Plant Mol Biol Rep (2012) 30:1303–1312
DOI 10.1007/s11105-012-0435-5

ORIGINAL PAPER

Phenotypic and Candidate Gene Analysis of a New Floury Endosperm Mutant (*osagpl2-3*) in Rice

Dapeng Zhang · Jianguo Wu · Yujiang Zhang · Chunhai Shi

Published online: 3 April 2012

Abstract A floury endosperm mutant, *osagpl2-3*, was isolated from the M_2 generation of japonica rice cultivar Nipponbare following ethyl methane sulfonate mutagenesis. The *osagpl2-3* mutant produced a white-core endosperm compared to the transparent endosperm of the wild type (WT). The results from scanning electron microscope showed that the *osagpl2-3* mutant grains comprised of round and loosely packed starch granules, some of which were compounded. The analysis for cooking and nutrition quality traits indicated that the values of gel consistency, gelatinization temperature, and rapid viscosity analysis profile of *osagpl2-3* grains were lower than those of the WT. Besides, the protein content, the contents of nine different amino acids, and the thermodynamic parameters of T_p and $\Delta T_{1/2}$ in *osagpl2-3* were also different from those of the WT. Genetic analysis revealed that *osagpl2-3* mutation was controlled by a single recessive gene. The *osagpl2-3* gene was mapped between InDel markers R1M30 and ID1-12 on rice chromosome 1. In the candidate region of the Nipponbare genome, an annotated gene, LOC_Os01g44220 which encodes a large subunit of putative ADP-glucose pyrophosphrylase named *OsAPL2* was considered the optimal candidate. Cloning and sequencing of LOC_Os01g44220 in different plants of the *osagpl2-3* mutants revealed a single nucleotide mutation (G→A) in the open reading frame region, which led to a substitution of an acidic amino acid Glu (E) by a basic amino acid Lys (K) accordingly. Furthermore, the mutant site is close to the functional domain which interacts with the ADP-Glc. In brief, these results suggested that the *osagpl2-3* is a new mutant of *OsAPL2*.

Keywords *osagpl2-3* · *flo6* · *OsAPL2* · Floury endosperm · ADP-glucose pyrophosphrylase · Map-based cloning · Rice

Abbreviations

AC	Amylose content
ADPG	ADP-glucose
AGPase	ADP-glucose pyrophosphorylase
ATP	Adenosine 5′ triphosphate
GC	Gel consistency
Glc-1-P	Glucose-1-phosphate
GT	Gelatinization temperature
ORF	Open reading frame
PC	Protein content
RVA	Rapid viscosity analysis
PPDK	Pyruvate orthophosphate dikinase

Introduction

Rice grain chalkiness refers to the opaque spots in the endosperm which can affect the quality, specially the appearance of the rice grain (Fitzgerald et al. 2000; Yamakawa et al. 2007). Chalky grains are predisposed to rupture during polishing leading to a decrease in the proportion of edible rice (Wan et al. 2007). Chalk in rice grains directly and indirectly contributes to both the proportion of chalkiness and broken grains, which are two of the major traits that most markets base on to dictate the price of rice (Fitzgerald et al. 2009). Floury endosperm is

Dapeng Zhang and Jianguo Wu contributed equally to this paper.

Electronic supplementary material The online version of this article (doi:10.1007/s11105-012-0435-5) contains supplementary material, which is available to authorized users.

D. P. Zhang · J. G. Wu · Y. J. Zhang · C. H. Shi (✉)
Agronomy Department, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310058, China
e-mail: chhshi@zju.edu.cn

