Plastidic pyruvate dehydrogenase complex E1 component subunit Alpha1 is involved in galactolipid biosynthesis required for amyloplast development in rice

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Introduction

Plastids arose from an ancestor of extant cyanobacteria that was engulfed by a eukaryote and exist in different forms including leukoplasts, chromoplasts and chloroplasts according to the requirements of specialized tissues in high plants (Jarvis and López-Juez, 2013; Sakamoto and Miyagishima, 2008; Yoon et al., 2004). The amyloplast, a terminally differentiated plastid, is a large organelle for starch biosynthesis and storage in the cereal endosperm cells. Starch accumulates inside amyloplasts and fills most of the intracellular spaces in the form of SGs that readily stain with iodine solution and can be observed with a light microscope (Matsushima et al., 2010). The morphology of SGs in the endosperm of Poaceae plants is diverse (Matsushima et al., 2010, 2013; Tateoka, 1962). For instance, each amyloplast in rice (Oryza sativa) endosperm synthesizes only compound SGs, whereas only simple SGs are produced in maize (Zea mays), barley (Hordeum vulgare) and wheat (Triticum aestivum). Simple SGs are classified as bimodal or uniform. Barley and wheat produce bimodal SGs consisting of both small and much larger simple SGs in the same cells. Maize develops uniform SGs of similar size. To clarify the molecular mechanisms underlying the formation of starch grains scientists have identified various mutants with abnormal SGs in cereal endosperm. For example, rice mutants substandard starch grain (ssg4) and ssg6 develop much larger starch grains in endosperm (Matsushima et al., 2014, 2016). Maize DEK5 protein which is homologous to rice SSG4 influences plastid envelope biogenesis. A dek5 mutant had larger and fewer amyloplasts and chloroplasts with multiple ultrastructural defects (Zhang et al., 2019). In addition, mutation of Opaque5 (encoding monogalactosyldiacylglycerol synthase) in maize affected total galactolipids necessary for plastid membranes, leading to opaque kernels phenotype (Myers et al., 2011). In rice, FSE1 encodes a phospholipase-like protein homologous to phosphatidic acid-prefering phospholipase A1 (PA-PLA1) and is crucial for synthesis of galactolipids required for amyloplast...
development (Long et al., 2018). Taken together, these studies identify the essential roles of amyloplast membranes for SG formation during cereal endosperm development.

Monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are the main constituents of plastid membranes in plant cells. MGDG is synthesized by MGDG synthase (EC 2.4.1.46) which transfers a galactose from UDP-galactose to sn-1,2-diacylglycerol (DAG) (Joyard and Douce, 1987); DGDG is synthesized by DGDG synthase (EC 2.4.1.241) by adding a galactose from UDP-galactose to MGDG (Heemskerk et al., 1990). The direct precursor, diacylglycerol (DAG) can be derived from two metabolic pathways, the prokaryotic and the eukaryotic pathways. In the prokaryotic pathway, the DAG fatty acid backbone is synthesized in the plastid and contains a 16-carbon unsaturated fatty acid at the sn-2 position and 18-carbon at the sn-1 position. In the eukaryotic pathway, the backbone is provided by the endoplasmic reticulum and contains an 18-carbon unsaturated fatty acid at the sn-2 position and an 18 or 16-carbon at the sn-1 position (Li-Beisson et al., 2017). In chloroplasts of higher plants, MGDG and DGDG account for 50% and 20% of total lipids, respectively, and play specific roles in photosynthesis. Unique characteristics of MGDG which does not form bilayers in mixtures with water at room temperature, and DGDG which could form bilayers, may be critical for organization and structure of thylakoid membranes (Lee, 2000). Moreover, galactolipids are directly bound to the photosynthetic reaction centres (Jordan et al., 2001; Loll et al., 2005, 2007). In addition to the important functions in photosynthetic membranes, the MGDG and DGDG play a key role in determining the structure and function of non-photosynthetic membranes. For instance, galactolipid constitutes more than 90% of the total polar lipids in maize seedling leaves, as well as 42% in developing endosperm. MGDG accounts for about one-third of the total galactosylated diacylglycerol (GDG) in endosperm and two-thirds in leaves (Myers et al., 2008; Gayral et al., 2011). Changes in polar lipid composition or content can result in organized granal stacks of thylakoid membranes and abnormal compound granules separated by amyloplast membrane. According to recent studies, the cereal amyloplast and chloroplast have the similar mechanisms for ER-plastid lipid trafficking, fatty acid and galactolipid biosynthesis (Dupont, 2008; Gayral et al., 2019). Moreover, genes encoding the enzymes and transporters involved in these metabolic processes are expressed in maize endosperm (Gayral et al., 2019). Therefore, it suggested that galactolipids produced in amyloplasts might affect starch synthesis and morphology of starch grains during cereal endosperm development, although the molecular mechanism remains largely unknown.

