm cells to be packed loosely and the kernel to be chalky. Such grains crack easily during milling, giving poor eating and cooking qualities [4-6].

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It has been shown in many studies that chalky and less transparent kernels contain more amylopectin and less amylose in the endosperm than do less chalky and more transparent kernels [7-9]. Eliminating chalkiness by regulating the amylopectin and amylose content ratios (by affecting biosynthesis) in the endosperm during the grain filling phase is therefore a key way of improving grain quality. The main enzymes involved in amylose biosynthesis are ADP-glucose pyrophosphorylase and granule-bound starch synthase I (GBSSI, encoded by the Waxy gene), and the enzymes involved in the biosynthesis and modification of amylopectin are ADP-pyrophosphorylase, soluble starch synthase, the starch-branching enzyme (BE), and the starch-debranching enzyme [10-12]. In higher plants, BE plays an essential role in amylopectin biosynthesis because it is the only enzyme that can add α-1,6-glucosidic linkages to polyglucans [13]. Three BE isoforms, BEI, BEIIa, and BEIIb, have been found in rice [10,14]. The rice mutant *amylose-extender*, which has a null mutation in *BEIIb*, has been found to alter the degree of polymerization (DP) of amylopectin, giving fewer short chains (DP ≤ 17) and more long chains (DP ≥ 18), the changes being related to the dose on the *amylose-extender* locus in the triploid endosperm cells. These results suggest that *BEIIb* might have critical effects on the amylopectin structure and the rheological properties of the starch [12]. Several floury endosperm rice mutants (*flo1–flo5*) have been isolated. Treating fertilized rice egg cells with the chemical mutagen N-methyl-N-nitrosourea gave mutants *flo1* and *flo2*, which had floury endosperms [15,16]. The *flo2* mutant has been found, through map-based cloning, to be a member of the tetratricopeptide repeat-motif protein family. The gene mutation decreases the grain size and decreases the starch quality (by decreasing the amylose content) and also changes the fine structure of the amylopectin [17]. The *flo3* mutant was produced through applying gamma-irradiation and ethyl methansulfonate treatment, and had a low 16 kDa globulin content in the endosperm [18]. The white-core floury endosperm mutants *flo4* and *flo5* were produced through T-DNA insertional mutagenesis and were found to have *pyruvate orthophosphate dikinase B* and *starch synthase IIIa* (SSIIIa) gene mutations, respectively [19,20]. The *flo4* mutant endosperm had a low amylose content, suggesting that *pyruvate orthophosphate dikinase B* might play a role in regulating carbon metabolism during the grain filling process [19]. DP analysis of amylopectin in *flo5* was performed, and the amounts of DP 6–8 and DP 16–20 in the mutant endosperm were found to be decreased but the amounts of DP 9–15 and DP 22–29 increased, indicating that

SSIIIa strongly affects the chain-length distribution of amylopectin biosynthesized in developing rice grains [20]. The floury endosperm mutant *flo6* had a completely floury white endosperm, but the *flo7* endosperm was floury and white only at the peripheries but not in the interior [21,22]. Map-based cloning demonstrated that *flo6* was an insertion mutation in the unknown function gene *Os03g0686900* [21] and that *flo7* was a deletion mutation in the unknown function gene *Os10g0463800* [22], suggesting that these genes may play vital roles in starch biosynthesis and granule formation in the endosperm during the grain-filling process.

Calcium ions (Ca^{2+}) are secondary messengers in plant cells, and are used when various environmental and developmental stimuli are sensed through temporal and spatial fluctuations or when the cytosolic Ca^{2+} concentration increases [23,24]. Calcium-dependent protein kinases (CDPKs) are a major family of calcium sensors that have been characterized in various plant species. CDPKs are Ser/Thr protein kinases that are encoded by multigene families [25,26]. CDPKs have four functional domains, an N-terminal variable domain, a catalytic kinase domain, an autoinhibitory domain, and a calcium-binding EF-hands regulatory domain [27]. It has been shown in many studies that CDPKs play important physiological roles in response to various environmental stresses and developmental processes [23,24,28-30]. Only in a few studies have CDPKs been shown to play a role in starch biosynthesis during the grain-filling process in rice. The rice *CDPK* called *SPK* (which shared 79% of its amino acid sequence with *OsCDPK1*) has been found to encode a sucrose synthase kinase. Expression of antisense *SPK* in transgenic rice produced watery grains because large amounts of sucrose had accumulated in the endosperm due to low sucrose synthase activity, resulting in inefficient sucrose degradation [31]. This indicates that SPK may be a regulator in the starch biosynthesis pathway.

In previous studies, we demonstrated that overexpression of a constitutively active truncated form of *OsCDPK1* (*OEtr-1*) in rice gave smaller seeds whereas RNA interference gene knockdown (*Ri-1*) mutants gave larger seeds [32]. In the study presented here, *OsCDPK1* was found to play pivotal roles in rice-seed development, in the physicochemical properties of the starch produced, and in the appearance of the endosperm.

2. Results

2.1. Ectopic Overexpression and Silencing of OsCDPK1 in Transgenic Rice Plants Gave Opaque and Ttransparent Endosperms,Respectively

We examined the 55-day-old plant phenotypes in the T4 transgenic lines further, and the results were consistent with our previous studies [32]. The *Ri-1, -2 and -3* plants were higher (mean value is 76.6±6.18 cm) and the *OEtr-1, -3 and -4* plants were shorter (52.4±3.58 cm) than WT plants (63.3±4.63 cm) (Figure 1A,B). At the grain filling stage, the WT and transgenic plants were transferred to the growth room under optimal temperature of a cycle of 25°C for 16 h light and 20°C for 8 h dark. Fifteen seeds randomly selected from each individual lines and then dehulled. We found that the dehulled *Ri-1* grains were longer and that the *OEtr-1* grains were shorter than the WT grains (Figure 1E,F). We also found that the dehulled *OEtr-1* grains were all of the floury-kernel phenotype (Figure 1C,E).

Table 1
The ratios of chalky grains in wild type (TNG67), *OEtr-1* and *Ri-1* lines growth under lower temperature (LT) (22 °C for 16 h light, 20 °C for 8 h dark) or higher temperature (HT) (31 °C for 16 h light, 28 °C for 8 h dark) during rice grain filling process.

Plant species	Wild type	*OEtr-1*	*OEtr-3*	*OEtr-4*	*Ri-1*	*Ri-2*	*Ri-3*
The ratios of chalky grains in LT (%)	16.2 ± 2.19	100	100	100	6.0 ± 0.61	6.7 ± 0.56	7.1 ± 0. 61
The ratios of chalky grains in HT (%)	63.6 ± 5.19	100	100	100	50.1 ± 3.61	51.2 ± 4.56	45.1 ± 3. 21

Mean values calculated from 100 independent seeds. All data are presented as mean ± SE. Statistical significant is determined by t-test.

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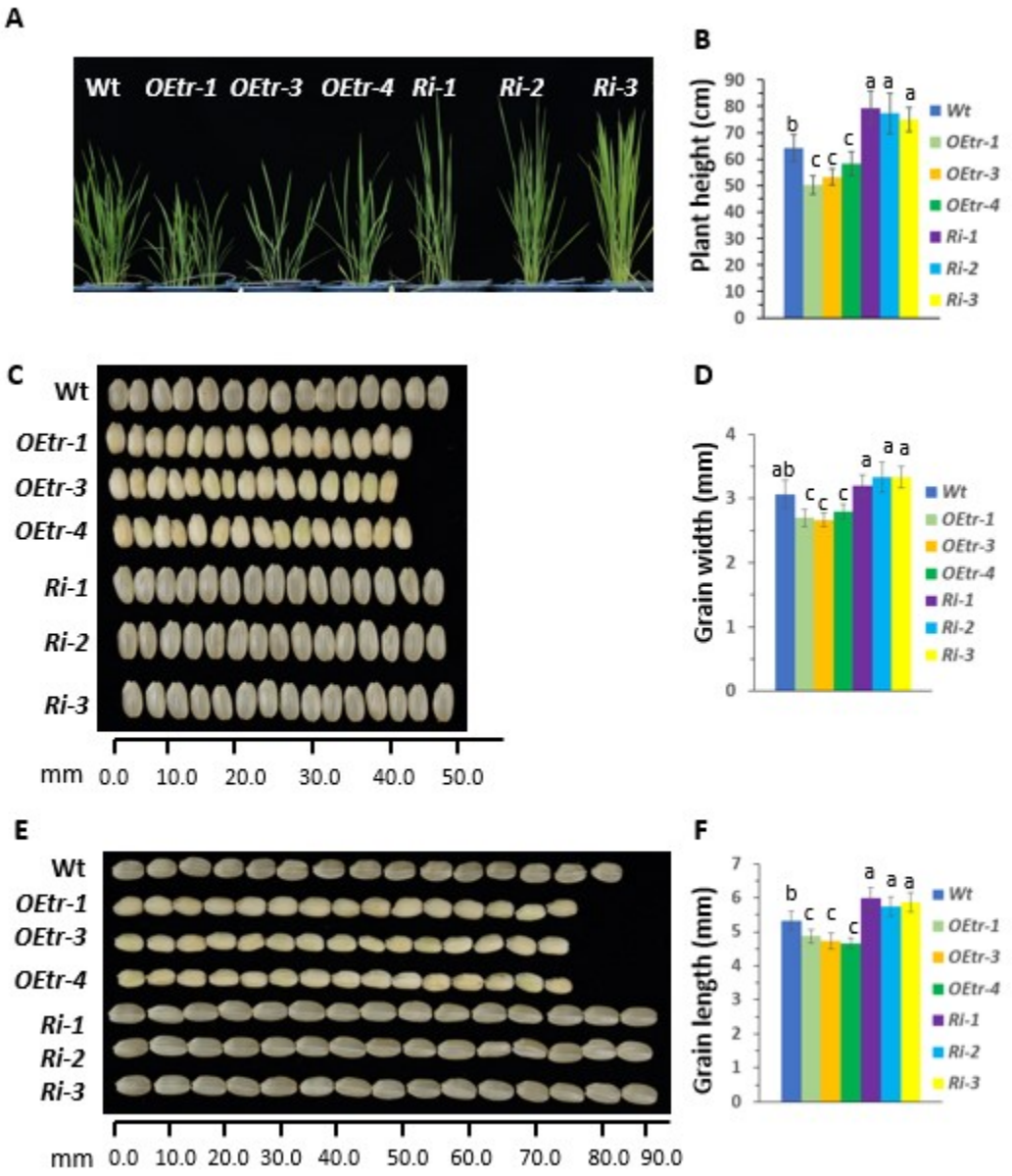