one phenotype of chalky rice with loosely packed starch granules in the center and normal outer layer of the endosperm. Five loci of rice floury endosperm (*flo*) have already been reported. The loci of *flo-1*, *flo-2*, and *flo-3* were located on chromosome 5, 4, and 4, respectively (Satoh and Omura 1981; Kumamaru et al. 1997; Nishio and Iida 1993). The *flo4* are mutants with insertional mutations in the *OsPPDKB* which is an important modulator of carbon flow for starch and lipid biosynthesis during grain-filling (Kang et al. 2005). The rice *SSIIIa* mutants named *flo5* are generated by T-DNA insertions in the coding sequence of the gene, resulting in a white-core floury endosperm (Ryoo et al. 2007). Recently, a mutant which lost the function of a gene that encodes a cell wall invertase required for carbon partitioning during early grain-filling named *gif1* has been reported, and the grains of this mutant also have the floury endosperm phenotype (Wang et al. 2008). Another mutant named *flo2* rice mutant has been reported, and its wild-type (WT) gene named *FLO2* which is a superior conductor of the novel regulatory cascade of endosperm organogenesis may also have important roles in the response to high-temperature stress (She et al. 2010). A new floury endosperm mutant named *flo(a)* whose gene was located on chromosome 4 by positional cloning was reported in 2010. Preliminary results showed that the protein of *FLO(a)* might play a significant role in rice starch biosynthetic pathways (Qiao et al. 2010). The deleted gene encoding a protein disulphide isomerase-like enzyme (*PDIL1-1*) in a rice somaclonal mutant T3612 that produces small grains with a floury endosperm was revealed by the positional cloning of the mutation (Han et al. 2011).

Starch biosynthesis enzymes in the cereal endosperm include AGPase, granule-bound starch synthase, soluble starch synthase, starch branching enzyme, starch debranching enzyme, and plastidial starch phosphorylase (Pho1) (Jeon et al. 2010; James et al. 2003). Based on the results from studies on *flo* serial mutants, we can know that the rice endosperm will develop abnormally if each of these essential enzymes in cereal starch biosynthesis is defective. ADP-glucose pyrophosphorylase (AGPase) catalyzes the first step of starch biosynthesis in plants (Hannah and Greene 1998). Observations from potato, maize, barley, and rice indicate that AGPase is a rate-limiting enzyme in starch biosynthesis (Hannah and Greene 1998; Denyer et al. 2004; Giroux et al. 2003; Dickinson and Preiss 1969). It is the enzymatic site for the regulation of storage polysaccharide accumulation in plants and bacteria, which is allosterically activated or inhibited by metabolites of energy flux (Dickinso and Preiss 1969; Furlong and Preiss 1969; Haugen et al. 1976; Preiss et al. 1975; Jin et al. 2005; Dickinson and Preiss 1969). AGPase is a tetramer that comprised two small and two large subunits, each of which is encoded by distinct genes (Okita et al. 1990). In rice, the AGPase gene family consists of two SSU genes (*OsAGPS1* and *OsAGPS2)* and four LSU genes (*OsAGPL1*, *OsAGPL2*, *OsAGPL3*, and *OsAGPL4)* (Akihiro et al. 2005; Lee et al. 2007; Ohdan et al. 2005; Jeon et al. 2010). The protein structure of AGPase in potato has also been reported (Jin et al. 2005). For cereals, it is largely extra-plastidial in the endosperm and it is functionally different from non-cereal plants (James et al. 2003). This could be due to a process that may have originated from a whole-genome-duplication event in an early ancestor of the grasses (Comparot-Moss and Denyer 2009).

Here, a new floury endosperm mutant was isolated and named *flo6* (*floury endosperm 6*) following the phenotype of floury endosperm. We report the phenotypic and candidate gene analysis of the *flo6* gene in rice. The *FLO6* gene which encodes a presumed rice AGPase large subunit *OsAPL2* was isolated and sequenced to ensure that it was responsible for *flo6* mutation. Thus, *flo6* mutant was renamed with *osagpl2-3* as a new mutant of *OsAPL2*.

Materials and Methods

Plants Materials

The *flo6* mutant was isolated from M_2 generation of rice cultivar Nipponbare (*Oryza sativa* L. ssp. *japonica*) by mutagenesis with ethyl methanesulfonate (EMS). Seeds (M_0) were presoaked in water for 16 h, dried, and then treated with 0.4 % EMS solution for 8 h. The treated seeds were then washed several times with distilled water. All seedlings (M_1) from the treated seeds were transplanted into the paddy field, and the seeds which constitute the M_2 generation were harvested at maturity (Thang et al. 2010). An F_2 population of grains derived from F_1 plants that were obtained from the cross between *flo6* mutant and normal rice cultivar R9703 (*O. sativa* L. ssp. *indica*) was used for genetic mapping. A total of 286 F_2 mutant individuals were identified from the population. In addition, 1,564 grains from F_1 plants derived from the cross between *flo6* and WT were used for genetic analysis.

