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ORIGINAL ARTICLE

A new SNP in cy*OsPPDK* gene is associated with floury endosperm in Suweon 542

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Abstract

Pyruvate orthophosphate dikinase (PPDK) is a component of glycolysis to mediate endosperm energy charge by adjusting the ratio of ATP to ADP and AMP that proposed to balance the flow of carbon into starch, protein, fatty acid and amino acid biosynthesis. However, these were inconsistent with the first report of a T-DNA insertional knockout mutant of the rice *PPDK* gene (*flo4*) showed that rice with inactivated *PPDK* gene failed to produce a opaque seeds. Therefore, the *PPDK* might have multifaceted functions in grain filling stage, which in some ways might depend on the direction of the reversible catalysis. Suweon 542 is a rice (*Oryza sativa* L.) mutant developed from *Oryza sativa* ssp. *japonica* cv. Namil. Suweon 542 has a milky-white floury endosperm suitable for dry filling, with low starch damage, low grain hardness, and fine flour particle size. The mutant locus on chromosome 5 controls the floury endosperm phenotype of Suweon 542. Fine mapping of this locus is required for efficient breeding of rice germplasm suitable for dry milling. In this study, whole genome of Suweon 542 and Milyang 23 were re-sequenced using Illumina HiSeq 2500. Co-segregation analysis of $F_{3:4}$ family populations derived from Suweon 542/Milyang 23 was performed using eight CAPS markers and phenotypic evaluation of the endosperm. The target region was mapped to a 33 kb region and identified to encode cytosolic pyruvate orthophosphate dikinase protein (cyOsPPDK). A G→A SNP in exon 8 of cy*OsPPDK* resulting in a missense mutation from Gly to Asp at amino acid position 404 was responsible for the floury endosperm of Suweon 542. qRT-PCR experiments revealed that *FLO4-4* was expressed to a considerably higher level in Suweon 542 than in Namil during the grain filling stage. Overall, fine mapping of *FLO4-4* and candidate gene analysis provided further insight into the floury endosperm of rice, and reveal a novel SNP in cy*OsPPDK* gene can affect the floury endosperm phenotype through active *PPDK* gene during grain filling stage.

Keywords *Oryza sativa* L. · Suweon 542 · *Floury4-4* · Floury endosperm · Dry milling

Introduction

Rice (*Oryza sativa* L.) flour is a popular food ingredient, as it is highly nutritious and gluten-free with a reduced risk of celiac disease and allergic reactions (Torbica et al. 2010; Heo et al. 2013). Moreover, rice is high in easily digestible protein and starch, and is highly hypoallergenic (Shih and Daigle 1999). Rice flour is, therefore, commonly

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used as a substitute for wheat (*Triticum aestivum* L.) flour in bread, as a coating for frying (Ashida et al. 2010), and formulated into various other foods (Leewatchararongjaroen and Anuntagool 2016). Hence, the physicochemical properties and molecular characterization of the floury endosperm mutant need to be analyzed and to provide a fine flour with ideal quality for actual breeding.

To facilitate the incorporation of rice flour in food products, it is essential to determine whether the physicochemical properties of the rice flour will affect the product quality, including the damaged starch content, particle size distribution, and milling method (Ngamnikom and Songsermpong 2011; Leewatchararongjaroen and Anuntagool 2016). Dry milling and wet milling are traditional processes to produce rice flour. The wet milling method involves overnight soaking of rice grains and yields a good-tasting flour with low damaged starch content and small particle size. However, this process is highly labor-intensive, requires a large volume of water, increases the risk of microbial growth in rice, and leads to severe deterioration of rice flour quality (Tong et al. 2017). On the other hand, the dry milling method yields a highly nutritious flour rich in protein, ash, and lipids, avoids wastage of water, and is a relatively simple method of flour production. However, dry milling produces higher damaged starch content due to high grain hardness, which is unacceptable to consumers (Ngamnikom and Songsermpong 2011). Addition of strengthening agents and specific treatments has been shown to improve the quality of rice flour (Hormdok and Noomhorm 2007; Yang and Tao 2008); however, the overuse of additives to enhance product quality is harmful to consumers' health (Tong et al. 2015). Therefore, new rice cultivars with low starch damage and low grain hardness suitable for dry milling need to be developed.

Mutagenesis has been used extensively to develop various rice cultivars with different endosperm characteristics. For instance, ethyl methane sulfonate (EMS), ethylene imine (EI), and *N*-methyl-*N*-nitrosourea (MNU) have been used to produce mutants with waxy, dull, sugary, and floury endosperm. MNU, gamma rays, and sodium azide (SA) have been successfully used to develop new Korean endosperm mutants, such as Seolgaeng with opaque endosperm (Hong et al. 2011), Baegjinju with dull endosperm (Hong et al. 2012), Goami 2 with high amylose, Nogwonchal with green-colored glutinous endosperm, and Suweon 472 with waxy, dull, floury, and white-core endosperm (Shin et al. 2009). Among these endosperm mutants, only those with a floury and white core contain round and loosely packed starch granules that grind easily (Mo et al. 2013). Therefore, floury endosperm with low grain hardness, low starch damage, and fine particle size is more suitable for dry milling than waxy, dull endosperm with normal grain hardness and high amylose content.