Figure 1. Plant heights and grain morphologies in the WT, and the *OEtr-1, -3* and *-4 (OEtrs)*, and the *Ri-1, -2* and *-3* (*Ris*) plants. After 5 d of flowering, rice plants were transferred to a growth chamber and grown at an optimal temperature (25 °C for 16 h light, 20 °C for 8 h dark). (A) Plant heights of the 55-day-old of the WT, *OEtrs*, and *Ris* plants. (B) Quantification of the plant heights. Each error bar is the standard error for 15 individual plants. (C) Endosperm appearances and grain widths for the WT, *OEtrs*, and *Ris* plants. Fifteen grains per line were positioned in a row and measured. (D) Quantification of the grain widths. Each error bar is the standard error for 50 individual grains. (E) Endosperm appearances and grain lengths for the WT,

OEtrs, and *Ris* plants. Fifteen grains per line were positioned in a row and measured. (F) Quantification of the grain lengths. Each error bar is the standard deviation (n = 50). Different letters above the bars indicate significant differences, identified by performing ANOVAs ($P < 0.01$).

We determined whether the temperature affected the endosperm appearance in the transgenic lines by growing rice plants at relatively low temperatures (LT)(22 °C for 16 h light, 20 °C for 8 h dark) and at relatively high temperatures (HT)(31 °C for 16 h light, 28 °C for 8 h dark) during grain filling process. The mature seeds were collected from the WT, and the *Ri-1*, *Ri-2* and *Ri-3* (*Ris*), and from the *OEtr-1, OEtr-3* and *OEtr-4* (*OEtrs*) plants, respectively. As shown in Figure 2, under LT and HT conditions, all the dehulled *OEtrs* grains had small floury endosperms (100% in the *OEtr-1, OEtr-3* and *OEtr-4* lines) regardless of the temperature at which the plants were grown. In contrast, under LH condition, most of the grains in WT and *Ris* lines displayed transparency phenotype; the ratios of chalky grains were 16.2% in the WT, and 6.0, 6.7 and 7.1% in the *Ri-1, Ri-2* and *Ri-3*, respectively (Figure 2A and Table 1). However, the higher temperature caused a significant increasing of the WT and *Ris* lines endosperms to be of the chalky phenotype; the ratios of chalky grains were 63.6% in the WT, and 50.1, 51.2 and 45.1% in the *Ri-1, Ri-2* and *Ri-3*, respectively (Figure 2A and Table 1). Illuminating the kernels with a backlight showed that the *OEtrs* grains all had opaque endosperms, whereas the WT and *Ris* grains had transparent endosperms at the LT condition (but the *Ris* endosperms were the most transparent) but increased opaque endosperms more than 50% in both WT and Ris lines (Figures 2B and S1). Cross-sections of the endosperms grown at the LT or HT (Figure 2C) indicate that the opaque kernels in the *OEtrs* lines had white cores at LT, but all displayed completely floury endosperms at HT treatment. These results suggest that *OsCDPK1* affects rice endosperm transparency by a temperature-dependent manner.

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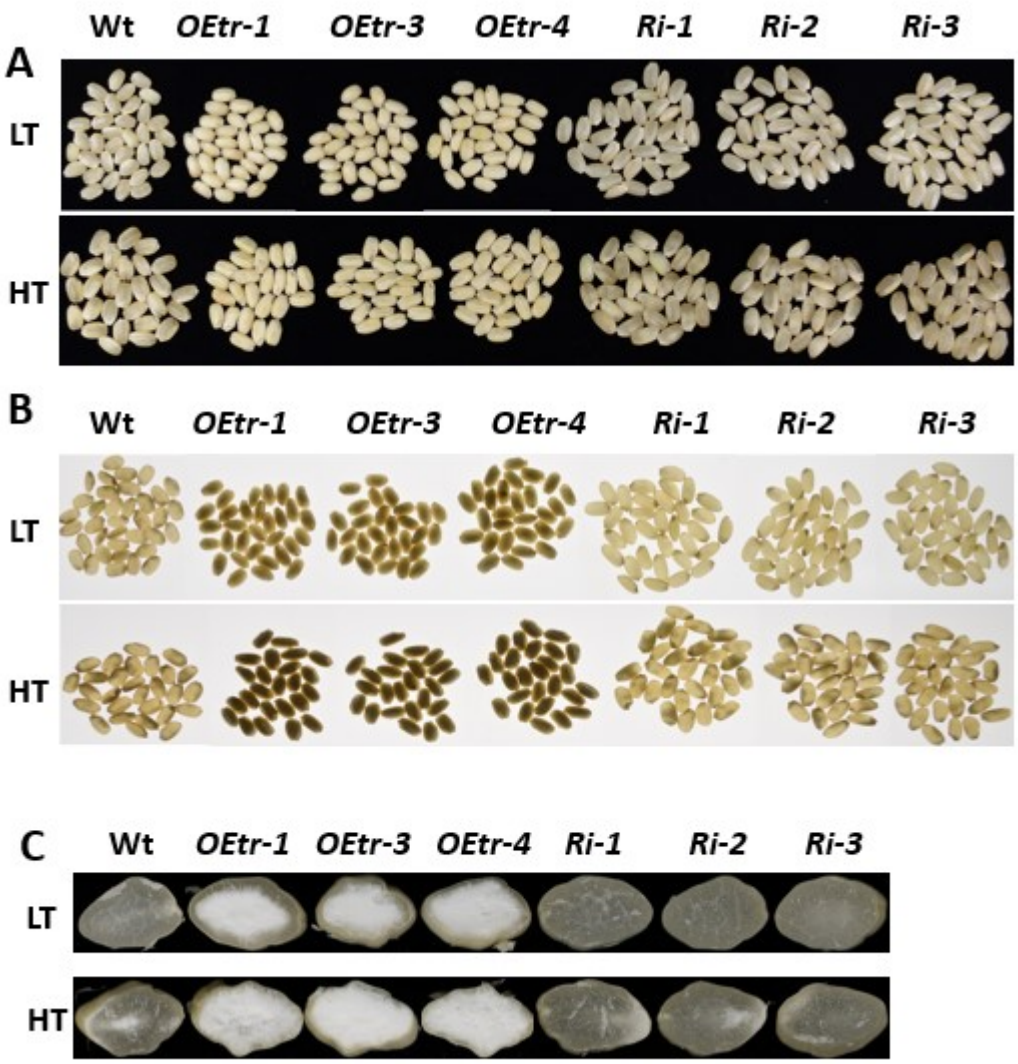

Figure 2. Effects of temperature on the appearances of the WT, *OEtrs*, and *Ris* endosperm. After 5 d of flowering, rice plants were transferred to a growth chamber and grown at a lower temperature (22 °C for 16 h light, 20 °C for 8 h dark) or a higher temperature (31 °C for 16 h light, 28 °C for 8 h dark). (A) and (B) Seeds harvested from the plants grown at the lower and higher temperatures, respectively, illuminated using (A) normal lighting and (B) backlighting. (C) Cross-sections of the endosperms of the seeds from plants grown at either lower or higher temperature. LT: lower temperature; HT: higher temperature.

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2.2. Effect of OsCDPK1 on Starch Granule Morphology in Rice Endosperm

Due to the similar transgenic plant phenotypes and grain morphology (Figures 1 and 2), the OsCDPK1-overexpressing line, *OEtr-1*, and the OsCDPK1-silencing line, *Ri-1*, were therefore selected for further studies. We examined the starch granule morphology in the WT and both *Ri-1* and *OEtr-1* transgenic seeds by analyzing the endosperm cross-sections by scanning electron microscopy. As shown in Figure 3, the three-dimensional structures of the starch granules in the endosperms were irregularly polygonal and polyhedral in all three grains types. The starch granules were large and tightly packed in the WT and *Ri-1* endosperms but small and loosely packed in *OEtr-1* endosperm (Figure 3B), suggesting that *OsCDPK1* affects the starch granule size and packing density in developing rice seed.