The pyruvate dehydrogenase complex (PDC) plays key roles in cellular anabolism and catabolism. It catalyses the irreversible reaction in which pyruvate provided by the glycolytic pathway is decarboxylated and oxidized sequentially to produce acetyl-CoA and NADH. As a large multienzyme complex, PDC from all living organisms contains three separate enzymes: pyruvate dehydrogenase (PDH; E1; EC1.2.4.1), dihydrolipoamide transacetylase (E2; EC 2.3.1.12) and dihydrolipoamide dehydrogenase (E3; EC 1.8.1.4) (Reed, 1974). Among them, E1 is a heterotetramer consisting of two subunits (a and b), whereas both E2 and E3 are single subunits with multiple binding domains (Faure et al., 2000). In addition to cytosolic PDC in prokaryotes, eukaryotes have at least one PDC localized in the mitochondria. However, plant cells contain different PDCs that are located within the mitochondria and plastids, respectively (Elias and Givan, 1979; Randall and Rubin, 1977; Reid et al., 1977; Rubin and Randall, 1977; Williams and Randall, 1979). The mitochondrial PDC (mtPDC) connects cytoplasmic glycolysis with the tricarboxylic acid cycle, whereas the plastidial PDC (ptPDC) supplies acetyl-CoA and NADH for de novo fatty acid biosynthesis (Camp and Randall, 1985; Ke et al., 2000; Rapp and Randall, 1980). In 1977, the ptPDC was first identified in leucoplasts of developing castor bean endosperm where it is responsible for fatty acid synthesis and storage (Reid et al., 1977). Subsequently, the ptPDC in green leaves of pea seedlings was reported and the complex enzyme activity was analysed (Williams and Randall, 1979). Finally, cloning and analysis of the Arabidopsis thaliana E1–α and β of the ptPDC complex revealed that plastidial E1 subunits show higher homology to the Porphyra purpurea chloroplast odpA and odpB proteins than to the Arabidopsis mitochondrial E1 sequences (Johnston et al., 1997). Despite the known role of ptPDC in fatty acid biosynthesis, knowledge of its effects on plant growth and development is limited.

In this study, we identified a rice flo19 mutant that displayed an opaque inner endosperm. FLO19 encodes a plastidic pyruvate dehydrogenase complex E1 component subunit α1 (ptPDC-E1-α1). Enzyme assays suggested that the flo19 mutant had much reduced ptPDC activity. Both the galactolipid and starch contents in flo19 seeds were remarkably decreased. Our results indicate that FLO19 in rice is involved in galactolipid biosynthesis which is essential for amyloplast development and starch biosynthesis. FLO19 overexpression resulted in increased grain weight, suggesting a possible application in rice improvement.

Results
Phenotypic characterization of the flo19 mutant
To better understand detailed mechanisms underlying starch biosynthesis in endosperm development, we performed a mutant screen of an ethyl methanesulfonate (EMS)-treated rice cultivar Diangengyou1 population and isolated a flo19 mutant which produced seeds with opaque inner endosperm, but translucent in the peripheral region (Figure 1a–d). Scanning electron microscopy (SEM) showed loosely packed and spherical starch granules and large numbers of small starch granules in the floury-white mutant endosperm compared with tightly packed and polyhedral starch granules in the wild type (Figure 1e–h). The flo19 mutant had a slower grain filling rate and reduced 1000-grain weight (Figure 1i; Table S1). Consistently, grain length, thickness and width were markedly reduced in the mutant (Table S1). Further analyses found lower contents of total starch and amylose in mutant kernels (Figure 1j,k); crude protein content was also lower, but lipid content was higher compared to the wild-type grain (Figure 1l, Table S1). Thus, FLO19 plays an important role in endosperm development and plant growth.

Abnormal SGs and defective starch biosynthesis in flo19 mutant endosperm
During endosperm development in rice, large numbers of compound SGs are formed filling the endosperm cells. To observe the structure of the compound SGs, semithin sections of developing endosperm at 6, 9, 12 days after flowering (DAF) were examined. In the central region of the wild-type endosperm, most amyloplasts were well developed and filled with densely packed, polyhedral and sharp-edged compound
granules (Figure 2a, i–iii), whereas two types of abnormal amyloplasts were readily observed in the flo19 mutant (Figure 2a, iv–vi). One type contained irregular single starch granules that weakly stained with iodine. The other type had scattered starch granules, some of which were well stained with iodine. Transmission electron microscopy of the amyloplasts at 6 and 9 DAF confirmed the semithin section results (Figure 2b). These results clearly indicated that formation of compound starch grains during endosperm development was significantly affected by the flo19 mutation.

The abnormal SG development likely affects starch biosynthesis. Real-time PCR analysis showed that expression levels of most genes related to starch synthesis were down-regulated in the flo19 mutant compared to the wild type (Figure 2c). The activity of ADP-glucose pyrophosphorylase (AGPase), a rate-limiting enzyme in starch biosynthesis, in developing endosperm at the nine DAF was significantly lower than in the wild type (Figure 2d).

Further, analysis of starch physicochemical properties showed that the flo19 had a higher proportion of amylopectin chains with a degree of polymerization (DP) between eight and 15 and a lower proportion with a DP between 16 and 25 than the wild type (Figure S2a). Moreover, the viscosity pattern of flo19 mutant pasting starch was similar to the wild type with increasing temperature, but the level was much lower (Figure S2b). In the wild type, starch solubilities in urea solution were similar to that of the flo19 mutant, with both beginning to gelatinize in 4 M urea and being completely gelatinized in 9 M urea (Figure S2c).

Therefore, the flo19 mutation caused defects in amyloplast development in rice.
development and starch biosynthesis during rice endosperm development.