Scanning Electron Microscopic Analysis

Grain samples for scanning electron microscopic analyses were directly dried to a critical point with liquid CO_2, mounted on SEM stubs, and then coated with gold palladium. The mounted specimens were observed under an SEM (Hitachi TM-1000 Tabletop Microscope).

Cooking and Nutrition Quality Analysis

The measurement of protein content (PC) and amylose content (AC), gel consistency (GC), thermodynamic characteristics (GT) of rice floury, floury rapid viscosity analysis (RVA) profile, and amino acid content were conducted according to the standard of the Ministry of Agriculture (1988), the National

Standard of the People's Republic of China (1999); Xu et al. (2004); Bao et al. (2004) and Wu et al. (2002), respectively.

DNA Extraction and PCR Analysis

The rice grains were sterilized by the method of Kato et al. (2010). Rice genomic DNA was extracted from the plants grown in 1/2 MS medium at trefoil stage. The DNA extraction and PCR reaction system for mapping were according to the method by Li et al. (2010).

Linkage Analysis and Genetic Mapping

A total of 350 markers (300 SSR markers and 50 InDel markers) well distributed on the 12 chromosomes were taken from the rice database Gramene (http://www.gramene.org/markers/microsat/) and Shen et al. (2004), respectively. The markers showing polymorphisms between Nipponbare and R9703 were selected to determine the approximate chromosomal position of *flo6* gene by using the bulked segregation analysis (BSA) method developed by Michelmore et al. (1991). Genetic mapping was according to the methods of Zhao et al. (2010) and Sui et al. (2012).

Development of New InDels Markers

Thirty-four InDel markers were designed in the present experiment by the methods of Li et al. (2010) and Wang et al. (2011). However, only one marker ID1-12 (forward, 5′-TAAGA GAGGTTGAAATGGAGTC-3′; reverse, 3′-ATTTATCT CAACAGACGGTTTT-5′) which has polymorphism between Nipponbare and R9703 was used for mapping the *flo6* gene.

Sequencing Analysis of the Candidate Region

The rice Gramene database (http://www.gramene.org/) was referred to for candidate gene annotation. The special attention was drawn to a putative gene, LOC_Os01g44220, for analyzing the annotation information in the candidate region. To sequence the LOC_Os01g44220, ten sets of primers were designed and synthesized (Supplementary Table 1). Specific DNA fragment were amplified from the genomic DNA of *flo6* mutant and WT and then cloned into TA cloning vector pMD19-T (Takara, Dalian, China) for sequencing.

In Silicon Analysis

The following proteomics tools: ProtParam (http://web.expasy.org/protparam/), TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), PredictProtein (http://www.predictprotein.org/), SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html), TargetP (http://www.cbs.dtu.dk/services/TargetP/), SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/), CPHmodels 3.0 server (http://www.cbs.dtu.dk/services/CPHmodels/), multiple sequence alignment tools ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and software SweetMollyGrace 1.3 were used to analyze the predicted protein parameters of the WT and *flo6*. The SNP database dbSNP BUILD 136 (http://www.ncbi.nlm.nih.gov/snp/) was used to analyze the SNP sites. SITF sequence (http://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html) was used to predict the effects of amino acid substitutions on protein function.

Results

Characterization of the *flo6* Mutants

Although no distinct morphological differences were found between the WT and the *flo6* mutant plants, some obvious differences can be found in the grains. The grains of the WT had transparent endosperms (Fig. 1a, f, g), while those of the *flo6* mutant had white-core endosperms (Fig. 1b, h, i). Cross-sectioning of the grains revealed that the outer part of *flo6* grain as well as that of the WT was normal. However, the interior of the mutant grain was floury-white which was not as that of the WT (Fig. 1j, k). The scanning electron microscopic results showed that the starch granules of the WT grains were packed, and the edges of starch granules in the cross-sectioning were sharp (Fig. 1l, m). The starch granules in the normal sections of the *flo6* mutant were similar to those of the WT (Fig. 1n), whereas those in floury endosperm were round and loosely packed and some starch granules were compounded by film coating (Fig. 1o). The morphological trails of the grains were also analyzed in present experiment (Table 1). The chalky area ratio of *flo6* mutant was much higher than that of the WT. The 1,000-grain weight, grain length, grain width, and grain thickness of *flo6* were lower than that of the WT. However, the length/width ratios of the *flo6* mutant grain and the WT were similar. These results suggested that the *flo6* mutation has significant effects on the rice grains resulting in decreased yields and reduced grain size. But the lack of significant difference in the length/width ratios between WT and *flo6* mutants indicated that the mutant gene generated no effects on the shape of grains.