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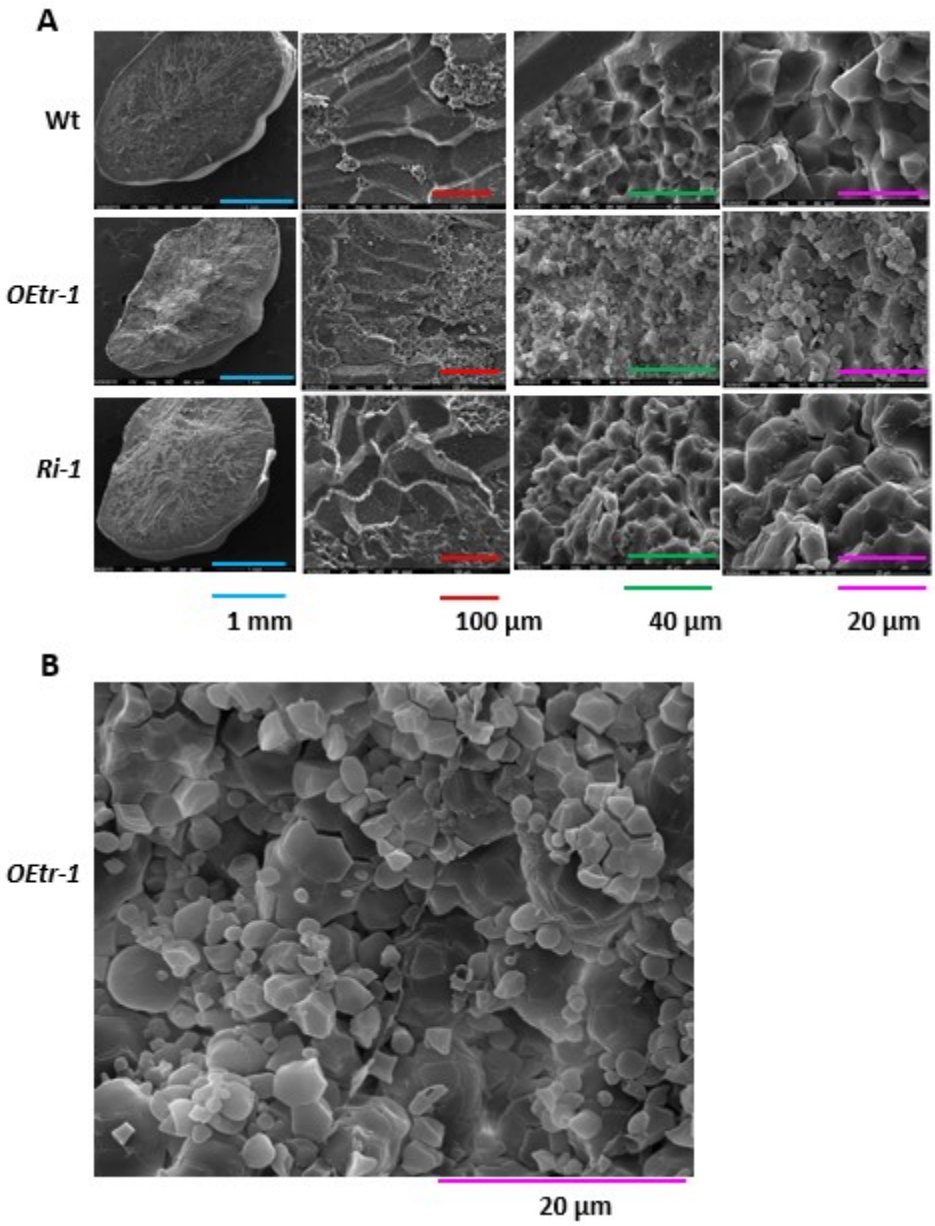

Figure 3. Scanning electron microscopy images of the structures of the starch granules in the rice endosperms. (A) The central areas of the cross-sections of mature endosperms from the WT plants (top panel), *OEtr-1* plants (middle panel), and *Ri-1* plants (bottom panel) were acquired using a scanning electron microscope. (B) The zoom in image from the 20 µm photo in *OEtr-1*. Scale bar colors: blue as 1 mm, red as 100 µm, green as 40 µm, purple as 20 µm.

2.3. Effects of OsCDPK1 on Starch Properties and Gelatinization in the Endosperm

We examined the apparent amylose content in the endosperm by reacting 20 mg of each rice endosperm powder sample with 1 N NaOH to gelatinize the starch. The amylose content was measured using a colorimetric method using an I_2/KI solution [12,33]. The *OEtr-1* samples had less affinity than the other samples for iodine and were light purple, whereas the *Ri-1* and WT sampler were dark blue and light blue, respectively (Figures 4A and S2). We examined the absorption spectra of the I_2/KI stained solutions and found strong absorbance between 480 and 720 nm for both the *Ri-1* and WT samples, with maxima at 620 nm, but stronger absorbance was found for *Ri-1* than for WT at the same wavelength (Figure 4B). *OEtr-1* absorbance was weaker and decreased as the wavelength increased. We compared the results to the absorbances of potato amylose standards to allow the apparent amylose contents to be determined. The apparent amylose contents of the WT, *OEtr-1*, and *Ri-1* samples were 19.53%, 16.82%, and 22.45%, respectively (Table 2). These results demonstrate that the *Ri-1* seed endosperms had higher amylose contents than the WT seed endosperms and that the *OEtr-1* seed endosperms had lower amylose contents than the WT seed endosperms. The WT, *OEtr-1*, and *Ri-1* amylose contents were different, so we investigated starch gelatinization at different urea concentrations (0–9 M). A 20 mg aliquot of a rice endosperm powder was mixed with 1 mL of urea solution, and the mixture was allowed to react for 24 h. The mixture was then centrifuged and the degree of gelatinization determined by measuring the sediment volume. Starch gelatinization started at urea concentrations of 3.0–4.0 M (Figures 4C and S3). In 4.0 M urea, the *Ri-1* sediment volume was 5.3% higher than the WT sediment volume, whereas the *OEtr-1* sediment volume was 36.4% lower than the WT sediment volume (Figure 4D). These results indicate that *OsCDPK1* affects the physicochemical properties of the starch in rice endosperms.

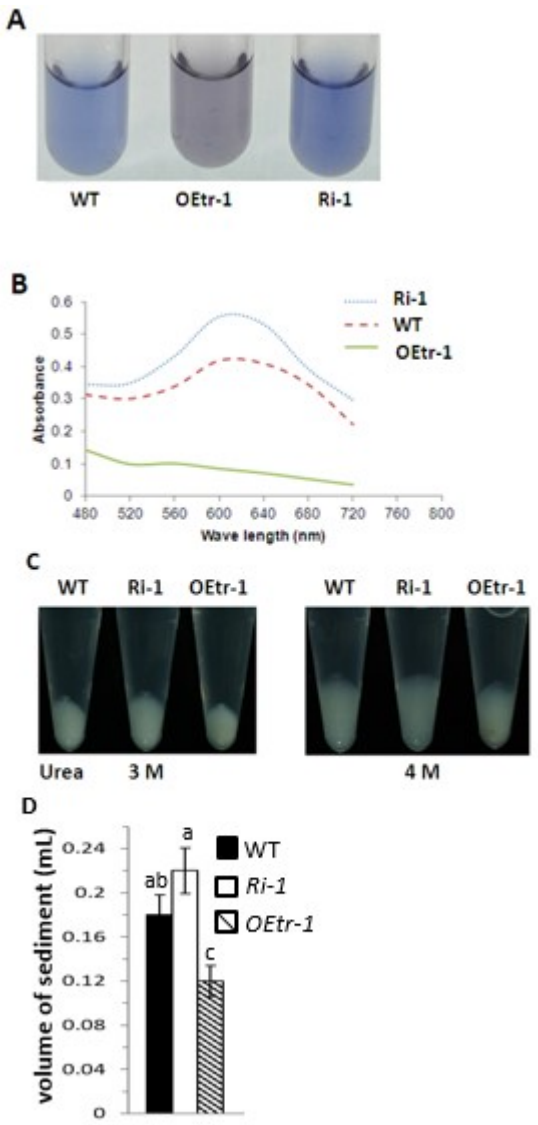

Figure 4. Iodine-staining and gelatinization properties of the starch in the rice endosperms. A 20 mg aliquot of endosperm powder was treated with 1 N NaOH as described in the materials and methods section. (A) Supernatants of the iodine-stained WT, *OEtr-1*, and *Ri-1* samples. (B) Starch–iodine absorbance spectra of the supernatants. (C) Effects of using 3.0 and 4.0 M urea solutions on the gelatinization characteristics of the WT, *OEtr-1*, and *Ri-1* endosperm starch. A 20 mg aliquot of rice powder was mixed in an Eppendorf tube with 1 mL of urea solution and the mixture was shaken for 24 h at 25 °C. The mixture was centrifuged and the volume of the gelatinized starch sediment was measured. (D) Quantification of the gelatinization volume. Different letters above the bars indicate

significant differences, identified by performing ANOVAs ($P < 0.05$). Each value is the mean ± the standard deviation of three independent measurements.

Table 2 Comparison of apparent amylose content in endosperm among Wt, *OEtr-1* and *Ri-1*.

Genotype	Amylose (%)
Wt (TNG67)	19.53±0.42
OEtr-1	16.82±0.39
Ri-1	22.45±0.57

Mean values calculated from three independent experiments. All data are presented as mean ± SE. Statistical significant is determined by t test

2.4. OsCDPK1 Expression Profiles in Developing Rice Seeds

Our results have demonstrated that *OsCDPK1* affects rice seed development. It is necessary to track changes in *OsCDPK1* gene expression during rice seed development. The *OsCDPK1::GUS* transgenic line was generated using a GUS reporter gene controlled by the *OsCDPK1* promoter (−1706 to +301 bp, i.e., a total of 2007 bp upstream of the translational start site) containing the first intron (607 bp, in the 5′-untranslated region) (Figure 5A). As shown in Figure 5B, strong GUS staining was observed in the ovaries and anthers before flowering but weaker staining was observed in the styles and lemma. At 1 DAF, strong GUS activity was found only in the ovaries and styles, weaker activity was found in the lemma, and no GUS activity was found in the anthers and stigma. Between 2 and 5 DAF, concentrated GUS staining was found in the rachilla and both ends of the developing seeds and weak staining was found in the lemma. The blue color gradually expanded from both ends toward the central parts of the developing seeds between 6 and 7 DAF, and at 8 DAF the entire seeds were thoroughly stained blue. Staining gradually decreased afterwards, but remained strong between 10 and 14 DAF, then decreased quickly after 14 DAF and had completely gone by 18 DAF. These results suggest that *OsCDPK1* was

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expressed in a particular temporal and spatial way, predominantly in the middle stage of rice seed development.