Molecular characterization of the FLO19 locus

To identify the underlying gene, the flo19 mutant was crossed with an indica variety IR36 and then self-pollinated to generate an F2 mapping population. With a total of 10 recessive individuals, the FLO19 locus was initially mapped to the short arm of chromosome 4 between the markers P1 and P9. Moreover, fine mapping was performed by using a total of 2000 recessive individuals and FLO19 was narrowed down to an 80.5 kb region between markers P4 and P5, which contains 12 open reading frames (ORFs) (Figure 3a). Sequence analysis showed that the 11th ORF (LOC_Os04g02900) in flo19 had a single nucleotide substitution of C to T in the second exon, leading to the transition of alanine to valine (Figure 3b).

A genetic complementation test performed to validate whether the mutation was responsible for the flo19 mutant phenotype generated 10 positive transgenic plants. All plants had restored transparent endosperm and starch granule morphologies similar to the wild type (Figure 3c,d and S3b, d). Notably, we investigated generated 10 positive transgenic plants. All plants had restored LOC_Os04g02900 is the underlying gene for the mutation was responsible for the flo19

Figure 2 Compound starch grain formation, expression profiles and enzymatic activity analyses of genes related to rice starch biosynthesis in WT and flo19 mutant seeds. (a) Semithin sections of the central part of WT (i–iii) and flo19 mutant (iv–vi) endosperm at 6, 9 and 12 DAF. Bars = 20 µm. (b) Transmission electron microscopy of the central part of WT and flo19 mutant endosperm at 6 (i–iii) and 9 DAF (iv–vi). Bars, 1 µm (i, b), 2 µm (ii–vi). (c) Relative expression levels of starch synthesis-related genes in developing endosperm at nine DAF. Rice Ubiquitin was used as an internal control. The expression level of each gene in the wild type was set to 1. Values are means ± SD, n = 3. *P < 0.05, **P < 0.01, Student’s t-test.
Figure 3  Map-based cloning of the FLO19 allele. (a) Fine mapping narrowed the FLO19 locus to an 80.5 kb region between markers P4 and P5 on rice chromosome 4 containing 12 predicted open reading frames (ORFs). The molecular markers and numbers of recombinants are shown. Black eclipse indicates the centromere (CEN). (b) Genomic structure and mutation site in LOC_0s04g02900. A single-base substitution mutation (C to T) in the flo19 allele detected in the second exon of LOC_0s04g02900 leads to the transition of alanine (Ala) to valine (Val). Black boxes and lines indicate exons and introns, respectively. White boxes represent 5' and 3' untranslated regions. ATG and TGA are the start and stop codons, respectively. (c) Genetic complementation. The floury endosperm phenotype of the flo19 mutant was rescued by expressing LOC_0s04g02900 driven by its native promoter. Bars, 2.5 mm. (d) SEM images of transverse sections of representative complemented grains. Bars, 10 μm. C1 to C3 are independent transgenic lines.

Figure 4  Expression pattern and subcellular localization of FLO19. (a, b) Relative expression levels of FLO19 in various plant tissues and developing endosperm at 6–21 DAF. Rice Ubiquitin was used as an internal control. Values are means ± SD, n = 3. (c) GUS staining of fLO19::GUS transgenic plant tissues; root (i), stem (ii), sheath (iii), panicle (iv), leaf blade (v), germinating seed (vi) and grain at nine DAF (vii). Bars, 1 cm (i–iii, v), 2 mm (iv, vi–vii). (d) Subcellular localization of FLO19 in rice protoplasts. Free GFP was used as a control. Bar, 5 μm. (e) Amyloplast localizations of FLO19 in pericarp cells at 4 (upper panel) and 6 DAF (lower panel). Bars, 5 μm.
Galactoglycerolipid biosynthesis is defective in the flo19 mutant

Galactoglycerolipid biosynthesis requires acetyl-CoA that is mainly derived from pyruvate oxidative decarboxylation catalysed by PDC in plastids (McHugh et al., 1995). We speculated that the flo19 mutation affected acetyl-CoA biosynthesis and subsequent galactoglycerolipid biosynthesis, resulting in abnormal membrane lipids in the amyloplasts. MGDG contents in total lipids extracted from developing seeds at 6, 9, 12 DAF and from brown grains were reduced by 25.5%, 31.7%, 45.6% and 26.3% in the flo19 seeds, respectively, compared with those from the wild type, concomitant with a decrease in DGDG by 28.0%, 11.2%, 1.1% and 41.2% (Figure 6a,b). As a result, the GDG contents in developing seeds of flo19 mutant were decreased by 26.4%, 24.8%, 32.1% and 32.3%, respectively (Figure 6c). All results showed that mutation of FLO19 altered the contents of membrane lipids in developing rice endosperm.

Overexpression of FLO19 increases grain size and grain yield

To determine whether FLO19 has application potential in rice improvement, the whole coding sequence of FLO19 driven by the maize Ubiquitin promoter was introduced into variety...
Nipponbare. qRT-PCR analysis showed that FLO19 was significantly elevated in positive lines compared with Nipponbare (Figure 7d). No significant differences were detected between these lines and Nipponbare in major agronomic traits, such as plant height, tiller number, panicle length and seed setting rate except grain weight per plant (Figure 7i-m; Table S2). However, the lines developed larger grains as grain lengths from transgenic lines OE-1, OE-2 and OE-3 were increased by 5.8%, 8.1% and 8.9% (Figure 7f) and grain widths increased by 6.4%, 4.9% and 5.1% (Figure 7g), respectively, compared with Nipponbare grains. Grain thickness of the three lines was unchanged (Figure 7h). As a result, 1000-grain weights of OE-1, OE-2 and OE-3 were increased by 15.7%, 13.2% and 13.8% (Figure 7e), and grain yield per plant was increased by 17.0%, 18.2% and 12.8%, respectively, compared with Nipponbare (Figure 7n). These results indicated that overexpression of FLO19 in rice led to larger and heavier grains and increased grain yield.