Seven floury endosperm genes (*FLO1-7*) have been identified in rice. The *flo1* mutant is characterized by high amylose content, and the underlying gene is located on chromosome 5 (Kaushik and Khush 1991). *FLO2* maps to chromosome 4, harbors a tetratricopeptide repeat motif, and mediates protein–protein interactions (Qiao et al. 2010; She et al. 2010); the *flo2* mutant exhibits significantly reduced grain size (She et al. 2010). The *flo3* mutation lowered the expression of 16-kDa allergenic polypeptide, and the responsible gene maps to chromosome 4 (Nishio and Iida 1993). The *flo4* and *flo5* mutants are characterized by a floury endosperm with a white core and a normal outer portion, and were generated via T-DNA insertion into the cy*OsP-PDK* gene on chromosome 5 (Kang et al. 2005) and *starch synthase IIIa* gene (*OsSSIIIa*) on chromosome 8 (Ryoo et al. 2007), respectively. The *FLO6* gene resides on chromosome 3 and encodes a 529 amino acid protein that interacts with starch isoamylase1 to regulate starch synthesis and compound granule formation in developing rice grains (Peng et al. 2014). The *flo7* mutation produces a floury, white endosperm only on its periphery and not in the inner portion, and affects a gene on chromosome 10 that encodes a regulatory factor affecting the peripheral development of endosperm (Zhang et al. 2016).

The rice mutant, Suweon 542 was developed via SA mutagenesis of *Oryza sativa* ssp. *japonica* cv. Namil (Shin et al. 2009). Suweon 542 has a milky-white opaque endosperm except for a thin peripheral area. Physicochemical analysis of Suweon 542 revealed a loosely packed structure of irregular and globular shaped starch granules, low protein content and grain weight, and high amylose content compared with its wild-type progenitor, Namil. During dry milling, Suweon 542 shows significantly lower grain hardness, finer particle size, loosely packed starch granules, and lower starch damage than that of Namil and other rice cultivars (Mo et al. 2013). Therefore, Suweon 542 is highly suitable for dry milling. Mo et al. constructed a rice linkage map from a population of 94 F_2 individuals derived from a cross between Suweon 542 and *Oryza sativa* ssp. *indica* cv. Milyang 23 using 70 SSR markers (4–8 markers per chromosome). Subsequently, 14 additional polymorphic SSR markers were added to the linkage map to narrow down the *flo7(t)* location to 19.33–19.73 Mbp region on chromosome 5 between the markers RM18624 and RM18639. However, the molecular mechanisms regulating floury endosperm in Suweon 542 are unknown. Fine mapping and molecular cloning of *FLO7(t)* are predicted to verify and elucidate the genetic framework of floury endosperm development in rice.

In this study, we performed resequencing analysis of Suweon 542 and Milyang 23 to fine map and clone the *FLO4-4* gene. Our results demonstrated that *FLO4-4*

encodes a cyOsPPDK protein. Co-segregation analysis and qRT-PCR experiments confirmed that *flo4-4* was responsible for the development of floury endosperm during the grain filling stage.

Materials and methods

Plant material

The rice mutant, Suweon 542, has an entirely milky-white opaque kernel except for a thin peripheral area of the grain and the maturity starch granules are packed loosely with irregular and rounded shape, was derived from SA mutagenesis of Namil (Fig. 1), a early maturing, high-yield, and non-glutinous Korean elite rice cultivar. The F_2 population of Suweon 542/Milyang 23 and parental lines were grown at the experimental farm of the Pusan National University to generate $F_{3:4}$ family populations for fine mapping *FLO4-4*. Total genomic DNA was extracted from fresh leaves of all genotypes using the NucleoSpin® Plant II kit (MACHEREY–NAGEL GmbH & Co.KG, Germany), according to the manufacturer's instructions.

Resequencing and marker development

Whole genome of Suweon 542 and Milyang 23 was re-sequenced with an average of 75-fold coverage using Illumina HiSeq 2500 Sequencing Systems Platform (Illumina Inc. USA). Raw sequence reads were aligned against the rice reference genome (IRGSP 1.0) (Kawahara et al. 2013). To fine map *FLO4-4*, SNPs without a missing value, a minor allele frequency (MAF) > 0.05, and genotype calls for both parental accessions were used. Highest quality homologous SNPs were selected to develop eight cleaved amplified polymorphic sequence (CAPS) markers using Oligo7.0 software to narrow down the target region.

Fine mapping of *FLO4-4*

To investigate the candidate region, F_2 lines heterozygous for the genomic region between RM18624 and RM18639 were grown to F_3 generation. The F_3 plants were then selfed to generated $F_{3:4}$ seeds. Parental lines and randomly selected 96 $F_{3:4}$ recombinants were dehulled for visual inspection of endosperm and genotyped with eight CAPS markers. Open reading frames (ORFs) and their functional products in the region were annotated according to the MSU Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/) based on the defined physical locations. PCRs were performed in a 20-µl volume containing 10 ng of DNA template, 10 pmol of each primer, 1× PCR buffer [50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100, and 1.5 mM $MgCl_2$], 0.2 mM of dNTPs, and 1 unit of *Taq* DNA polymerase (Nurotics, Korea). PCRs were performed with an MJ Research PTC-100 thermocycler (Waltham, MA, USA) using the following conditions: initial denaturation at 94 °C for 5 min, followed by 36 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were digested using restriction enzymes (New England Biolabs), according to the manufacturer's instructions. The digestion of PCR products encompassing CAPS markers was detected using WatCut program (http://watcut.uwaterloo.ca/template.php?act=snp_new). The digestion products were separated on 3% polyacrylamide gels using 6 M urea and 1X TAE at 80 volts and visualized using Molecular