Cooking and Nutrition Quality of *flo6* Grains

The AC of the WT was slightly lower than that of the *flo6* mutant, and no significant difference between them was detected (Fig. 2a). The values of GC, GT, and RVA profile of the *flo6* mutant were lower than that of the WT (Fig. 2b, e and Table 2), and the thermodynamic parameters T_p and $\Delta T_{1/2}$ of the WT and *flo6* mutant were also different (Table 2). PC

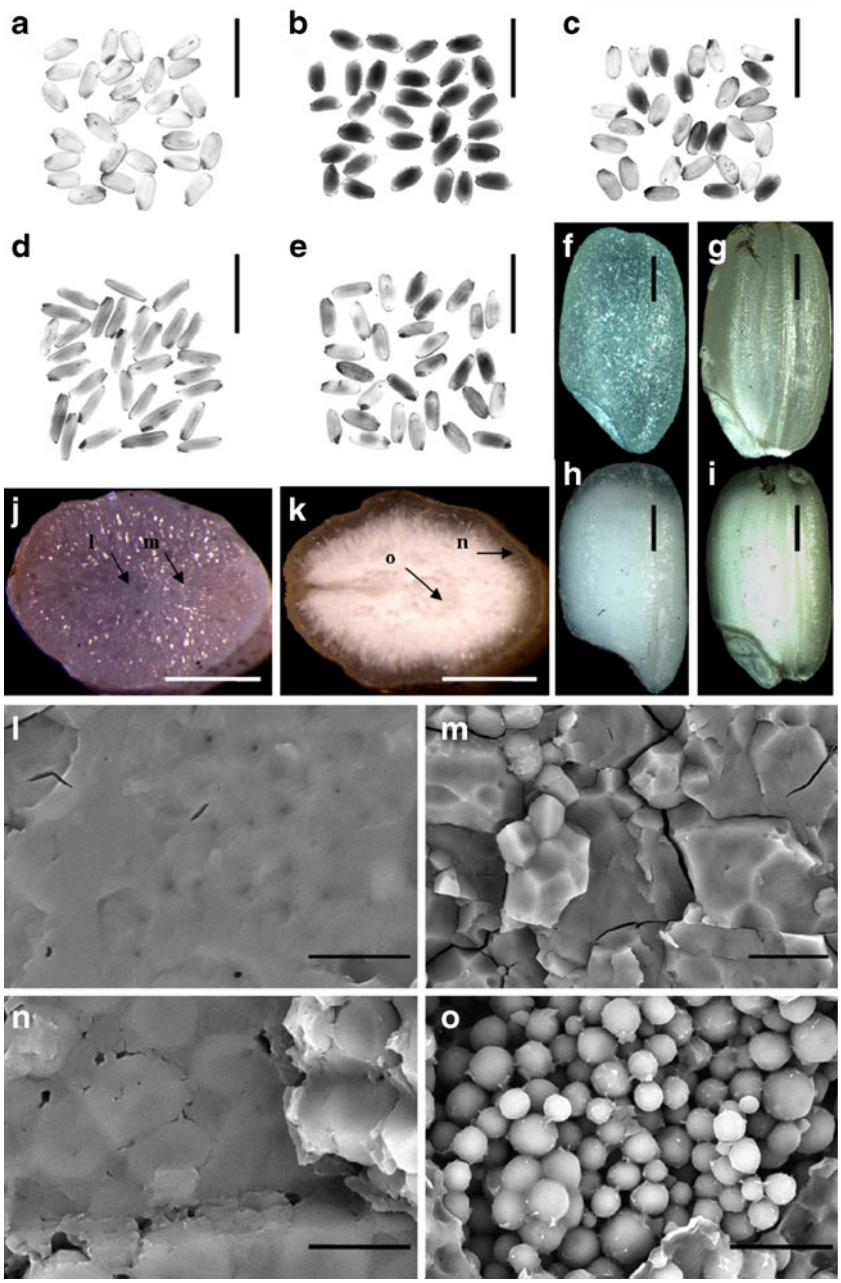

Fig. 1 Phenotypes of *flo6* kernels. **a**, **b** Brown rice of wild-type and *flo6*. **c** Brown rice of F_2 crossed by WT and *flo6*. **d** Brown rice of *indica* variety R9703. **e** Brown rice of F_2 crossed by *flo6* and *indica* variety R9703. **f**, **h** Milled rice of WT and *flo6*. **g**, **i** Brown rice of WT and *flo6*. **j**, **k** Crossed sections of WT and *flo6*. **l**, **m** SEM analysis of normal sections indicated by *arrow* in ***j***. **n**, **o** SEM analysis of mutant sections indicated by *arrow* in **k**. *Bars* **a**–**e**=10 mm; **f**–**k**=1 mm; **l**, **n**=5 μm; **m**, **o**=10 μm

was higher in the *flo6* mutant than the WT (Fig. 2c), as was also observed in mutant *flo-4* (Kang et al. 2005). The relative content analysis of 17 amino acids revealed differences between the WT and the *flo6* mutant in nine of them, and all of them, except for Ser and Met, were higher in *flo6* than those in the WT (Fig. 2d). These results above indicated that the mutant gene did not change the AC but had an obvious effect on GC, GT, and RVA profile of rice floury endosperm, suggesting that the WT gene may play an important role in rice cooking quality and could affect the nutritional quality of rice.

Table 1 Grain morphology data of *flo6*

Type	Chalky ratio (%)	Thousand-grain weight (g)	Grain length (mm)	Grain width (mm)	Grain thickness (mm)	Length/width ratio
WT	3.5	21.40±0.25	5.22±0.27	2.85±0.16	1.95±0.09	1.84±0.01
flo6	100	19.70±0.29*	4.86±0.24*	2.70±0.13*	1.82±0.13*	1.80±0.10

Values are mean ± SD except for the percentage of chalky grains: length, width, and thickness of grain, n=50; length/width ratio, n=30

*P<0.01; **P<0.05 (significant differences)