Variation in the promoter sequence of FLO19 affects its expression and grain length

Given that overexpression of FLO19 increases grain size and weight, we next explored the potential association between grain length and natural genetic variation of FLO19. We investigated the sequences of the FLO19 coding region and regions from 2 kb upstream to 1 kb downstream in 2013 germplasm accessions based on sequence variation data in the 3K rice genome panel. We identified 100 polymorphic sites and performed association studies, which uncovered a significant association between the 4 bp in the FLO19 promoter and grain length \( (P = 9.2e-04; \text{Figure S10a,b}) \). Haplotype pFLO19\(^2\) was associated with long grain length in GJ cultivars, whereas the pFLO19\(^2\) was associated with short grain length (Figure S10c). A transient expression assay of a luciferase reporter gene driven by different promoters of FLO19 showed that the pFLO19\(^2\) more effectively enhanced the expression of the reporter gene than pFLO19\(^2\) (Figure 8e). Consistently, we further observed that the FLO19 expression levels were obviously higher in accessions with pFLO19\(^2\) haplotype than those with pFLO19\(^2\) haplotype (Figure 8f). Taken together, a rare natural deletion in the FLO19 promoter region seemed to cause shorter grain length by influencing FLO19 expression.

**Discussion**

ptPDC-E1-\(\alpha\)1 has a role in starch biosynthesis and amyloplast development in rice seeds

In this study, we identified a floury endosperm flo19 mutant that exhibits an opaque interior central endosperm phenotype, as well as decreased plant height, fewer tillers, slower rate of grain filling, lower grain weight and reduced seed setting rate (Figure 1a-i and S1a-c; Table S1). Total starch and amylose contents were significantly reduced in the flo19 mutant compared with the wild type (Figure 1j,k). Pasting properties and amylopectin structure were also affected (Figure S2a,b). The starch granule phenotype of the mutant was also altered, with the smaller, scattered starch granules showing weak iodine staining, compared with the densely packed, polyhedral starch granules in the wild type (Figure 2a,b). Thus, mutation of the FLO19 gene had a major influence on starch biosynthesis and amyloplast development during rice endosperm development.

Further qRT-PCR experiment showed that most of the starch synthesis genes analysed were significantly down-regulated in 9-DAF seeds of the flo19 mutant compared with wild type (Figure 2c). Among these genes, AGPL and AGPS are primarily responsible for the first committed step of starch biosynthesis in higher plants.
Mutation of OsAGPS2b causes a shrunken rice endosperm due to a significant reduction in starch synthesis (Lee et al., 2007; Yano et al., 2008). Therefore, the decreased starch content in flo19 may at least partially result from down-regulated OsAGPS2b expression and decreased AGPase activity (Figure 2d). In addition, BEs, DBEs and SSS cooperatively control the amylopectin biosynthesis in the cereal endosperm (Tian et al., 2009). Mutations of them have major changes in amylopectin composition (Fujita et al., 2006; Kubo et al., 1999; Nakamura et al., 2005; Ryoo et al., 2007; Satoh et al., 2003). Thus, the changes in amylopectin composition in flo19 may be ascribed to the combined effects of altered expression of OsSSI, OsSSIIa, OsSSIIb, OsBEI and OsISA1. Taken together, the altered expression levels of starch synthesis genes could affect starch synthesis.