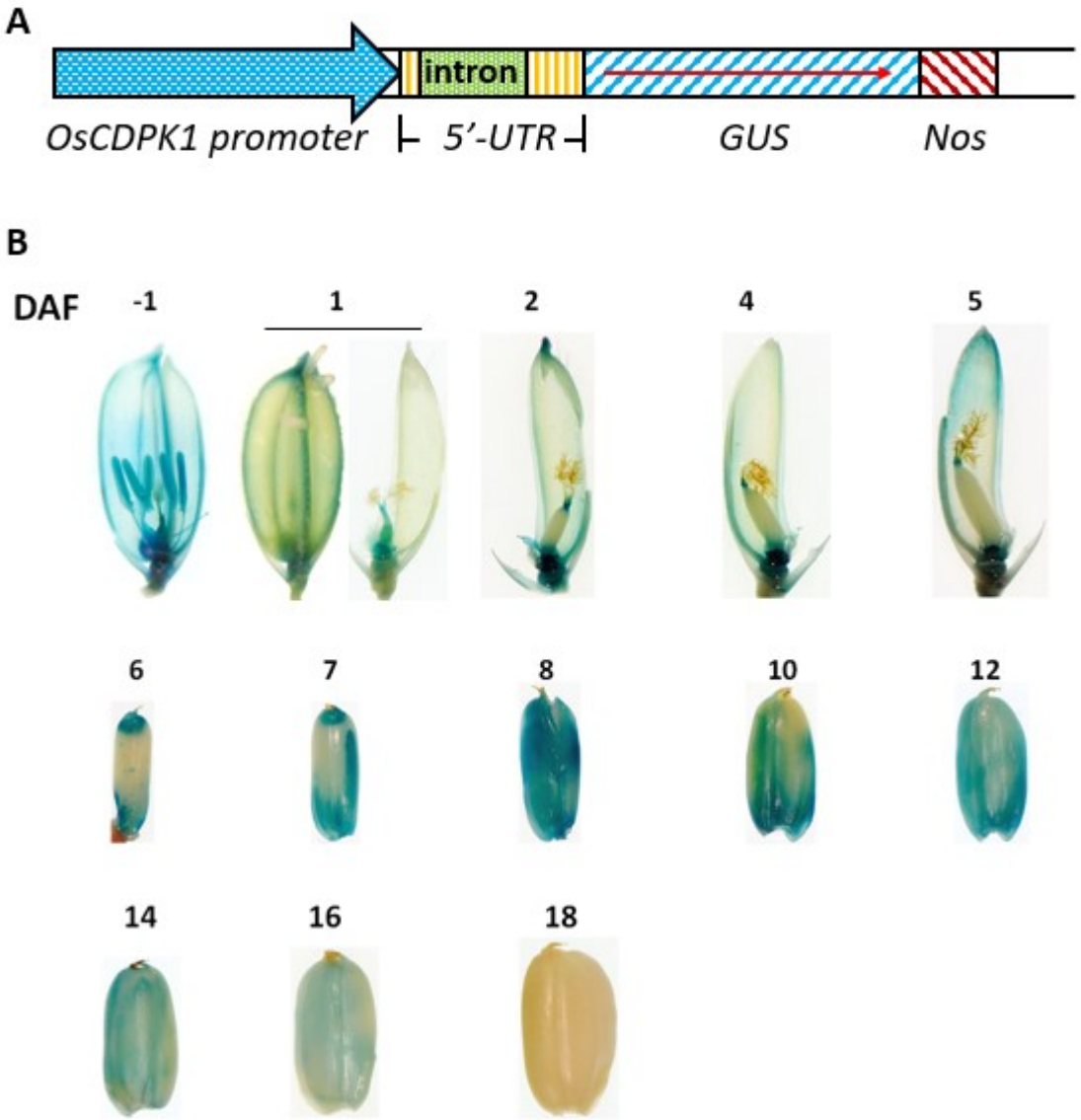

Figure 5. Histochemical GUS (β-glucuronidase) activity staining in flowers and developing rice seeds. (A) Map of the *OsCDPK1::GUS* expression construct. (B) Rice spikelets were collected before flowering and 1–18 days after flowering (DAF). The lemma and palea were partially or completed removed from each spikelet or developing seed before staining. The stained spikelets or immature grains were preserved in 70% ethanol and photographed. −1 DAF means before flowering.

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2.5. Effects of OsCDPK1 on the Levels of Starch-Biosynthesis-Related Genes in Developing Rice Seeds

We further investigated the roles of *OsCDPK1* in rice-seed development by analyzing the expression patterns of twelve genes involved in starch biosynthesis. These genes were granule-bound starch synthase (*OsGBSSI*), starch synthase (*OsSSI*, *OsSSIIa*, *OsSSIIb*, *OsSSIIc*, *OsSSIIIa*, and *OsSSIIIb*), branching enzyme (*OsBEI*), ADP-glucose pyrophosphorylase large subunit (*OsAGPLI*, *OsAGPLII*, and *OsAGPLIII*), and a small subunit of ADP-glucose pyrophosphorylase (O*sAGPSIIb*). The developing WT, *Ri-1*, and *OEtr-1* rice seeds at 5 and 12 DAF were analyzed. Total RNA was isolated from the dehulled embryo-less half seeds and subjected to quantitative RT-PCR. The relative expression levels of *OsAGPLI*, *OsAGPSIIb*, *OsGBSSI*, *OsSSIIc*, and *OsSSIIIa* were significantly up-regulated in *Ri-1* and down-regulated in *OEtr-1* at 12 DAF but there was no significant difference at 5 DAF compared with the genes in WT plants (Figure 6). Expression of the seven other genes in the transgenic lines was not significantly different at 5 or 12 DAF from expression in the WT plants. These results suggest that *OsCDPK1* might be involved in regulating starch-biosynthesis-related genes in the mid-development stage of the rice seed.

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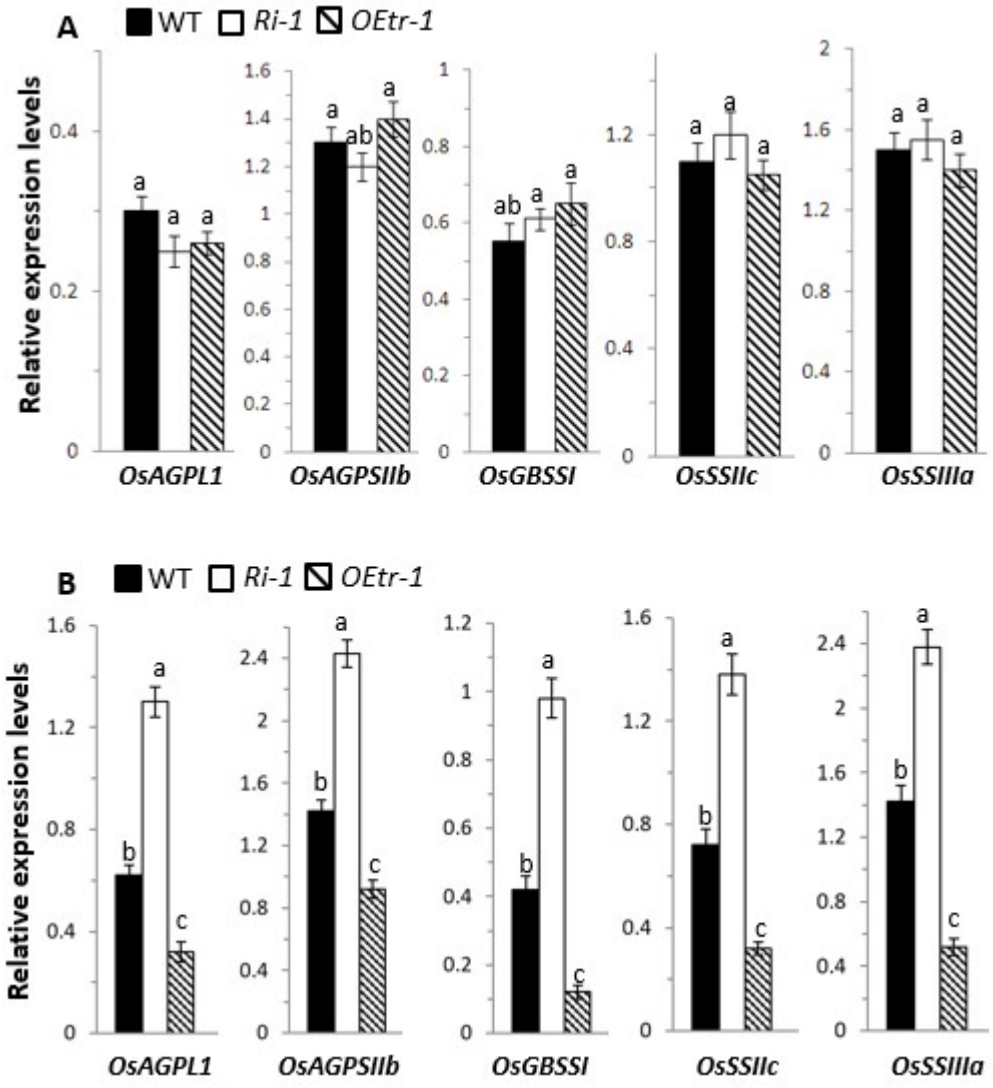

Figure 6. Expression of starch-biosynthesis-related genes during seed development in the WT, *Ri-1*, and *OEtr-1* plants. Total RNA was isolated from developing endosperms 5 and 12 days after flowering (DAF) from the WT, *Ri-1*, and *OEtr-1* plants and subjected to RT-PCR analysis. The relative expression levels of each gene were normalized to the expression level of the internal control *OsActin*. Different letters above the bars indicate significant differences, identified by performing ANOVAs ($P < 0.01$). Each value is the mean ± the standard deviation of three independent measurements. *OsAGPLI*: ADP-glucose pyrophosphorylase large subunit I; *OsAGPSIIb*: ADP-glucose pyrophosphorylase small subunit IIb; *OsGBSSI:* granule-bound starch synthase I; *OsSSIIc:* starch synthase IIc; *OsSSIIIa:* starch synthase IIIa. Primer sets and gene accession numbers are listed in Supplementary Table S1.