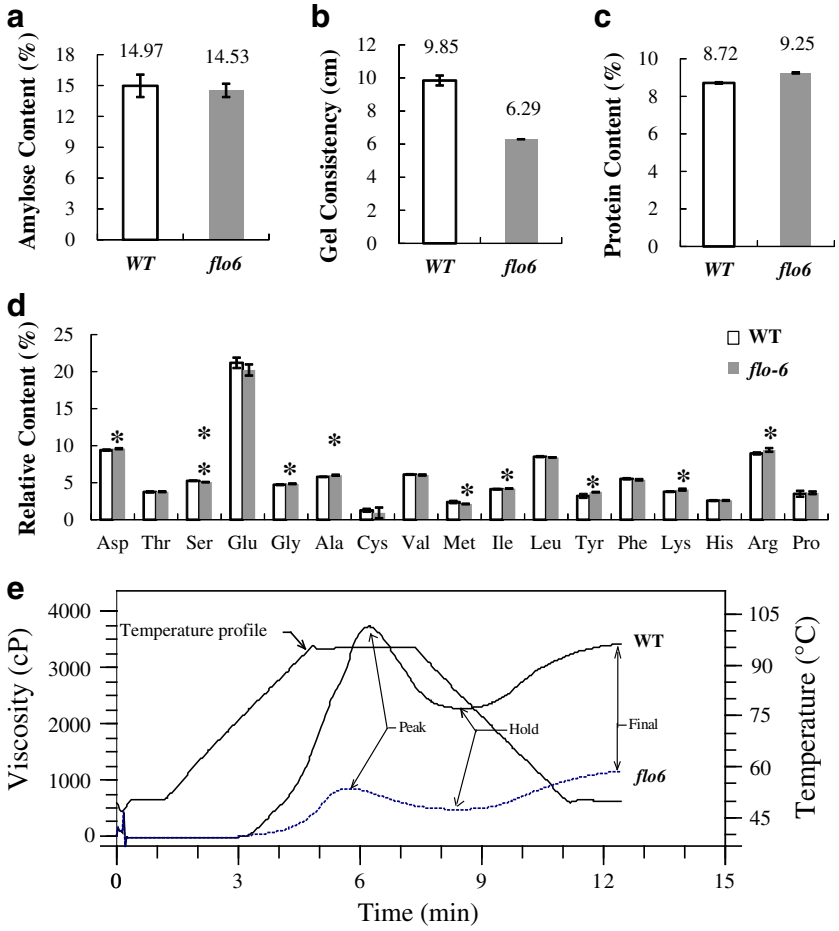

Fig. 2 Quality traits parameter of WT and *flo6*. **a–c** AC, GC, and PC of WT and *flo6* (n=3). **d** Relative content of amino acids of WT and *flo6* (**P*<0.05; ***P*<0.01. n=2). **e** Floury RVA curve of WT and *flo6*

Genetic Mapping and Map-Based Cloning of *flo6* Gene

To determine whether *flo6* was controlled by a single gene or multiple genes, the *flo6* mutant was crossed with WT plants. The *indica* rice R9703 (Fig. 1d) was crossed with flo6 mutant for the construction of gene mapping population (Fig. 1e). A total of 1,217 wild-type grains and 347 *flo6* grains were obtained from the F_1 plants. The segregation of normal grains and mutant grains (Fig. 1c) was fitted to a 3:1 segregation ratio (P=0.89>0.01). The 3:1 segregation ratio of normal grains to mutant grains indicated that the *flo6* phenotype was controlled by a single recessive gene.

In order to determine the chromosomal location of the *flo6* gene, 158 molecular markers (SSR markers and InDel markers) which showed polymorphisms between Nipponbare and R9703 were selected to conduct linkage analysis by BSA (Fig. 3a). It was observed that the InDel marker R1M30 and SSR marker RM3475 which are located on the long arm of rice chromosome 1 showed close linkage with the *flo6* gene (Fig. 3b, c). Using 286 *flo6* individuals from the F_2 mapping population, the markers R1M30 and RM3475 were mapped on the left and right of *flo6* locus with genetic distances of 8.6 and 3.5 cM, respectively (Fig. 4a, b).

Table 2 Thermodynamics character of rice floury of WT and *flo6*

Type	GT (°C)	T_o (°C)	T_p (°C)	T_c (°C)	$\Delta T_{1/2}$ (°C)	ΔH_g (J/g)
WT	65.59±0.01	50.08±0.24	72.47±0.16	87.44±0.30	7.70±0.25	–10.03±0.36
flo6	62.14±0.06*	50.42±0.12	69.84±0.30*	86.77±0.18	9.56±0.19**	–9.67±0.15

Values are mean ± SD (n=2)

**P*<0.01; ***P*<0.05 (significant differences)

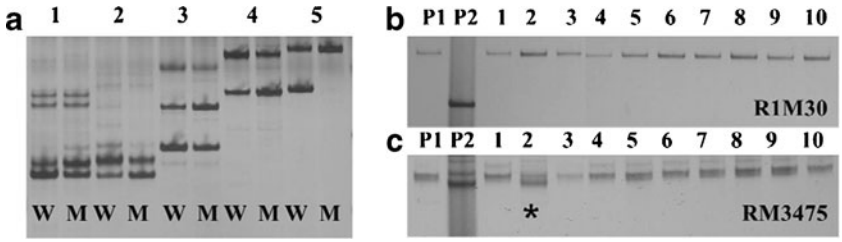

Fig. 3 Results of BSA and identification of recombinants in F_2 population. **a** The results of BSA of five polymorphism markers in chromosome 1, *1~5* represent markers RM581, RM23, R1M7, RM493, and R1M30, respectively. *W* wild-type pool and *M* mutant type pool. **b** The In/Del marker R1M30 is linked to the *flo6* locus in primary mapping and without recombinants in the ten individuals of F_2 population. *P1 flo6* mutant and *P2 indica* rice R9703, *1~10* represent mutant individuals from F_2 population. **c** The SSR marker RM3475 is linked to *flo6* locus and used for identification of recombinants in F_2 population. *Asterisk* recombinants