ptPDC-E1-α1 is a functional component of ptPDC

It was reported that the PDC is a multienzyme complex with three primary components: pyruvate dehydrogenase (E1), dihydrolipoamide transacetylase (E2) and dihydrolipoamide dehydrogenase (E3) in higher plants. Among them, E1 is a heterotetramer
Figure 8 Haplotype analysis of FLO19 associated with grain length. (a) Local Manhattan plot (upper panel) and LD heatmap (lower panel) in the region from 2 kb upstream to 1 kb downstream of LOC_Os04g02900 across the 3K rice genome panel. Red and blue dots indicate the significant and non-significant levels of polymorphic sites associated with the grain length, respectively. Arrow indicates the position of nucleotide variation at 284 bp upstream of the LOC_Os04g02900 transcription start site. (b) Exon–intron structure of LOC_Os04g02900 and DNA polymorphism in the gene. (c) Comparison of grain length between haplotypes pFLO19⁺ and pFLO19⁻ in a rice accession panel. Values are means ± SD. (d) Genotypic analysis of the rice accession panel for groups xian/indica (XI), geng/japonica (GJ), AUS, temperate japonica (GJ-tmp), tropical japonica (GJ-trp), subtropical japonica (GJ-subtmp), and admix (GJ-adm). (e) Promoter activity assay. Values are means ± SD, n = 3. P values from Student’s t-test are indicated. (f) Expression levels of FLO19 in representative rice varieties with two different haplotypes. Total RNA was isolated from rice endosperm at seven DAF. Values are means ± SD, n = 3.
composed of two α- and two β-subunits in mtPDC. Similar to the mtPDC-E1, the ptPDC-E1 could also form a heterotetramer in vitro (Johnston et al., 2000). When the pre-ptPDC-E1-α and β were imported and processed by isolated pea chloroplasts, E1-α and E1-β monomer, αβ heterodimer, and α2β2 heterotetramer were identified by size exclusion chromatography and immunoprecipitation. However, neither hetero- nor homodimers were formed when pre-ptPDC-E1-β was imported and processed in the absence of pre-ptPDC-E1-α, suggesting that the assembly of the ptPDC-E1 is in the form of an α2β2 heterotetramer. Moreover, the most likely pathway to the final α2β2 heterotetrameric enzyme proceeds through an αβ-heterodimer in plastids (Johnston et al., 2000). In our study, three potential ptPDC-E1 subunits were identified in rice based on phylogenetic analysis, including one ptPDC-E1-α and two ptPDC-E1-βs (Figure S5). To confirm the heteromeric structure of the ptPDC-E1 subunits in rice, Y2H, BIFC and Co-IP experiments were performed (Figure 5a–c). These results revealed that the ptPDC-E1-α, β1 and β2 could interact with each other both in vitro and in vivo. Furthermore, an IP-MS assay confirmed that ptPDC-E1-α is an integral part of the ptPDC (Figures 5d and S9). Enzymatic activity assays showed that ptPDC activity in the flo19 mutant was markedly decreased compared with the wild type in both seedlings and developing endosperm (Figure 5e,f). Taken together, we proposed that ptPDC-E1-α might form heterocomplexes with ptPDC-E1-βs, which act cooperatively in amyloplast development and starch biosynthesis.

**FLO19 is involved in galactolipid biosynthesis required for amyloplast development in rice endosperm**

Higher plants possess both prokaryotic and eukaryotic pathways responsible for glycerolipid biosynthesis. In the two parallel pathways, plastidial fatty acids (FA) provide key substrates for glycerolipid biosynthesis. De novo synthesized FAs either directly enter the prokaryotic pathway in plastids or are exported to the endoplasmic reticulum (ER) to be assembled into glycerolipids by the eukaryotic pathway. The ptPDC catalyses the irreversible reaction in which pyruvate provided by the plastidial glycolysis was decarboxylated and oxidized sequentially to produce acetyl-CoA and NADH for de novo FA biosynthesis (Chu et al., 2000; Rapp and Randall, 1980). As a result, lipid biosynthesis is tied to glycolytic fluxes in plastids. For instance, FLO19 is regulated in a flo19 mutant with the wild type in both seedlings and developing endosperm (Figures 5d and S9). Enzymatic activity assays showed that ptPDC activity in the flo19 mutant was markedly decreased compared with the wild type in both seedlings and developing endosperm (Figure 5e,f). Taken together, we proposed that ptPDC-E1-α might form heterocomplexes with ptPDC-E1-βs, which act cooperatively in amyloplast development and starch biosynthesis.

**FLO19 has potential application in rice breeding**

Overexpression of FLO19 in Nipponbare increased grain weight, length and width (Figure 7e–g). Previous studies have shown that natural variations in the regulatory region of genes can affect agronomic traits through downstream gene expression (Liu et al., 2015; Takashige et al., 2013). We therefore investigated the association between grain length and sequence variation in FLO19. There were three significant polymorphic sites associated with grain length (Figure 8a), two in the introns and one in the promoter region. A promoter activity assay indicated that the pFLO19b haplotype was associated with stronger normalized luciferase activity than pFLO19a (Figure 8e), suggesting that the variation in the promoter sequence affected its expression level and contributed to variation in grain length among rice varieties. Based on these findings, we also determined that the four-nucleotide sequence polymorphism of FLO19 promoter can be utilized for germplasm improvement through either genome editing or traditional selective breeding.

As a quantitative trait, seed size is regulated by numerous genetic factors. Extensive studies have identified many genes related to seed size in rice, such as GS3 (Fan et al., 2006; Mao et al., 2010), qGL3/GL3.1 (Qi et al., 2012; Zhang et al., 2012), G55 (Li, Fan, et al., 2011; Li, Wang, et al., 2011), qGS5/GW5 (Liu et al., 2017; Shomura et al., 2008; Weng et al., 2008) and GW8 (Wang et al., 2012). Expression analysis revealed that mRNA levels of GWS were significantly up-regulated in FLO19 overexpression lines, but GS3 was down-regulated (Figure S11a). GWS acts in the brassinosteroid signalling pathway to positively regulate grain width and weight in rice (Liu et al., 2017; Shomura et al., 2008; Weng et al., 2008), while GS3, a major QTL for grain length, is a negative factor for grain length by regulating cell division (Fan et al., 2006; Mao et al., 2010). Thus, up-regulated GWS and down-regulated GS3 may contribute to the enlarged seed size in FLO19 overexpression lines. Furthermore, qRT-PCR analysis of cell cycle-related genes was also performed. As a result, the mRNA levels of CYCA2.1, CYCD4.1 and CDBK2 were significantly up-regulated in FLO19 overexpression lines, while CYCB1.1, CYCB2.1, CYCB2.2, CYCD2.1 and CYCD4.2 showed no obvious difference compared with Nipponbare (Figure S11b). Thus, up-regulation of these cell cycle-related genes may increase cell division rate, leading to an increased grain size in FLO19 overexpression lines.