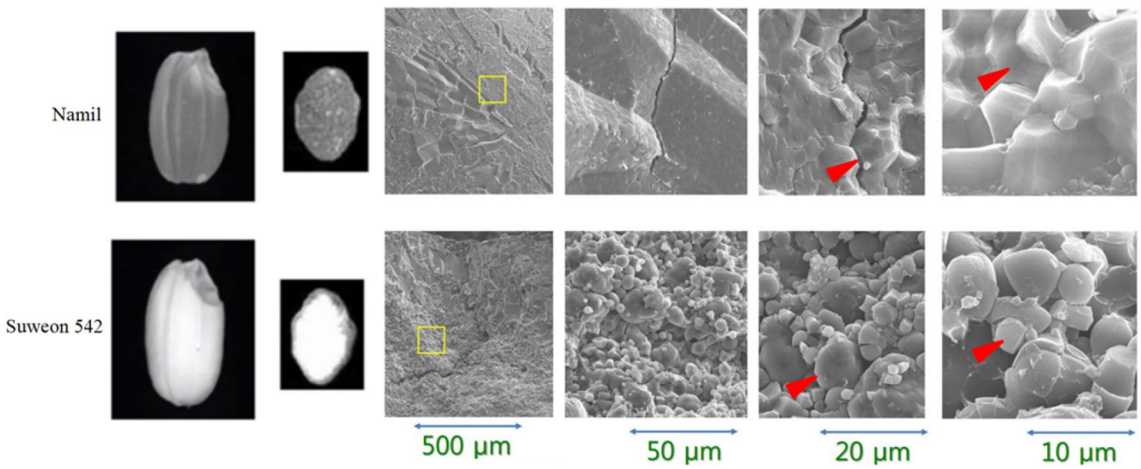

Fig. 1 Phenotypic analyses of the Suweon 542 and Namil. **a** Brown rice and transverse sections of Suweon 542 and Namil. **b** Electron microscope visualization of mature endosperm. The Suweon 542 is packed loosely with compound starch granules, as indicated by red arrow. The yellow box represents the magnified region. (Color figure online)

Imager® Gel Doc™ XR System (Bio-Rad Laboratories, Inc. USA).

Cloning of *FLO4-4* and identification of the mutation site

The coding sequence of cy*OsPPDK* in Suweon 542 and Namil were compared using CLC Sequence Viewer 7. To verify the mutation site, a derived CAPS (dCAPS) marker containing *Hinf*I restriction site in the floury endosperm mutant Suweon 542 was developed using dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/). A 161 bp fragment containing the mutation site was PCR amplified from Suweon 542 and Namil using the primers F: CCCAGGTGATGC AGGTGAG and R: GCACAGGTCTTGGACATTGC. PCR products were digested with *Hinf*I (New England Biolabs) in a 15-µl volume containing 5 µl of PCR product, 1.5 µl 10X NEBuffer, 0.5 µl *Hinf*I, and 8 µl ultrapure water, and incubated at 37 °C for 2 h. The digestion products were separated on a 4% agarose gel. The dCAPS marker was also used for co-segregation analysis of $F_{3:4}$ families and 48 Korean rice cultivars with their endosperm phenotype. Complete genomic DNA of the cy*OsPPDK* gene was cloned in three overlapping segments using primers designed on the basis of the cy*OsPPDK* gene sequence of Nipponbare (Table S1).

Total RNA isolation and qRT-PCR analysis

Total RNA was isolated from rice grains of Namil and Suweon 542 at 10 days after flowering (DAF) using RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturers' instructions. Genomic DNA was removed with DNase I (QIAGEN, Hilden, Germany), and reverse transcription was performed using RNA to cDNA EcoDry Premix Kit (Clontech, Mountain View, CA, USA). qRT-PCR was performed using QuantiNova SYBR Green RT-PCR kit (QIAGEN, Hilden, Germany) with Rotor-Gene Q instrument (QIAGEN, Hilden, Germany) and the following PCR conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 10 s, and 60 °C for 20 s. Fold change was calculated relative to Namil. Fold induction data represent mean ± SD of three biological replicates. Rice *Actin 1* (*OsACT1*; Os03g0718150) was used as an internal control. The qRT-PCR primer (qOsPPDKB) was designed based on the coding sequence of *FLO4-4*. Primer sequences used for qRT-PCR are listed in Table S1.

Results

Fine mapping of the floury endosperm locus

Whole genome of Suweon 542 was re-sequenced and aligned against that of the *indica* cultivar Milyang 23 as a reference. A total of 2,604 SNPs were identified in the target region. Using the genomic sequence between RM18624 and RM18639 as a template, eight CAPS markers were developed (Table 1) and used to genotype 60 floury and 36 normal individuals derived from $F_{3:4}$ recombinant families. Multiple comparisons were conducted between the genotypes of these recombinants and the phenotypes of their offspring. If the CAPS marker genotype of floury endosperm individuals matched with that of Milyang 23 or heterozygous genotype, due to the candidate gene was confirmed to control by a recessive gene, the CAPS marker flanking region was not as the location for target gene. On the other hand, among individuals with wild-type endosperm, if the CAPS marker genotype was same as that of Suweon 542, it demonstrated that the target gene was rejected as the location for target gene too. Individuals with heterozygous genotype for CAPS5–8 and a floury endosperm, and those with Suweon 542 genotype for CAPS1–4 and normal endosperm were identified (Fig. 2a). The target region was mapped to the BAC clones OJ1174_H11 and BAC OSJNBb0006J12 in a 33 kb region flanked by the markers CAPS8 and RM18639.