3. Discussion

The amylose content of endosperm is an important parameter determining the eating quality of rice, which is negatively related with stickiness but positively related to rice grain hardness [34,35]. However, the upstream signaling regulators that control the amylose content of the endosperm are still largely unidentified. Our results indicate for the first time that the protein kinase OsCDPK1 is functionally negatively correlated with the amylose content, endosperm transparency, and seed size in developing rice seed.

The *OEtr-1* and *Ri-1* seeds had some distinct features compared with the WT seeds. For example, the *OEtr-1* grains were smaller, had lower amylose contents, and had more floury endosperms than the WT grains, and the *Ri*-1 grains were larger, had higher amylose contents, and had more transparent endosperms than the WT grains (Figures 1-4). This indicates that the *OsCDPK1* function is closely associated with the rice endosperm starch properties. The *OsCDPK1::GUS* expression profile in the developing rice grains gradually increased immediately after flowering and reached a maximum between 7 and 14 DAF (Figure 5). The *OsCDPK1::GUS* expression timing in the developing rice seeds was similar to that found in a study by Ohdan et al. [36], in which 27 different genes involved in starch biosynthesis were examined during rice-seed development. In that study, all the genes had been differentially expressed before 15 DAF. In this study, the GUS staining was found throughout the endosperm at 12 and 14 DAF of developing seeds, and was more intensified in the interior region at 12 DAF (Figure S4), suggesting that *OsCDPK1* first affects the expression of starch biosynthesis-related genes and later affects the starch composition and endosperm appearance. The starchy endosperm in the *OEtr-1* grains had a low amylose content and an opaque floury appearance, and the starch granules were small and loosely packed (Figures 1-3), indicating that the *OsCDPK1* roles were closely associated with the structures and qualities of the starch granules during the grain filling process. Moreover, *OsCDPK1::GUS* staining was found throughout the developing endosperm at 7-16 DAF (Figures 5B and S4). These results suggest that the expression of some starch-biosynthesis-related genes in the endosperm cells may be affected by *OsCDPK1*, followed by changing the amylose content and resulting in the opaque endosperm in the *OEtr-1* grains.

Several mutations of rice genes involved in starch biosynthesis have been found to alter the structures and properties of the starch produced. For example, the *SSIIIa* mutation (*flo5*) was found to increase the amylose content, alter the amylopectin structure, and cause the endosperm to have a white core. The *waxy* mutant (a mutation in *GBSSI*) produced an amylose-free, floury endosperm. The *amylose-extender* mutation in *BEIIb* altered the fine structure of amylopectin and gave a floury endosperm. A mutation in *BEI* (starch-branching enzyme I) caused the amylopectin fine structure to change but did not appear to affect the endosperm appearance. Our data show that the expression of some starch-biosynthesis-related genes was affected by *OsCDPK1*, similar to the results of previous studies. During the middle phase (12 DAF) of endosperm development, examples of genes affected were *OsAGPLI*, *OsAGPSIIb*, *OsGBSSI*, *OsSSIIc*, and *OsSSIIIa*, which were significantly up-regulated in the *Ri-1* and down-regulated in *OEtr-1* (Figure 6). Changes in the expression of these genes caused the *OEtr-1* endosperm to have a low amylose content and a floury appearance, whereas the *Ri-1* endosperm had a high amylose content and was more transparent (Figures 1, 2 and 4 and Tables 1 and 2). These results support the idea that *OsCDPK1* acts as an upstream regulator that is closely associated with starch biosynthesis. Some regulators have been found to regulate the expression of genes encoding key starch-biosynthesis enzymes, and null mutations in these regulators usually alter the amylopectin fine structure, the starch composition, the starch granule morphology and size, and the endosperm appearance. For instance, some starch-biosynthesis-related genes were found to be affected in five independent mutants, *flo2* (a tetratricopeptide repeat motif protein) [17], *flo4* (*pyruvate orthophosphate dikinase B*) [19], *flo7* (an unknown protein) [22], *osbzip58* (a bZIP transcription factor) [37], and *osbt1* (an ADP-glucose transporter) [38]. All these effects decreased the amylose content and changed the amylopectin composition. The endosperm appearance was affected differently in different parts, e.g., *flo2* had endosperm with a floury kernel, *flo4* and *osbzip58* and *osbt1* gave white-core phenotypes, and chalkiness was only found in the peripheral endosperm of *flo7*. Similarly, the low amylose content of the *OEtr-1* endosperm was expressed in a floury morphology (Figures 1 and 4). The results of previous studies and this study together indicate that a low-amylose content of rice endosperm gives a chalky or floury phenotype, suggesting that the amount of amylose present is an important factor affecting the quality and appearance of the starchy endosperm. This raises the question of whether rice can be

engineered to have an endosperm with a high amylose content and therefore a transparent appearance. Here, we have provided direct evidence that silencing *OsCDPK1* (*Ri-1*) increases the amylose content of the endosperm, making the starchy grain more transparent (Figures 1 and 4 and Tables 1 and 2). These results will be useful in developing rice-breeding strategies aimed at maintaining (or even improving) grain quality in rice to cope with global warming. The transgenic lines *OEtrs* and *Ris* could also be ideal materials for investigating the mechanisms controlling rice seed size and starch biosynthesis.

The temperature also strongly affects amylose synthesis during rice grain development. In previous studies, a lower temperature increased the expression of the *Waxy* gene and protein and increased the amylose content in developing rice endosperm [39] but a higher temperature had the opposite effects [39,40]. Moreover, during the rice grain-filling process, a temperature higher than the optimum usually causes impaired starch accumulation, resulting in loosely packed starch granules and small air spaces between them, giving high proportions of opaque chalky/floury grains. These effects decrease the market value because the rice will have poor milling qualities (being easily broken), poor cooking and eating qualities, and a poor appearance [41,42]. It has recently been found that a high temperature (33 °C for 12 h light, 28 °C for 12 h dark) also induced the expression of three α-amylase genes, *Amy1A*, *Amy3C*, and *Amy3D*, in developing endosperm, causing the grains to be chalky, probably because of starch degradation and the accumulation of soluble sugars in the endosperm [43]. In contrast, we found that the *Ri-1* endosperm was more transparent at both a low temperature (22 °C for 16 h light, 20 °C for 8 h dark) and a high temperature (31°C for 16 h light, 28 °C for 8 h dark) than that of the WT during the grain-filling process (Figure 2 and Table 1). Our results will be useful in developing rice-breeding strategies aimed at maintaining (or even improving) grain quality in rice selected to cope with global warming. Our results also improve our understanding of the molecular mechanisms involved in amylose biosynthesis.

It has been found in several studies that chalky/floury endosperm might be caused by loosely packed small round starch granules formed in developing rice seeds[9,19,20,38]. The scanning electron microscopy images indicate that the WT and *Ri-1* endosperm contained closely packed polyhedral starch granules but that the white core of the *OEtr-1* endosperm contained loosely packed small starch granules. The starch granules in the *OEtr-1* endosperm were polyhedral (like

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in the WT and *Ri-1* endosperm) rather than small and round as in most chalky/floury mutants (Figure 3B). It is therefore likely that *OsCDPK1* plays a role in starch biosynthesis and negatively affects the sizes but not the shapes of the starch granules.

We previously found that *OsCDPK1* plays a role that feedback inhibition of GA biosynthesis through down-regulating *GA3ox2* and *GA20ox1* [32]. In this study, we put forward a model in which *OsCDPK1* plays key roles in negatively controlling the grain size, amylose content, and endosperm appearance, and also affects the physicochemical properties of the starch (Figure 7). Milled *OsCDPK1*-gene-silenced *Ri-1* grains were larger than WT grains, and there were numerous densely packed polyhedral starch granules accompanied by a high amylose content and a transparent endosperm. In contrast, the *OEtr-1* grains were smaller and contained loosely packed small but still polyhedral starch granules. The *OEtr-1* grains had lower amylose contents and opaque white-cored endosperms. Notably, the phenotypes of the grain size and the floury endosperm in *OEtr-1, -2* and *-3* were consistent and were unaffected by the temperature during the grain-filling process (Figures 1 and 2). *OsCDPK1* therefore plays pleiotropic roles in rice reproductive developmental processes, in a negative sense. Our results indicate that *Ri-1* and *OEtr-1* could be ideal materials for investigating the mechanisms controlling rice seed size and starch biosynthesis, and could also be valuable reference samples for rice breeding aimed at simultaneously improving grain yield and quality.

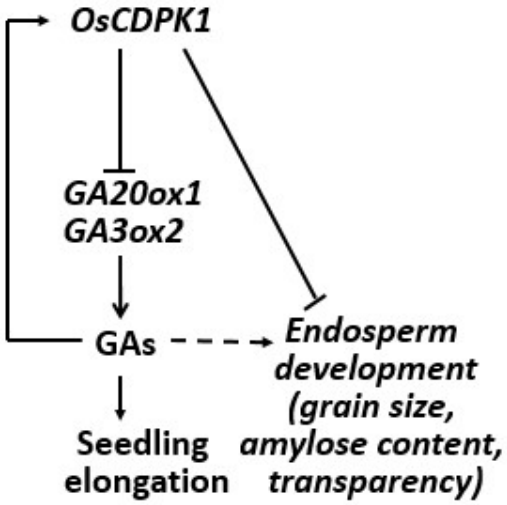

Figure 7. Proposed roles of *OsCDPK1* in the interconnecting GA biosynthesis and signaling pathways and endosperm developmental processes. The dashed arrow indicates the predicted

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pathway. The solid arrows and lines indicate the pathway directly supported by our previous studies and this study. The model is described in detail in the text.