On the other hand, starch is the major component of rice seeds and largely determines the grain weight. We hypothesize that higher starch biosynthesis in the overexpression lines caused the increased grain size and weight. Consistent with this, the expression of type I starch synthesis genes was significantly up-regulated in a rs1 mutant, resulting in the larger seed size and increased 1000-grain weight. RS1 is a rice AP2/EREBP family transcription factor that negatively regulates endosperm starch biosynthesis (Fu and Xue, 2010). Surprisingly, the expression pattern of starch synthesis-related genes observed in FLO19 overexpression lines was very similar to that of FLO16 overexpression lines (Figure S12). This latter gene encodes a NAD-dependent cytosolic malate dehydrogenase and has a critical role in starch biosynthesis and seed development in rice (Teng et al., 2011). These data suggested a functional connection between galactolipid accumulation and starch synthesis in plant endosperm. Together, FLO19 is involved in galactolipid biosynthesis required for amyloplast development in rice endosperm.
2019). Expression levels of five sucrose synthase genes (OsSUS1, OsSUS2, OsSUS4, OsSUS5, OsSUS7) in the FLO19 overexpression lines were significantly higher than in Nipponbare (Figure S12b). Sucrose is principally produced in the source leaves and transported to the vascular tissue of seeds, then converted to glucose-1-phosphate (Glu-1-P) by invertase, sucrose synthase (SuSy), UDPG pyrophosphorylase (UGPase) and hexokinase, prior to use in starch synthesis (Bahaji et al., 2014). GRAIN INCOMPLETE FILLING 1 (GIF1) is a member of cell-wall invertase required for sucrose unloading in the ovular and stylar vascular tissues. Overexpression GIF1 had larger and heavier grains compared to the wild type (Wang et al., 2008). Therefore, up-regulated expression of OsSUSs shown in our study might increase sucrose unloading capacity and starch biosynthesis in the endosperm of the FLO19 overexpressing lines, resulting in an increase in grain weight. Our study indicates that FLO19 can increase grain size and yield potential through promoting starch biosynthesis in overexpression lines. However, how does a starch biosynthesis-related gene regulates seed size is still a question to be addressed.

Summary, our study suggests that FLO19 plays a key role in amyloplast development and starch biosynthesis by regulating the glycolipid biosynthesis in rice endosperm and its natural variation or overexpression provides a potential avenue for improving grain size and therefore grain yield in rice.

Materials and methods
Plant materials and growth conditions
The flo19 mutant was produced by EMS treatment of cultivar Diangengyou 1 (Oryza sativa, geng/japonica). An F2 population was derived from a cross between the flo19 mutant and xian/ indica cultivar IR36 for mapping. All plants were grown in experimental fields at Tuqiao and Pailou experimental stations of Nanjing Agricultural University. Developing seeds at 6 to 30 days after flowering (DAF) were harvested for subsequent experiments.

Microscopy
Transverse sections of mature seeds were coated with gold and observed with a Hitachi S-3400N scanning electron microscope (Kang et al., 2005). For ultrathin and semithin section experiments, developing seed sections (approximately 1 mm) were stained with iodine-potassium iodide (I2-KI) for 5 s and photographed using a light microscope (Nikon ECLIPSE80i, Tokyo, Japan).

Analysis of the starch characters, physicochemical properties and galactolipid contents in seeds
Total starch concentrations were determined with a Megazyme starch assay kit (Megazyme, Wicklow, Ireland). The contents of amylose, protein and lipid were estimated by a published method (Kang et al., 2005; Liu et al., 2009). The chain length profile of amylopectin was analysed by fluorescence-activated capillary electrophoresis (FACE) (Nakamura, 2002; Shea et al., 1998). Starch pasting properties were determined with a Rapid Visco Analyzer (RVA-Tec Master, Perten, Sweden). 0–9 M urea was used to detect the swelling and gelatinization properties of starch (Nishi et al., 2001). Galactolipid content of grains was measured by a Plant Galc ELISA Kit (Kete, Jiangsu, China).

Map-based cloning of the FLO19 gene
Map-based cloning was performed using the F2 population derived from a cross between flo19 and IR36. Flourey endosperm seeds from the F2 population were selected for genomic DNA extraction. The FLO19 locus was first mapped to an interval between the markers P1 and P9 on the short arm of chromosome 4 with more than 180 polymorphic simple sequence repeat (SSR) markers. For fine mapping of FLO19, 2000 individuals with the recessive phenotype were used and genotyped with newly designed molecular markers (listed in Table S5).

Plasmid construction and rice transformation
For complementation tests, a 5817 bp genomic fragment, including the putative promoter and the entire coding region of FLO19, was amplified and recombined into the pCUbi1390 vector to create a complementary plasmid. To obtain FLO19 knockout lines, a 20-bp guide RNA was cloned into the CRISPR/Cas9 expression vector following a published method (Miao et al., 2013). To construct the overexpression vector, the entire coding sequence of FLO19 was amplified and cloned into pCUbi1390 driven by the maize Ubiquitin promoter. The complementary plasmid was transferred into the flo19 mutant by Agrobacterium tumefaciens-mediated transformation. Other binary vectors were introduced into Nipponbare as described (Qu et al., 2005). Positive transformants were identified by PCR. Primers are listed in Table S5.