Table 1 Primer sequences of CAPS markers that developed for fine mapping

Marker name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Restriction enzyme name	PCR product (bp)	Digestion in mutant type (bp)	Digestion in wild type (bp)
CAPS1	ACGGACATGGGTAGTGGTTAC	AGTAATGCGGTGCCAAAAG	*Sca*I	455	157 + 298	455
CAPS2	GGACGGAGGGAGTATGTG	CGATCACGACCAGGATAAG	*Ssp*I	374	289 + 85	374
CAPS3	CGTTTCAGTGTCAGATTC	CTAAAGCTATCAGAGGATTG	*Bcl*I	459	159 + 300	459
CAPS4	CACACACACACTGACAGTTG	TCTCCCTCGCTATCACTG	*BsrB*I	674	342 + 332	674
CAPS5	ACGGGCACGGATAGTGACTAC	GTGAGTTTGTGACAGGTA AACGC	*Sac*II	159	55 + 56 + 48	55 + 104
CAPS6	AACGCAAAGCCCCAAAAG	CACTGCCACCCACATGAAG	*BsrB*I	566	314 + 252	566
CAPS7	AGCTCCTTCTCAATCTTGG	CATCTCTTCCACCATCTCTC	*Stu*I	360	115 + 245	360
CAPS8	TGAACCATGGTGAAGACTC	AGCTCAGGCCTTGTTAGG	*Dra*I	446	162 + 284	446

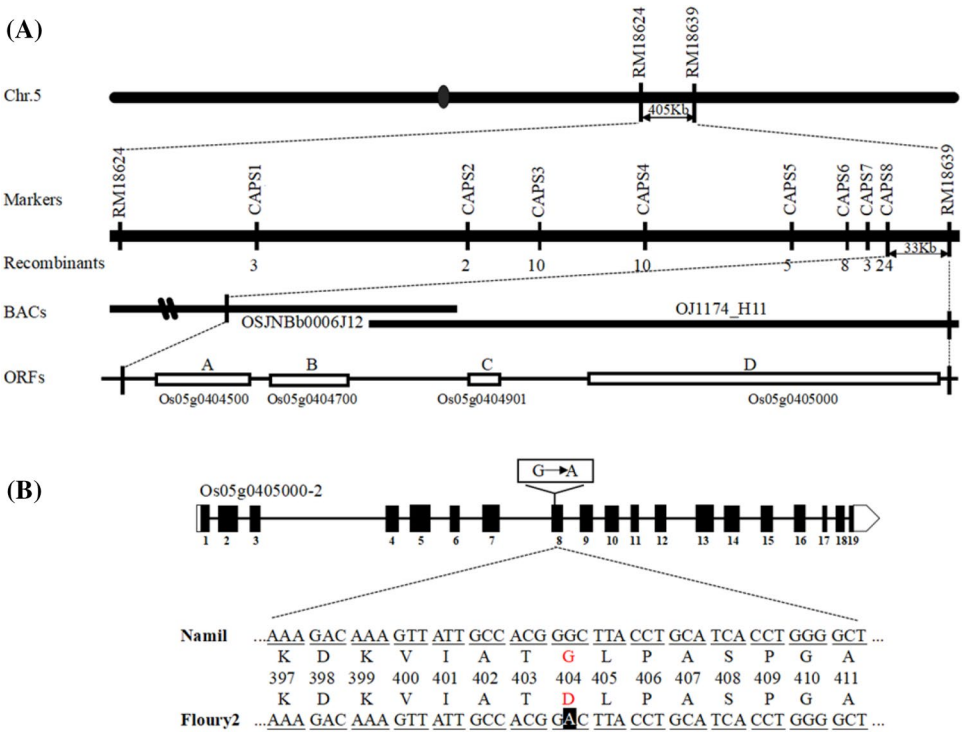

Fig. 2 Map-based cloning the *flo4-4* mutant. **a** Fine mapping of the *flo4-4* locus. The molecular markers and number of recombinants are shown. The 33 kb virtual contig, composed of overlapping 2 BAC clones, was delimited by e-Landings of two significant markers on the reference rice genome, 'Os-Nipponbare-Reference-IRGSP-1.0'. **b** *flo4-4* gene structure and cDNA sequence comparison showing a nucleotide mutant (G–A) within exon 8 where Gly-404 of the wild was induced to Asp-404 of the *flo4-4*. White boxes represent untranslated regions, black boxes represent coding regions, and solid lines represent introns