4. Materials and Methods

4.1. Plant Materials

In our previous studies [32], eight independent T1 transgenic lines that carried single transgene in rice was subjected to ectopic overexpression of a constitutively active truncated form of *OsCDPK1* (*OEtr*) in rice seedlings, which all gave a semi-dwarf phenotype that produced small seeds. By contrast, five independent T1 transgenic plants exhibited single copy of transgene was subject to *OsCDPK1* gene silencing (*Ri*) by RNA interference, which all gave a slender-like phenotype during seedling development and subsequently produced large seeds. Due to the consistency of the phenotypic traits in the eight independent lines of *OEtrs* (lines *OEtr-1, -3,- 4, -6, -7, -8, -9* and *-15*), and in the five independent transgenic lines of *Ris* (lines *Ri-1 - -5*) [32]. According to these results, in this study, the T4 transgenic plants from *OEtr-1, OEtr-3* and *OEtr-4,* and *Ri-1, Ri-2* and *Ri-3,* whose carrying homozygous transgenes were selected to explore the roles of *OsCDPK1* in rice grain development.

4.2. Callus Induction

Rice *Oryza sativa* L. cv Tainung 67 was used in the study. Immature seeds were de-hulled, sterilized with 2.4% NaOCl for 30 min, then washed thoroughly with sterile water. The seeds were then incubated on N6 agar medium [44] containing 10 μM 2,4-D to induce calli to form. Calli derived from the scutella were transferred to fresh N6 agar medium containing 2,4-D for one month and was subjected to *Agrobacterium*-mediated gene transformation.

4.3. Primers

The nucleotide sequences of all the primers used in the real-time polymerase chain reaction (qRT-PCR) amplification are shown in Supplementary Table S1.

4.4. Construction of OsCDPK1::GUS Expression Vectors

The *OsCDPK1::GUS* expression vector was constructed by amplifying a 2,007 bp DNA fragment containing the *OsCDPK1* promoter and its 5′-untranslated region (which contained a 607 bp intron) (Figure 1A) by PCR using the forward primer *OsCDPK1-5P* (5'-ATACTGCAGTGGTCTTATT AGGTAAGGCC-3') and the reverse primer *OsCDPK1-3B* (5'-ATAGGATCC TCCAAGAACTCCTTATGCAA-3'). The DNA fragment was cleaved using *Pst*I and *Bam*HI, and then cloned into vector *pBX-2* as described previously [45]. The *OsCDPK1::GUS* construct was linearized by digesting it with *Pst*I, then inserted into the *Pst*I site of the *pSMY1H* binary vector [45], then it was subjected to *Agrobacterium*-mediated gene transformation.

4.5. Plant Transformation

Recombinant binary plasmids were introduced into *Agrobacterium tumefaciens* strain EHA101 by electroporation, and rice calli were transformed as described previously [45].

4.6. Histochemical Staining of GUS Activity in Developing Rice Grains

Rice spikelets collected before flowering and 1–18 day after flowering (DAF) were subjected to GUS activity staining to assess OsCDPK1 gene expression profiles in developing rice grains. The lemma and palea were partially or completely removed from each spikelet (developing seed) before the staining process, then the spikelets were incubated in a 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide solution (in 100 mM sodium phosphate containing 10 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 0.1% Triton X-100, at pH 7.0)

at 37 °C in the dark for 4 h. The stained spikelets or immature grains were then preserved in 70% ethanol and rinsed with water before being photographed.

4.7. Quantitative RT-PCR

Developing seeds were collected from wild-type (WT), *Ri-1*, and *OEtr-1* at 5 and 12 DAF, respectively. Total RNA was isolated from developing endosperms by using TRIzol reagent (Invitrogen, Carlsbad, California, USA) and then DNA contamination was removed using a TURBO DNA-free kit (Ambion, Foster, USA). A 5 μg aliquot of the total RNA was used to synthesize first strand cDNA using M-MuLV reverse transcriptase (New England Biolabs) and oligo (dT) primer. Quantitative RT-PCR was performed using an Eco Real-Time PCR System (Illumina, San Diego, CA, USA) following the manufacturer's instructions. Gene-specific primer sets (Table S1) localized at the 3'-untranslated regions for each gene examined were selected to allow assessment of the extent to which the starch-biosynthesis-related genes in WT, *OEtr-1*, and *Ri-1* were expressed. The relative expression levels were normalized to expression in the internal control, *OsActin 1*. All reactions were performed in triplicate.

4.8. Analysis of the Gelatinization Properties of the Starch

A 20 mg aliquot of rice endosperm powder derived from de-embryonic seeds was mixed with 1 mL of urea solution at a concentration of between 0 and 9 M, and the mixture was shaken vigorously for 24 h at room temperature. The mixture was then centrifuged, and the volume of the gelatinized starch sediment was measured.

4.9. Apparent Amylose Content Analysis

A 20 mg aliquot of rice endosperm powder was gelatinized by adding 2 mL of 1 N NaOH and incubating the mixture at 25 °C for 24 h. Then, 4 mL of 1 N CH_3COOH was added, the mixture was mixed well, and 4 mL H_2O was added. A 0.8 mL aliquot of the solution was mixed with 0.2 mL I_2/KI (0.2%/2%), then 4 mL H_2O was added. The apparent amylose content was measured

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using the colorimetric method described by [33]. Absorbance at 620 nm was measured, and the apparent amylose content was determined by comparing the absorbance to a calibration curve prepared using potato amylose standards.

4.10. Scanning Electron Microscopy

Dehusked rice grains were cut transversely and analyzed using a scanning electron microscope (Quanta 200; FEI, Hillsboro, OR, USA) following the manufacturer's instructions.

5. Conclusions

Our results demonstrated that *OsCDPK1* plays key roles in negatively controlling the grain size, amylose content, and endosperm appearance, and also affects the physicochemical properties of the starch. Milled *OsCDPK1*-gene-silenced *Ri-1* grains were larger than WT grains, and there were numerous densely packed polyhedral starch granules accompanied by a high amylose content and a transparent endosperm. In contrast, the *OEtr-1* grains were smaller and contained loosely packed small but still polyhedral starch granules. The *OEtr-1* grains had lower amylose contents and opaque white-cored endosperms. Moreover, the grain phenotypes in *OEtrs* were unaffected by the temperature during the grain-filling process. *OsCDPK1* therefore plays pleiotropic roles in rice reproductive developmental processes, in a negative sense. Our results indicate that *Ri-1* and *OEtr-1* could be ideal materials for investigating the mechanisms controlling rice seed size and starch biosynthesis, and for rice breeding to improve grain yield and quality.

Author Contributions

Conceptualization, Shin-Lon Ho; Data curation, Jian-Zhi Jiang and Shin-Lon Ho; Investigation, Jian-Zhi Jiang, Chun-Hsiang Kuo, Bo-Hong Chen and Mao-Kei Chen; Methodology, Choun-Sea Lin; Supervision, Shin-Lon Ho; Writing – original draft, Shin-Lon Ho.

Acknowledgments

This work was supported by grants from the Ministry of Science and Technology of the Republic

of China (grant no. MOST 105-2313-B-415-009-, MOST 106-2313-B-415-006- and MOST 107-2313-B-415-006-).

Conflicts of Interest

The authors declare no conflict of interest

References

1. Rabiei B.; Valizadeh M.; Ghareyazie B.; Moghaddam M.; Ali A.J. Identification of QTLs for rice grain size and shape of Iranian cultivars using SSR markers. *Euphytica* **2004**, *137,* 325-332.
2. Bao J.; Jin L.; Xiao P.; Shen S.; Sun M.; Corke H. Starch physicochemical properties and their associations with microsatellite alleles of starch synthesizing genes in a rice RIL population. *J. Agric. Food Chem.* **2008**, *56,* 1589-1594.
3. Fitzgerald M.A.; Lisle A.J.; Martin M. Chalky and translucent rice grains differ in starch composition and structure and cooking properties. *Cereal Chem.* **2000**, *77*, 627-632.
4. Jiang H.; Dian W.; Wu P. Effect of high temperature on fine structure of amylopectin in rice endosperm by reducing the activity of the starch branching enzyme. *Phytochemistry* **2003***, 63*, 53-59.
5. Morita S.; Shiratsuchi H.; Takanashi J.; Fujita K. Effect of high temperature on grain ripening in rice plants: analysis of the effects of high night and high day temperatures applied to the panicle and other parts of the plant. *Jpn. J. Crop Sci.* **2004**, *73*, 77-83.
6. Counce P.A.; Bryant R.J.; Bergman C.J.; Bautista R.C.; Wang Y.J.; Siebenmorgen T.J.; Moldenhauer K.A.; Meullenet J.F.C. Rice milling quality, grain dimensions, and starch branching as affected by high night temperatures. *Cereal Chem.* **2005***, 82*, 645-648.
7. Patindol J.; Wang Y.J. Fine structures and physicochemical properties of starches from chalky and translucent rice kernels. *J. Agric. Food Chem.* **2003***, 51*, 2777-2784.
8. Yamakawa H.; Hirose T.; Kuroda M.; Yamaguchi T. Comprehensive expression profiling of rice grain filling-related genes under high temperature using DNA microarray. *Plant Physiol.* **2007**, *144*, 258-277.