Enzyme assays
An NADH-coupled assay was used to measure plastid pyruvate dehydrogenase complex (ptPDC) activity as described previously with minor modifications (Hoppe et al., 1993). For assays, 0.1 g 9 DAF endosperm was prepared and homogenized on ice with 1 mL buffer containing 50 mM Hepes-KOH (pH 7.4), 2 mM MgCl2 and 12.5% (v/v) glycerol. After centrifuging at 20 000 g for 10 min at 4 °C, the supernatant was collected for enzyme assay. The reaction mixture consisted of 50 mM Hepes-NaOH (pH 8.2), 10 mM MgCl2, 0.25 mM diphosphothiamine, 1 mM NAD+, 1 mM EDTA, 20 mM diethiothreitol, 0.5 mM CoA and 3 mM pyruvate in a total volume of 1 mL. The reaction was initiated by adding 50 μL of crude extraction and incubated at 25 °C. Formation of NADH was monitored at 340 nm, in which the increasing absorbance values were detected with a TECAN microplate spectrophotometer (Infinite 200 PRO, Switzerland). For seedling ptPDC activity, chloroplasts of 10-day-old rice seedlings were isolated and purified. Then, the intact chloroplasts were lysed and centrifuged to obtain the stromal fraction (Li et al., 2011). Activities of ptPDC were determined in the stromal fraction using the above method. Proteins were quantified by Bradford assays.

Multiple sequence alignment and phylogenetic analysis
The NCBI database was adopted to search FLO19 homologs. The obtained protein sequences were submitted to the ClustalW (Larkin et al., 2007) for multiple sequence alignment. The phylogenetic tree was produced with MEGA7.0 using the neighbour-joining method with 1000 bootstrap replicates.

RNA extraction and real-time PCR analysis
Total RNAs were extracted using an RNA Prep Pure Plant Kit (TIANGEN Biotech, Beijing, China). About 2 μg of total RNA was
used for first-strand synthesis in a 40 µL volume with Oligo (dT)18 primer and PrimeScript Reverse Transcriptase (TaKaRa, Dalian, China). Real-time PCR was performed using Bio-Rad T100TM real-time PCR system (California) with the SYBR Green I mix (TaKaRa, Cat# RR420A). The rice Ubiquitin gene was amplified as the internal control. All primers of this analysis are given in Table S5.

**Subcellular localization**

The coding sequences (CDSs) of FLO19, ptPDC-E1-b1 and ptPDC-E1-b2 without stop codons were amplified by PCR and each was inserted into the XbaI-BamHI site of pAN580 vector driven by the CaMV35S promoter, respectively. Recombinant plasmids were separately introduced into the rice protoplasts following the method described by Chen et al. (2006). In addition, these CDSs were cloned into a binary vector of pCAMBIA1305-GFP vector under the control of the CaMV 35S promoter. The generated constructs were transferred into Agrobacterium strain EHA105 and then expressed in Nicotiana benthamiana leaf epidermal cells (Waadt and Kudla, 2008). A Leica TCS SP8 laser-scanning confocal microscope was used to examine the GFP fluorescent signal.

**GUS staining**

The putative promoter region of FLO19 (2271 bp upstream of ATG) was amplified by PCR and inserted into the BamHI/HindIII sites of pCAMBIA1381Z. The binary vector was introduced into Nipponbare callus using Agrobacterium tumefaciens-mediated transformation. After PCR screening of the resulting transgenic plants, 10 positive lines were identified and T2 transgenic progeny were subjected to GUS staining (Jefferson et al., 1987). Images of various tissues were obtained with a stereoscope (Leica Application Suite 3.3, Germany).

**Yeast two-hybrid (Y2H) and bimolecular fluorescent complementation (BiFC) assays**

For yeast two-hybrid assays, the CDS of FLO19 (ptPDC-E1-α1) was recombined into the pAD507 or pGBKT7 vectors as well as other rice plastidial pyruvate dehydrogenase subunits (ptPDC-E1-β1 and ptPDC-E1-β2). The resultant plasmids were transformed into yeast strain AH109. Yeast cells were cultivated at 30 °C for 2 days in selective medium containing 0.67% (w/v) yeast nitrogen base, 2% (w/v) glucose and auxotrophic amino acids needed for growth. For BiFC assays, CDSs of ptPDC-E1-α1, ptPDC-E1-β1 and ptPDC-E1-β2 without stop codons were inserted into the PacI and Spel sites of p2YC and p2YN, respectively. The resulting constructs were transformed into Agrobacterium strain EHA105 and transiently expressed in tobacco leaves (Waadt and Kudla, 2008). The same confocal microscope was used to detect the fluorescent signals.

**Co-IP assays in N. benthamiana**

The CDSs of ptPDC-E1-α1, β1 and β2 without stop codons were cloned into pCAMBIA1300-221-Flag, pCAMBIA1300-221-Myc and pCAMBIA1305-GFP vectors, respectively, to generate the expression cassettes of 35S: E1-α1-Flag, 35S: E1-β1-Myc and 35S: E1-β2-GFP. The constructs were co-transformed into N. benthamiana for transient expression. Total protein was isolated with extraction buffer (50 mM Tris-MES, pH 7.5, 1 mM MgCl2, 0.5 M Suc, 10 mM EDTA, 5 mM DTT, 0.1% [v/v] Nonidet P-40 and 1% Complete Protease Inhibitor Cocktail) and were incubated with GFP-Trap magnetic beads (D153-10; MBL) for 2 h at 4 °C with shaking. After washing with the extraction buffer five times, the IP samples were eluted in a reducing buffer, followed by SDS-PAGE and immunoblot analyses using anti-GFP (dilution 1 : 5000, ab32146, Abcam), anti-Flag (dilution 1 : 5000, M1857-5, MBL) and anti-Myc (dilution 1 : 3000; M20002, Abmart) antibodies, respectively.