Analysis of candidate gene for floury endosperm

Based on the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/viewer/gbrowse/irgsp1/?name=chr02%3A1..105000), four genes were predicted in the critical 33 kb region (Fig. 2a). Among these, Os05g0404500 was annotated as encoding a hypothetical protein, Os05g0404700 was a functional gene similar to the methyl-binding protein gene *MBD1*, Os05g0404901 encoded a conserved hypothetical protein, and Os05g0405000 was annotated as *PPDK* gene. Sequencing of all four candidates in Suweon 542 and the wild-type Namil revealed a G/A SNP in exon 8 of *PPDK* (Fig. 2b). In rice, the *PPDK* encodes a cytosolic and a chloroplast-targeted PPDK protein (cy*Os*PPDK and ch*Os*PPDK, respectively). To analyze the transcript type of *PPDK* in developing grains of rice, *PPDK* of Suweon 542 and Namil was sequenced using the primer combinations PF2/PR3 (cy*OsPPDK*) and PF3/PR3 (ch*OsPPDK*) (Kang et al. 2005) and three additional primer pairs (Table S1). Full-length genomic sequence of cy*OsPPDK* was identified as 7556 bp with a cDNA sequence of 2649 bp containing 19 exons and encoding an 882 amino acid protein. The SNP in exon 8 resulted in Gly to Asp missense mutation at amino acid position 404 in cy*OsPPDK* (Fig. 2b). Comparison of cy*OsPPDK* sequences of Suweon 542 and Namil with those of Nipponbare sequence revealed 9 SNPs and 6 InDels (insertion and deletion) in the intron region and 2 SNPs in the coding region with synonymous mutation, except the SNP in the coding region between Suweon 542 and Namil (Table S2). The novel recessive floury gene was named *floury4-4* (*FLO4-4*). The full-length coding sequence of *FLO4-4* of Suweon 542 and Namil were deposited to the GenBank, and are represented by the accession numbers MG267058 and MG267056, respectively.

Co-segregation and expression analyses

The site of mutation in *flo4-4* was confirmed using a dCAPS marker with *Hinf*I restriction site. *Hinf*I digested the mutant Suweon 542 allele (Fig. 3, lane 2), but not the Namil allele (Fig. 3, lane 3). For screening $F_{3:4}$ family populations using the dCAPS marker, 18 plants with Suweon 542 phenotype and 18 with Namil phenotype were selected. As shown in Fig. 3a, all plants with the Suweon 542 phenotype were digested with *Hinf*I, whereas those with the Namil phenotype were not. Fourteen $F_{3:4}$ plants were heterozygous and exhibited the Namil phenotype, suggesting that the floury endosperm is controlled by a recessive gene. Additionally, none of the 48 Korean rice cultivars with the wild-type phenotype digested with *Hinf*I (Fig. 3b). Overall, co-segregation analysis suggested that *FLO4-4* is responsible for floury endosperm in rice. The dCAPS marker was also used to conduct an association analysis to estimate the *FLO4-4* gene effect of variation in floury grains percentage of the 94 F_2 samples by crossing Suweon 542 and Milyang 23 (Mo et al. 2013) which as a kind gift from Mo's lab in RDA (Rural Development Administration, Republic of Korea). The dCAPS marker exhibited the highest *F* value and explained