9. Fu F.F.; Xue H.W. Coexpression analysis identifies rice starch regulator1, a rice AP2/EREBP family transcription factor, as a novel rice starch biosynthesis regulator. *Plant Physiol.* **2010**, *154*, 927-938.

10. Mizuno K.; Kimura K.; Arai Y.; Kawasaki T.; Shimada H.; Baba T. Starch branching enzymes from immature rice seeds. *J. Biochem.* **1992**, *112*, 643-651.

11. Smith A.M.; Denyer K.; Martin C. The synthesis of the starch granule. Annu. Rev. Plant Physiol. *Plant Mol. Biol.* **1997**, *48*, 67-87.

12. Nishi A.; Nakamura Y.; Tanaka N.; Satoh H. Biochemical and genetic analysis of the effects of amylose-extender mutation in rice endosperm. *Plant Physiol*. **2001**, *127*, 459-472.

13. Singh B.K.; Preiss J. Starch branching enzymes from maize. *Plant Physiol.* **1985**, *79*, 34-40.

14. Nakamura Y.; Takeichi T.; Kawaguchi K.; Yamanouchi H. Purification of two forms of starch branching enzyme (Q-enzyme) from developing rice endosperm. *Physiol. Plant.* **1992**, *84*, 329-335.

15. Satoh H.; Omura T. New endosperm mutations induced by chemical mutagens in rice, *Oryza sativa* L. *Jpn. J. Breed.* **1981**, *31*, 316-326.

16. Kaushik R.P.; Khush G.S. Genetic analysis of endosperm mutants in rice *Oryza sativa* L. *Theor. Appl. Genet*. **1991**, *83*, 146-152

17. She K.C.; Kusano H.; Koizumi K.; Yamakawa H.; Hakatae M. et al. A novel factor FLOURY ENDOSPERM 2 is involved in regulation of rice grain size and starch quality. *Plant Cell* **2010**, *22*, 3280-3294.

18. Nishio T.; Iida S. (1993) Mutant having a low content of 16-kDa allergenic protein in rice (Oryza sativa L.). *Theor. Appl. Genet.* **1993**, *86*, 317-321.

19. Kang H.G.; Park S.; Matsuoka M.; An G. White-core endosperm floury endosperm-4 in rice is generated by knockout mutations in the C_4-type pyruvate orthophosphate dikinase gene (*OsPPDKB*). *Plant J.* **2005**, *42*, 901-911.

20. Ryoo N.; Yu C.; Park C.S.; Baik M.Y.; Park I.M.; Cho M.H.; Bhoo S.H.; An G.; Hahn T.R.; Jeon J.S. Knockout of a starch synthase gene *OsSSIIIa/Flo5* causes white-core floury endosperm in rice (Oryza sativa L.). *Plant Cell Rep.* **2007**, *26*, 1083-1095

21. Peng C.; Wang Y.H.; Liu F.; Ren Y.L.; Zhou K.N.; Lv J.; Zheng M.; Zhao S.L.; Zhang L.; Wang C.M.; Jiang L.; Zhang X.; Guo X.P.; Bao Y.Q.; Wan J.M. *FLOURY ENDOSPERM6* encodes a CBM48 domain-containing protein involved in compound granule formation and starch synthesis in rice endosperm. *Plant J.* **2014**, *77*, 917-930.

22. Zhang L.; Ren Y.L.; Lu B.Y.; Yang C.Y.; Feng Z.M.; Liu Z. et.al. *FLOURY ENDOSPERM7* encodes a regulator of starch synthesis and amyloplast development essential for peripheral endosperm development in rice. *J. Exp. Bot*. **2016**, *67*, 633-647.

23. Sanders D.; Pelloux J.; Brownlee C.; Harper J.F. Calcium at the crossroads of signaling. *Plant Cell* **2002**, *14*, S401-S417.

24. Harper J.F.; Breton G.; Harmon A. Decoding Ca^{2+} signals through plant protein kinases. *Annu. Rev. Plant Biol.* **2004**, *55*, 263-288.

25. Mori I.C.; Murata Y.; Yang Y.; Munemasa S.; Wang Y.F.; Andreoli S.; et.al. CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca^{2+}-permeable channels and stomatal closure. *PLoS Biol*. **2006**, *4*, e327.

26. Ray S.; Agarwal P.; Arora R.; Kapoor S.; Tyagi A.K. Expression analysis of calcium-dependent protein kinase gene family during reproductive development and abiotic stress conditions in rice (*Oryza sativa* L. ssp. indica). *Mol. Genet. Genomics* **2007**, *278*, 493-450.

27. Ludwig A.A.; Romeis T.; Jones J.D. CDPK-mediated signalling pathways: specificity and cross-talk. *J. Exp. Bot*. **2004**, *55*, 181-188.

28. Romeis T.; Ludwig A.A.; Martin R.; Jones J.D.G. (2001) Calcium-dependent protein kinases play an essential role in a plant defence response. *EMBO J.* **2001**, *20*, 5556-5567.

29. Abbasi F.; Onodera H.; Toki S.; Tanaka H.; Komatsu S. OsCDPK13, a calcium-dependent protein kinase gene from rice, is induced by cold and gibberellin in rice leaf sheath. *Plant Mol. Biol.* **2004**, *55*, 541-552.

30. Wan B.; Lin Y.; Mou T. Expression of rice Ca(2+)-dependent protein kinases (CDPKs) genes under different environmental stresses. *FEBS Lett.* **2007**, *581*, 1179-1189.

31. Asano T.; Kunieda N.; Omura Y.; Ibe H.; Kawasaki T.; Takano M.; Sato M.; Furuhashi H.; Mujin T.; Takaiwa F.; Wu C.C.; Tada Y.; Satozawa T.; Sakamoto M.; Shimada H. Rice SPK, a calmodulin-like domain protein kinase, is required for storage product

accumulation during seed development: phosphorylation of sucrose synthase is a possible factor. *Plant Cell* **2002**, *14*, 619-628.

32. Ho S.L.; Huang L.F.; Lu C.A.; He S.L.; Wang C.C.; Yu S.P.; Chen J.; Yu S.M. Sugar starvation- and GA-inducible calcium-dependent protein kinase 1 feedback regulates GA biosynthesis and activates a 14-3-3 protein to confer drought tolerance in rice seedlings. *Plant Mol. Biol*. **2013**, *81*, 347-361.
33. Juliano B.O. A simplified assay for milled-rice amylose. *Cereal Sci. Today* **1971**, *16*, 334-340.
34. Cameron D.K.; Wang Y.J. A better understanding of factors that affect the hardness and stickiness of long-grain rice. *Cereal Chem*. **2005**, *82*, 113-119.
35. Li H.; Prakash S.; Nicholson T.M.; Fitzgerald M.A.; Gilbert R.G. The importance of amylose and amylopectin fine structure for textural properties of cooked rice grains. *Food Chem*. **2016**, *196*, 702-711.
36. Ohdan T.; Francisco Jr P.B.; Sawada T.; Hirose T.; Terao T.; Satoh H.; Nakamura Y. Expression profiling of genes involved in starch synthesis in sink and source organs of rice. *J. Exp. Bot*. **2005**, *56*, 3229-3244.
37. Wang J.C.; Xu H.; Zhu Y.; Liu Q.Q.; Ca X.L. OsbZIP58, a basic leucine zipper transcription factor, regulates starch biosynthesis in rice endosperm. *J. Exp. Bot*. **2013**, *64*, 3453-3466. doi:10.1093/jxb/ert187
38. Li S.; Wei X.; Ren Y.; Qiu J.; Jiao G.; Guo X.; Tang S.; Wan J.; Hu P. *OsBT1* encodes an ADP-glucose transporter involved in starch synthesis and compound granule formation in rice endosperm. *Sci. Rep*. **2017**, *7*, 40124. doi:10.1038/srep40124
39. Sano Y.; Maekawa M.; Kikuchi H. Temperature effects on the Wx protein level and amylose content in the endosperm of rice. *J. Hered*. **1985**, *6*, 221-222
40. Asaoka M.; Okuno K.; Sugimoto Y.; Kawakami J.; Fuwa H. Effect of environmental temperature during development of rice plants on some properties of endosperm starch. *Starch – Stärke* **1984**, *36*, 189-193.
41. Lyman N.B.; Jagadish S.V.K.; Nalley L.L.; Dixon B.L.; Siebenmorgen T. Neglecting rice milling yield and quality underestimates economic losses from high-temperature stress. *PLoS ONE* **2013**, *8*, e72157.

42. Shi W.; Yin X.; Struik P.C.; Solis C.; Xie F.; Schmidt R.C.; Huang M.; Zou Y.; Ye C.; Jagadish S.V.K. High day- and night-time temperatures affect grain growth dynamics in contrasting rice genotypes. *J. Exp. Bot*. **2017**, *68*, 5233-5245. doi:10.1093/jxb/erx344

43. Nakata M.; Fukamatsu Y.; Miyashita T.; Hakata M.; Kimura R.; Nakata Y.; Kuroda M.; Yamaguchi T.; Yamakawa H. High temperature-induced expression of rice α-amylases in developing endosperm produces chalky grains. *Front. Plant Sci*. **2017**, *8*, 2089. doi: 10.3389/fpls.2017.02089

44. Chu C.C.; Wang C.C.; Sun C.S.; Hsu C.; Yin K.C.; Chu C.Y.; Bi F.Y. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Sci. Sin*. **1975**, *5*, 659–668.

45. Ho S.L.; Tong W.F.; Yu S.M. Multiple mode regulation of a cysteine proteinase gene expression in rice. *Plant Physiol.* **2000**, *122*, 57-66.