**IP and mass spectrometry**

The ptPDC-E1-α1 coding sequence without stop codon was cloned into XbaI and BamHI sites of the pCAMBIA1305-GFP vector to generate the 35S: ptPDC-E1-α1-GFP. The construct was transformed into Agrobacterium strain EHA105 and then introduced into the flo19 mutant as described (Qu et al., 2005). Transgenic leaf (5 g of fresh weight) expressing free GFP or ptPDC-E1-α1-GFP was separately homogenized in ice-cold IP buffer (50 mM Tris-MES, pH 7.5, 1 mM MgCl2, 0.5 M Suc, 10 mM EDTA, 5 mM DTT, 0.1% [v/v] Nonidet P-40 and 1× Complete Protease Inhibitor Cocktail) and centrifuged at 20 000 g for 10 min at 4 °C to remove the cell debris. The supernatants were further subjected to IP using the m-MACS GFP-tagged protein isolation kit (Miltenyi Biotec) following the manufacturer’s instructions, with minor modification in the column washing step, in which wash buffer 1 was replaced with the IP buffer containing 0.2% (v/v) Nonidet P-40.

For mass spectrometry analysis, the immunoprecipitated proteins were resolved by SDS–PAGE and stained with Coomassie brilliant blue (CBB). The lanes were cut from the polyacrylamide gel, followed by dehydration and digestion with trypsin and subjected to liquid chromatography–tandem mass spectrometry analysis using a Q-Exactive Mass Spectrometer (Thermo Fisher Scientific). Data were analysed using the Mascot server (version 2.3) in-house (http://www.matrixscience.com/) and compared with proteins registered in The Institute for Genomic Research (http://rice.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/).

**Haplotype analyses of FLO19**

The 3K rice genome array was used to determine the association of variation of the FLO19 allele with grain length. The analysis was performed using the generalized Lagrange multiplier method provide by TASSEL software, with the principal component analysis as covariates. A linkage disequilibrium heatmap was drawn using the package LDBlockShow-1.36 (Dong et al., 2020).

**Promoter activity assay**

For promoter activity assays, the putative promoter sequences of the pFLO19β and pFLO19α haplotypes (2000 bp upstream of ATG) from rice accessions were amplified by PCR and inserted into the KpnI site of pGreenII 0800-LUC vector, respectively. Recombinant plasmids were separately transformed into rice protoplasts following the method described by Chen et al. (2006). After incubation at 28 °C for 18 h, Firefly luciferase (LUC) and Renilla luciferase (REN) activities were measured by the Dual-Luciferase Reporter Assay System (Promega, E1910), and LUC activity was normalized to REN activity. All primers for this analysis are given in Table S5.

**Accession numbers**

Sequence data for this article can be found in the GenBank/EMBL databases under the following accession numbers: FLO19 (LOC_Os04g02900), ptPDC-E1-β1 (LOC_Os12g42230), ptPDC-E1-β2 (LOC_Os03g44300) and Ubiquitin (LOC_Os03g13170).
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Conflict of interest
The authors declare no conflicts of interests.

Author contributions
JW and YHW conceived the research plan and supervised the experiments. JL and XT completed most of the experiments and JL wrote the manuscript. JW and YHW revised the manuscript. YW, XJ, HZ, XZ, YR, HD, YLW, ED, YZ, MY, SX, XB, PZ, SL, XL, YT and LJ participated in the experiments. All authors reviewed and approved this submission.

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Supporting information

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

Figure S1 Characteristics of WT and flo19 mutant plants.
Figure S2 Physicochemical properties of starch in mature seeds of WT and flo19 mutant.
Figure S3 Seed size and grain yield of the wild type, flo19 and complemented transgenic plants.
Figure S4 Analysis of LOC_Os04g02900 knockout lines generated by CRISPR/Cas9.
Figure S5 Phylogenic tree of pPDC-E1 and homologs.
Figure S6 Multiple sequence alignment of FLO19 and its homologs.
Figure S7 Expression patterns of pPDC-E1-β1 and β2 in plant tissues and developing endosperm at 6-21 DAF in wild type.

Figure S8 Subcellular localization of pPDC-E1 subunits.
Figure S9 Western blot analysis of p35S::GFP transgene and p35S::OsPDC-E1-α1-GFP transgene.
Figure S10 Haplotype analysis of FLO19 associated with grain length in geng/japonica accessions.
Figure S11 Quantitative real-time PCR analysis of seed size-related genes and cell cycle-related genes of Nipponbare and FLO19 overexpression lines.
Figure S12 Expression levels of starch biosynthesis-related genes in Nipponbare and FLO19 overexpression lines.

Table S1 Agronomic traits of WT and the flo19 mutant
Table S2 Agronomic traits of FLO19 overexpression lines
Table S3 Phenotypic segregation in the F2 population
Table S4 Indels and SNPs in a 6.05 kb region of LOC_Os04g02900
Table S5 Primers used in this study