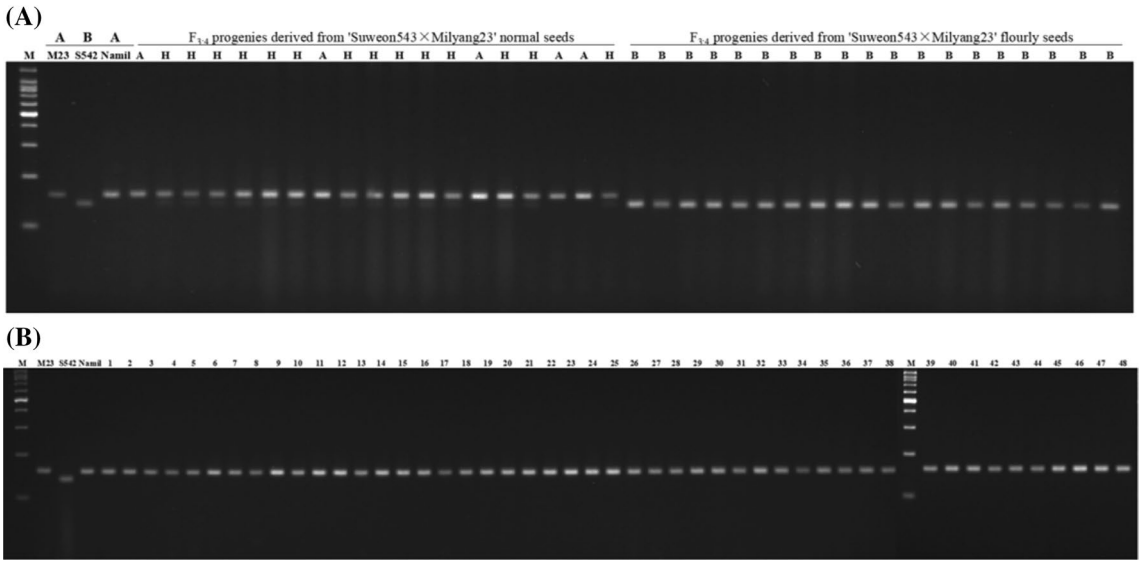

Fig. 3 Co-segregation analysis of the *flo4-4* genotype with floury endosperm phenotype. **a** Verification of the dCAPS marker and tagging the *flo4-4* locus using a part of F3 individuals. **b** Verification of the dCAPS marker and tagging the *flo4-4* locus using Korean rice cultivars. **a** and **b** are homogeneous of Suweon 542 and Milyang 23, respectively. H is heterozygote. M: size marker (100 bp), S542 (Suweon 542), M23 (Milyang 23). (1) Anmi, (2) Aranghyangchal, (3) Baegjinju1, (4) Baekogchal, (5) Boramchal, (6) Boramchan, (7) Borami, (8) Boseog, (9) Boseogchal, (10) Boseogheugchal, (11) Cheongnam, (12) Chindeul, (13) Chucheong, (14) Dabo, (15) Danmi, (16) Danpyeong, (17) Deuraechan, (18) Dodamssal, (19) Dongjin, (20). Dongjin1, (21) Dongjinchal, (22) Geonganghongmi, (23) Geonyang 2, (24) Goami, (25) Goami 2, (26) Goami 4, (27) Haepum, (28) Haiami, (29) Hanam, (30) Hanmaeum, (31) Heugjinmi, (32) Heughyang, (33) Heugjinju, (34) Heugnam, (35) Heugseol, (36) Hongjinju, (37) Hopum, (38) Hwanggeumnuri, (39) Hwaseong, (40) Hwawang, (41) Hwayeong, (42) Hyangnam, (43) Hyeonpum, (44) Ilmi, (45) Ilpum, (46) Jeogjinju, (47) Jeogjinjuchal, (48) Heughyangchal

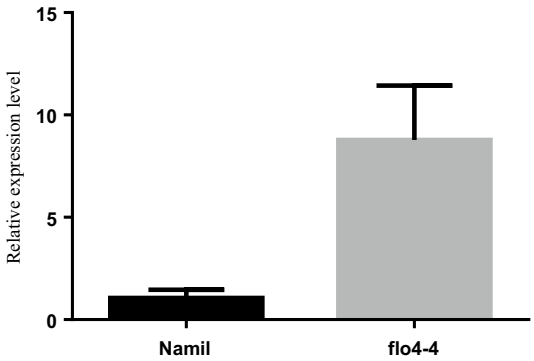

Fig. 4 Expression analysis of *flo4-4* between Namil and Suweon 542 at 10 DAF. Values shown are mean ± SD ($n = 3$)

92.9% of the variation in floury grain percentage among 94 F_2 progenies derived from Suweon 542 × Milyang 23 cross with an additive effect of 47.6% and co-segregation analysis detected 23 homozygous for Milyang 23 (Table S3), indicating that the *flo4-4* mutant allele of Suweon 542 was responsible for floury endosperm in rice.

Next, qRT-PCR was used to investigate the transcript type of *FLO4-4* in rice during the grain filling stage. Relative expression level of *FLO4-4* was much higher in Suweon 542 than in Namil (Fig. 4), indicating that the expression of *FLO4-4* was related with grain filling, and the difference in floury texture of grains was attributable to the *FLO4-4* allele. Altogether, these data suggest that *FLO4-4* is the candidate locus responsible for floury endosperm in rice.

Discussion

In this study, we fine mapped the gene responsible for the floury endosperm phenotype of the rice mutant, Suweon 542. The *FLO4-4* locus mapped to a 33 kb region on chromosome 5 between the markers CAPS8 and RM18639 encompassed within the BAC clones OSJNBb0006J12 and OJ1174_H11 (Fig. 2). The floury endosperm was caused by a SNP in exon 8 of the cy*OsPPDK* gene underlying the *FLO4-4* locus.

Previously, the genes *FLO1* and *FLO4* have been mapped to rice chromosome 5. The *FLO1* gene was identified via trisomic analysis; however, detailed information regarding the location of *FLO1* is lacking (Kaushik and Khush 1991). The white-core endosperm mutant allele of *flo4* was generated using insertional mutagenesis of the cy*OsPPDK* (Kang et al. 2005), which is also located within the BAC clone OJ1174_H11. Comparative sequencing of the putative region harboring the floury endosperm gene in Namil and Suweon 542 identified a SNP (G–A) (Fig. 2) and mapped

the mutant *flo4-4* allele to the same region as *flo4* in cy*OsPPDK*. Despite the same location of *flo4-4* and *flo4* alleles, the mutant phenotype of *flo4-4* is characterized with an entirely milky-white opaque kernel except for a thin peripheral area of the grain (Mo et al. 2013), whereas that of *flo4* is characterized with a white-core endosperm and a normal outer portion (Kang et al. 2005). Chalky mutant lines are classified as 'milky-white' with an entirely opaque grain except for the peripheral area, whereas lines with a white core are opaque only in the central region of the grain. Compared with the wild type, the *flo4* mutant exhibits higher protein, lower amylose, reduced kernel weight, and increased total lipid content, whereas the *flo4-4* mutant exhibits lower protein and slightly higher amylose (Kang et al. 2005; Mo et al. 2013). This variation in physicochemical characteristics of *flo4-4* and *flo4* mutants suggests that these lines are divided into two groups. We speculate that the *flo4-4* might be controlled by another mutated allele of cy*OsPPDK* gene.

Comparative sequence analysis of Namil and Suweon 542 revealed a recessive point mutation (G→A) in exon 8 of the cy*OsPPDK* allele of Suweon 542, which resulted in a missense mutation from Gly to Asp at amino acid 404 (Fig. 2). This result was consistent with our speculation that the mutation site in *flo4-4* was different from that of *flo4*, which was generated via T-DNA insertion into the fifth intron of cy*OsPPDK* (Kang et al. 2005). To our knowledge, *flo4-4* is a novel mutant allele of cy*OsPPDK* responsible for floury endosperm in rice.