Supplementary information

Table S1 Primers used in real time RT-PCR

Name	Sequence
OsAGPLI-RT5	5'-CGCTGATGCC ACAGATCTCT-3'
OsAGPLI-RT3	5'-GCCATGTGGAATCAACGAGC-3'
OsAGPLII-RT5	5'- GCATAGATAGGCCTTGGAAT -3'
OsAGPLII-RT3	5'-TACGCTATCCGTCTTGAATC -3'
OsAGPLIII-RT5	5'-CAGCCATGACCATTTGCGGC-3'
OsAGPLIII-RT3	5'-TAGATTCACGAGACAATGAT-3'
OsAGPSIIb-RT5	5'- GCAACCAATCACCACTGTAC-3'
OsAGPSIIb-RT3	5'- GGCAGCATGGAATAAACCAC-3'
OsGBSSI-RT5	5'-AGATCTACATATGGAGTGAT -3'
OsGBSSI-RT3	5'-GCCTATATAATTGGCTATAG -3'
OsSBEI-RT5	5'-GATTTCTTGATCAGGAGCAA -3'
OsSBEI-RT3	5'-ATAGGTACATAGCAACTGCT-3'
OsSSI-RT5	5'-ATCAACCATATGTCATGTAA-3'
OsSSI-RT3	5'-GTACAAAGTTTTCATTCCGC-3'
OsSSIIa-RT5	5'-TATAGCTATAGCCTCCCTGA-3'
OsSSIIa-RT3	5'-TCAACGCACAGTACGGTCAG -3'
OsSSIIb-RT5	5'-CCTCTGGATCCCGCCGTGGA-3'
OsSSIIb-RT3	5'- GCAGCGTGCCAGTCATCGTG-3'
OsSSIIc-RT5	5'- CTCGACGGTTGTTCAGTCAT-3'
OsSSIIc-RT3	5'-CACAGGAAGATGTAAGCCAT-3'
OsSSIIIa-RT5	5'- AACGGTGGCAAGAGAAAGCA -3'
OsSSIIIa-RT3	5'- GTTAATTTGTATCTGCAGCT -3'
OsSSIIIb-RT5	5'- ACACATGGATTTTGGGAAGT -3'
OsSSIIIb-RT3	5'-TTCTTTCTGTCTAAAGAATG -3'

Gene accession number*:*
OsAGPLI: ADP-glucose pyrophosphorylase large subunit I (D50317)
OsAGPLII: ADP-glucose pyrophosphorylase large subunit II (U66041)
OsAGPLIII: ADP-glucose pyrophosphorylase large subunit III (AK069296)
OsAGPSIIb: ADP-glucose pyrophosphorylase small subunit IIb (XM_015756738)
OsGBSSI: granule-bound starch synthase I (X62134)
OsSBEI: Starch branching enzyme I (AF136268)
OsSSI: starch synthase I *(D16202)*
OsSSIIa: Starch synthase IIa *(AF419099)*
OsSSIIb: Starch synthase IIb *(AF395537)*
OsSSIIc: starch synthase IIc (AF383878)
OsSSIIIa: starch synthase IIIa (AY100469)
OsSSIIIb: Starch synthase IIIb *(AF432915)*

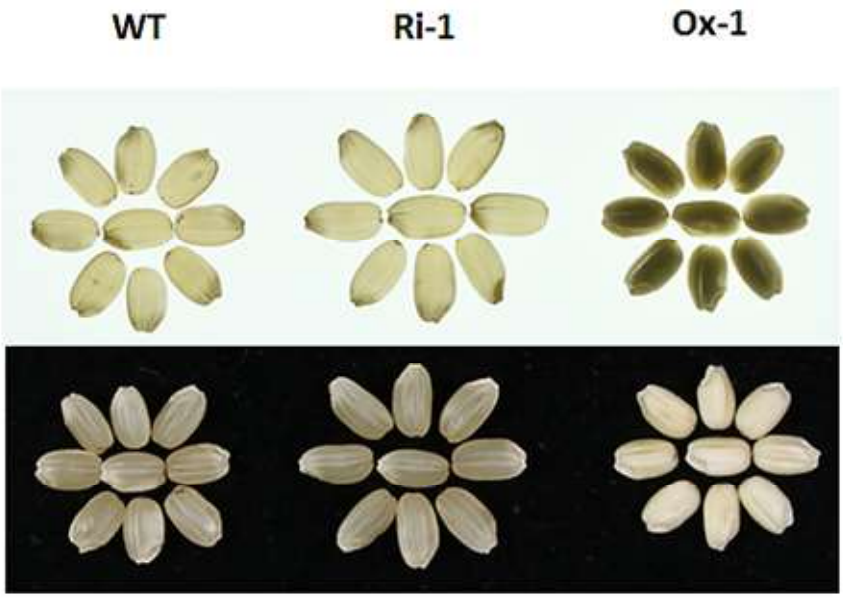

Figure S1. Comparison of the transparent appearance of endosperm between Wt, *OEtr-1* and *Ri-1* grains. Rice grains are illuminated with backlight (top panel) or normal lighting (bottom panel) conditions.

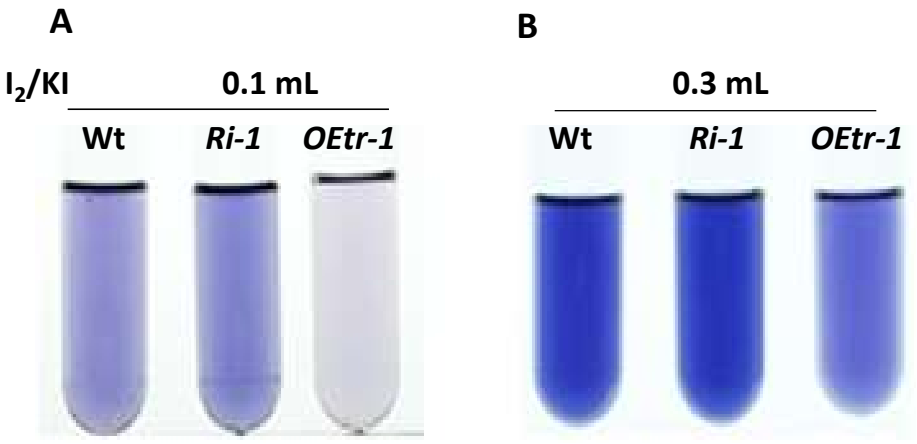

Figure S2. Iodine-staining of endosperm starch in Wt, *OEtr-1* and *Ri-1*. Twenty milligrams of endosperm powder were treated with 1N NaOH as described in materials and methods. Supernatants of the starch samples was mixed with (A) 0.1 mL or (B) 0.2 mL I_2/KI solution.

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Peer-reviewed version available at Int. J. Mol. Sci. 2018, 19, 3247; doi:10.3390/ijms19103247

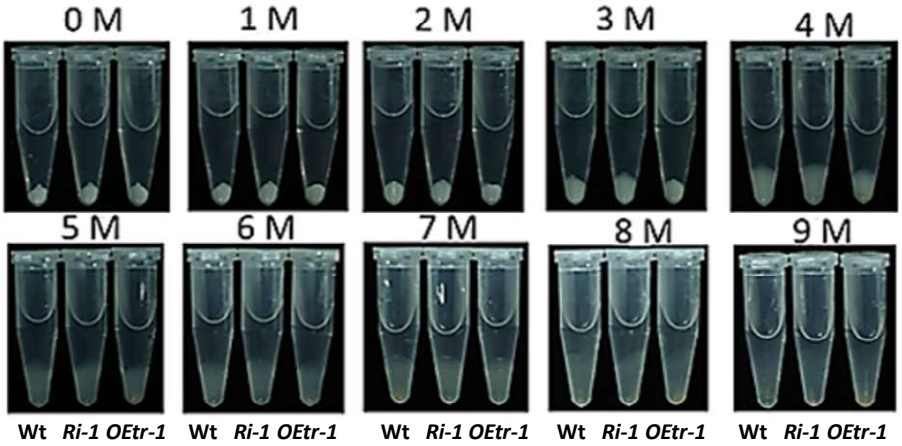

Figure S3. Effect of various concentration of urea on the gelatinization properties of endosperm starch in Wt, *OEtr-1* and *Ri-1*. Twenty milligrams of rice powder in an eppendorf tube was mixed with 1 mL of urea solution and shaken for 24 h at 25°C. After centrifugation, the volume of the gelatinization of starch swollen sediment was measured and recorded.

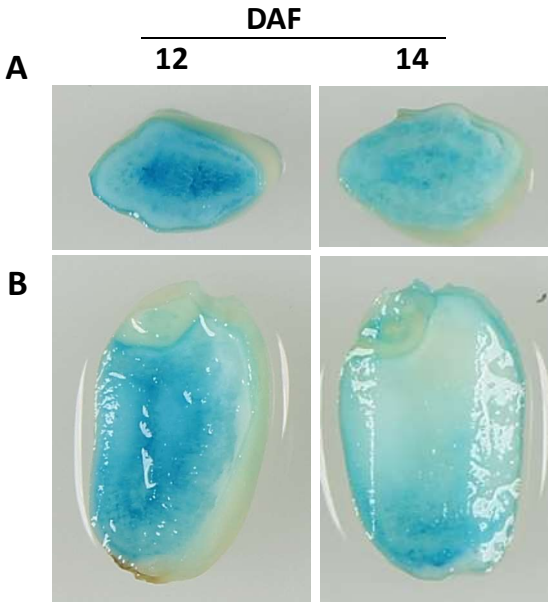

Figure S4. Histochemical GUS (β-glucuronidase) activity staining in cross and longitudinal section developing rice seeds. Immature rice seeds were collected at 12 and 14 DAF, respectively. The lemma and palea was completed removed carefully from the immature seeds. The cross section (A) and longitudinal section (B) of the seeds were cutted with a knife. The cutted of immature grains were stained and preserved in 70% ethanol and photographed. DAF: days after flowering.