PCR-based analysis of SNPs is a powerful and efficient tool for plant geneticists. Among PCR-based markers, CAPS markers are the most widely used for SNP detection (Neff et al. 2002). The SNP identified in *FLO4-4* did not overlap with a CAPS marker. Therefore, we developed a dCAPS marker, whereby a mismatch in the primer was used to create a polymorphism overlapping the mutation, to verify the SNP in *flo4-4* by digesting the PCR products of $F_{3:4}$ population and 48 Korean rice cultivars (Fig. 3). Co-segregation analysis of the *flo4-4* genotype with floury endosperm phenotype indicated that the mutation in *FLO4-4* was indeed responsible for floury endosperm in rice. The dCAPS marker developed in this study can be used for marker-assisted breeding for accurate and reliable screening of rice germplasm for floury endosperm. In addition to rice, PPDK mutations in maize (*Zea mays*) and wheat have also been associated with the development of opaque kernels (Meyer et al. 1982; Aoyagi and Bassham 1984; Aoyagi et al. 1984; Gallusci et al. 1996; Kang et al. 2005).

Gene expression can be either constitutive or specific to a particular growth stage, leading to a specific phenotype (He et al. 2017). *OsPPDK* encodes cy*OsPPDK* and ch*OsPPDK*. The ch*OsPPDK* mRNA is constitutively expressed in photosynthetic organs, whereas cy*OsPPDK* is expressed only in developing grains. In maize, the cy*PPDK* is expressed during kernel development and begins to accumulate 11–14 DAF, reaching a peak approximately 20 DAF (Gallusci et al. 1996). In wheat, *PPDK* expression in developing grains is induced approximately 9 DAF, reaching a maximum approximately 24 DAF (Aoyagi et al. 1984). In rice, cy*OsPPDK* is expressed in developing rice grains after pollination and reaching the highest amount of PPDK protein at 10 days post-pollination, then both PPDK protein level and activity are rapidly down-regulated via the combined posttranslational mechanisms of threonyl-phosphorylation and protein degradation at about 20 DAF (Chastain et al. 2006). However, our results indicated that the expression of *FLO4-4* was consistently higher in the floury endosperm mutant line than that in the wild type at 10 DAF and the report inconsistent with the first report of a T-DNA insertional knockout mutant of the rice *PPDK* gene (*flo4*) showed that rice with inactivated PPDK gene failed to produce an opaque seeds (Kang et al. 2005). We predicted the functional effect of amino acid substitutions in FLO4-4 by PROVEAN tool (Choi et al. 2012). The results demonstrated that the single amino acid changes in G→D on the protein of FLO4-4 will affect the protein function. A possible explanation was that the post-translational mechanisms of threonyl-phosphorylation is disrupted by the mutation site from C to T in the exon of *FLO4-4*, or the mutant effect was stronger than the normal threonyl-phosphorylation effect of *FLO4-4* at the middle and late stages. Finally, the releasing cy*Os*PPDK increasing lipid synthesis and resulting in the mutant phenotype of floury endosperm.

Overall, we identified a novel SNP (G–A) in the coding region of *FLO4-4*, whose expression during the grain filling stage was responsible for the development of an entirely milky-white opaque kernel except for a thin peripheral area of the grain and provided a valuable material for actual breeding. During dry milling, the milky-white chalky endosperm with a loosely packed structure of starch granules was responsible for a fragile grain, with lower starch damage, and finer flour particle size than white-core endosperm mutants and the wild-type parent (Ashida et al. 2009), that made the grains attractive for use in the food industry. To further understand the interrelation of cy*OsPPDK*, inhibitor, activator and amylopectin-synthesizing enzymes in rice endosperm, more detailed analysis of the coordinated actions of these produce floury endosperm related characteristics will be needed.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

References

Aoyagi K, Bassham JA (1984) Pyruvate orthophosphate dikinase mRNA organ specificity in wheat and maize. Plant Physiol 76(1):278

Aoyagi K, Bassham JA et al (1984) Pyruvate orthophosphate dikinase gene expression in developing wheat seeds. Plant Physiol 75(2):393

Ashida K, Iida S et al (2009) Morphological, physical, and chemical properties of grain and flour from chalky rice mutants. Cereal Chem 86(2):225–231. https://doi.org/10.1094/CCHEM-86-2-0225

Ashida K, Araki E et al (2010) Flour properties of milky-white rice mutants in relation to specific loaf volume of rice bread. Food Sci Technol Res 16(4):305–312. https://doi.org/10.3136/fstr.16.305

Chastain CJ, Heck JW et al (2006) Posttranslational regulation of pyruvate, orthophosphate dikinase in developing rice (*Oryza sativa*) seeds. Planta 224(4):924. https://doi.org/10.1007/s00425-006-0259-3

Choi Y, Sims GE et al (2012) Predicting the functional effect of amino acid substitutions and indels. PLOS ONE 7(10):e46688. https://doi.org/10.1371/journal.pone.0046688

Gallusci P, Varotto S et al (1996) Regulation of cytosolic pyruvate, orthophosphate dikinase expression in developing maize endosperm. Plant Mol Biol 31(1):45–55. https://doi.org/10.1007/BF00020605

He J, Shao G et al (2017) Fine mapping and candidate gene analysis of qTAC8, a major quantitative trait locus controlling tiller angle in rice (*Oryza sativa* L.). PLOS ONE 12(5):e0178177. https://doi.org/10.1371/journal.pone.0178177

Heo S, Lee SM et al (2013) Effect of dry- and wet-milled rice flours on the quality attributes of gluten-free dough and noodles. J Food Eng 116(1):213–217. https://doi.org/10.1016/j.jfoodeng.2012.11.017

Hong H, Moon H et al (2011) A lodging tolerant, opaque rice cultivar 'Seolgaeng'. Korean J Breed Sci 43(6):532–537

Hong H-C, Choi H-C et al (2012) A lodging-tolerance and dull rice cultivar 'Baegjinju'. Korean J Breed Sci 44(1):51–56

Hormdok R, Noomhorm A (2007) Hydrothermal treatments of rice starch for improvement of rice noodle quality. LWT Food Sci Technol 40(10):1723–1731. https://doi.org/10.1016/j.lwt.2006.12.017

Kang HG, Park S et al (2005) White-core endosperm floury endosperm-4 in rice is generated by knockout mutations in the C4-type pyruvate orthophosphate dikinase gene (OsPPDKB). Plant J 42(6):901–911. https://doi.org/10.1111/j.1365-313X.2005.02423.x

Kaushik RP, Khush GS (1991) Genetic analysis of endosperm mutants in rice *Oryza sativa* L. Theor Appl Genet 83(2):146–152. https://doi.org/10.1007/BF00226243

Kawahara Y, de la Bastide M et al (2013) Improvement of the *Oryza sativa* Nipponbare reference genome using next generation sequence and optical map data. Rice 6(1):4. https://doi.org/10.1186/1939-8433-6-4

Leewatchararongjaroen J, Anuntagool J (2016) Effects of dry-milling and wet-milling on chemical, physical and gelatinization properties of rice flour. Rice Sci 23(5):274–281. https://doi.org/10.1016/j.rsci.2016.08.005

Meyer AO, Kelly GJ et al (1982) Pyruvate orthophosphate dikinase from the immature grains of cereal grasses. Plant Physiol 69(1):7

Mo Y-J, Jeung J-U et al (2013) Agronomic and genetic analysis of Suweon 542, a rice floury mutant line suitable for dry milling. Rice 6(1):37. https://doi.org/10.1186/1939-8433-6-37

Neff MM, Neff JD et al (2002) dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. Plant J 14(3):387–392. https://doi.org/10.1046/j.1365-313X.1998.00124.x

Ngamnikom P, Songsermpong S (2011) The effects of freeze, dry, and wet grinding processes on rice flour properties and their energy consumption. J Food Eng 104(4):632–638. https://doi.org/10.1016/j.jfoodeng.2011.02.001

Nishio T, Iida S (1993) Mutants having a low content of 16-kDa allergenic protein in rice (*Oryza sativa* L.). Theor Appl Genet 86(2):317–321. https://doi.org/10.1007/BF00222095

Peng C, Wang Y et al (2014) FLOURY ENDOSPERM6 encodes a CBM48 domain-containing protein involved in compound granule formation and starch synthesis in rice endosperm. Plant J 77(6):917–930. https://doi.org/10.1111/tpj.12444

Qiao Y, Lee S-I et al (2010) Fine mapping and candidate gene analysis of the floury endosperm gene, FLO(a), in rice. Mol Cells 29(2):167–174. https://doi.org/10.1007/s10059-010-0010-6

Ryoo N, Yu C et al (2007) Knockout of a starch synthase gene OsSSIIIa/Flo5 causes white-core floury endosperm in rice (Oryza sativa L.). Plant Cell Rep 26(7):1083–1095. https://doi.org/10.1007/s00299-007-0309-8

She K-C, Kusano H et al (2010) A novel factor FLOURY ENDOSPERM2Is involved in regulation of rice grain size and starch quality. Plant Cell 22(10):3280

Shih F, Daigle K (1999) Oil uptake properties of fried batters from rice flour. J Agric Food Chem 47(4):1611–1615. https://doi.org/10.1021/jf980688n

Shin YS, Park CS et al (2009) Article: characteristics of endosperm starch of the rice mutant lines induced by sodium azide. Korean J Breed Sci 41(2):84–91

Tong L-T, Gao X et al (2015) Effects of semidry flour milling on the quality attributes of rice flour and rice noodles in China. J Cereal Sci 62:45–49. https://doi.org/10.1016/j.jcs.2014.12.007

Tong L-T, Zhu R et al (2017) Soaking time of rice in semidry flour milling was shortened by increasing the grains cracks. J Cereal Sci 74:121–126. https://doi.org/10.1016/j.jcs.2017.01.011

Torbica A, Hadnađev M et al (2010) Rheological, textural and sensory properties of gluten-free bread formulations based on rice and buckwheat flour. Food Hydrocoll 24(6):626–632. https://doi.org/10.1016/j.foodhyd.2010.03.004

Yang Y, Tao W-Y (2008) Effects of lactic acid fermentation on FT-IR and pasting properties of rice flour. Food Res Int 41(9):937–940. https://doi.org/10.1016/j.foodres.2007.10.011

Zhang L, Ren Y et al (2016) FLOURY ENDOSPERM7 encodes a regulator of starch synthesis and amyloplast development essential for peripheral endosperm development in rice. J Exp Bot 67(3):633–647. https://doi.org/10.1093/jxb/erv469