For further mapping of the *flo6* loci, 32 InDel markers were developed in the region restricted by markers R1M30 and RM3475 based on the differences of genomic DNA sequences between Nipponbare (*japonica* cultivar) and 93-11 (*indica* cultivar). However, only one InDel marker named ID1-12 which showed polymorphism between *flo6* mutant and R9703 was used for further mapping of the *flo6* gene. The results from genotyping of 286 *flo6* individuals of F_2 mapping population showed that the InDel marker ID1-12 was located between *flo6* locus and RM3475, away from *flo6* with a genetic distance of 1.3 cM (Fig. 4b). Thus, the *flo6* was mapped in an interval restricted by R1M30 and ID1-12 with a physical distance of 1,055 kb.

Attempts were made to develop polymorphic SSR or InDel markers in the 1,055-kb region. However, no polymorphic markers were found due to poor polymorphisms between genomic DNA of Nipponbare and R9703. It was thus not necessary to analyze all candidate genes in this large region since the average physical distance per centimorgan is observed to be 244 kb for the rice genome (Chen et al. 2002). Therefore, we analyzed the annotated genes approximately 500 kb upstream of ID1-12 in the rice Gramene database (http://www.gramene.org/) based on the 1.3 cM genetic distance at which ID1-12 was away from the *flo6* gene.

There are 86 open reading frames (ORFs) in this region (Supplementary Table 2). Only one is a relation gene of starch synthesis, and 18 of the 86 ORFs are annotation as hypothetical protein. We will choose the relation gene of starch synthesis for sequencing, first by analyzing the information of annotation of the presumption genes. If no mutation in the locus that we chose, then we will analyze the other ORFs by the methods of bioinformatics or construct a new population of gene mapping to narrow the candidate region. An annotated gene, LOC_Os01g44220 which encodes putative ADP-glucose pyrophosphrylase large subunit named *OsAPL2*, was considered the optimal candidate of *Flo6*. The reasons is that the starch granules of the endosperm in the homozygous *OsAPL2* defective mutants were smaller in size and rounder in shape when compared to those from the wild-type endosperm (Lee et al. 2007). The function of *OsAPL2* is starch synthesis which is expressed at the late stages of seed development (Akihiro et al. 2005). Thus, the locus LOC_Os01g44220 was therefore amplified from the genomic DNA of different plants of the *flo6* mutant and the WT by PCR and then sequenced (Fig. 4c). Results from DNA sequencing revealed a single nucleotide mutation (G→A) of the tenth exon in ORF region of LOC_Os01g44220 which led to a single amino acid substitution from acidic amino acid Glu (E) to basic amino acid Lys (K) accordingly (Fig. 4d). We analyzed the SNP sites of nucleotide sequence of the candidate gene *OsAGPL2* (NCBI accession NP_001043654, definition Os01g0633100); there are 95 SNP sites in this locus and the mutation site of (G→A) does not belong to them.

Differences of Protein Parameter and Multiple Sequence Alignment

In order to elucidate effect of the single nucleotide mutation (G→A) on the *Flo6* gene, some protein prediction parameters of the WT and the *flo6* mutants were analyzed using proteomic tools. The results showed both differences between the physical–chemical parameters and between the secondary and tertiary protein structures of the WT and the *flo6* (Supplementary Table 3 and 4; Supplementary Fig. 1). The multiple sequence alignment of *OsAPL1*, *OsAPL2*, *OsAPL3*, *Escherichia coli*, *Solamun tuberosum_L*, and *Zea mays_L2* were data-mined using the ClustalW2 program. The mutant site is close to the functional domain which interacts with ADP-Glc (Fig. 5). The substitution from acidic amino acid Glu (E) to basic amino acid Lys (K) brings a change in total electric charge around this functional domain (Supplementary Table 3).

Discussion

In cereals, the principal storage reserve is starch, which constitutes approximately 78 % of the dry weight of the grain, and one key regulatory step that controls the flux of carbon into starch is catalyzed by AGPase (Nagai et al. 2009; Singh and Juliano 1977). The overexpression of

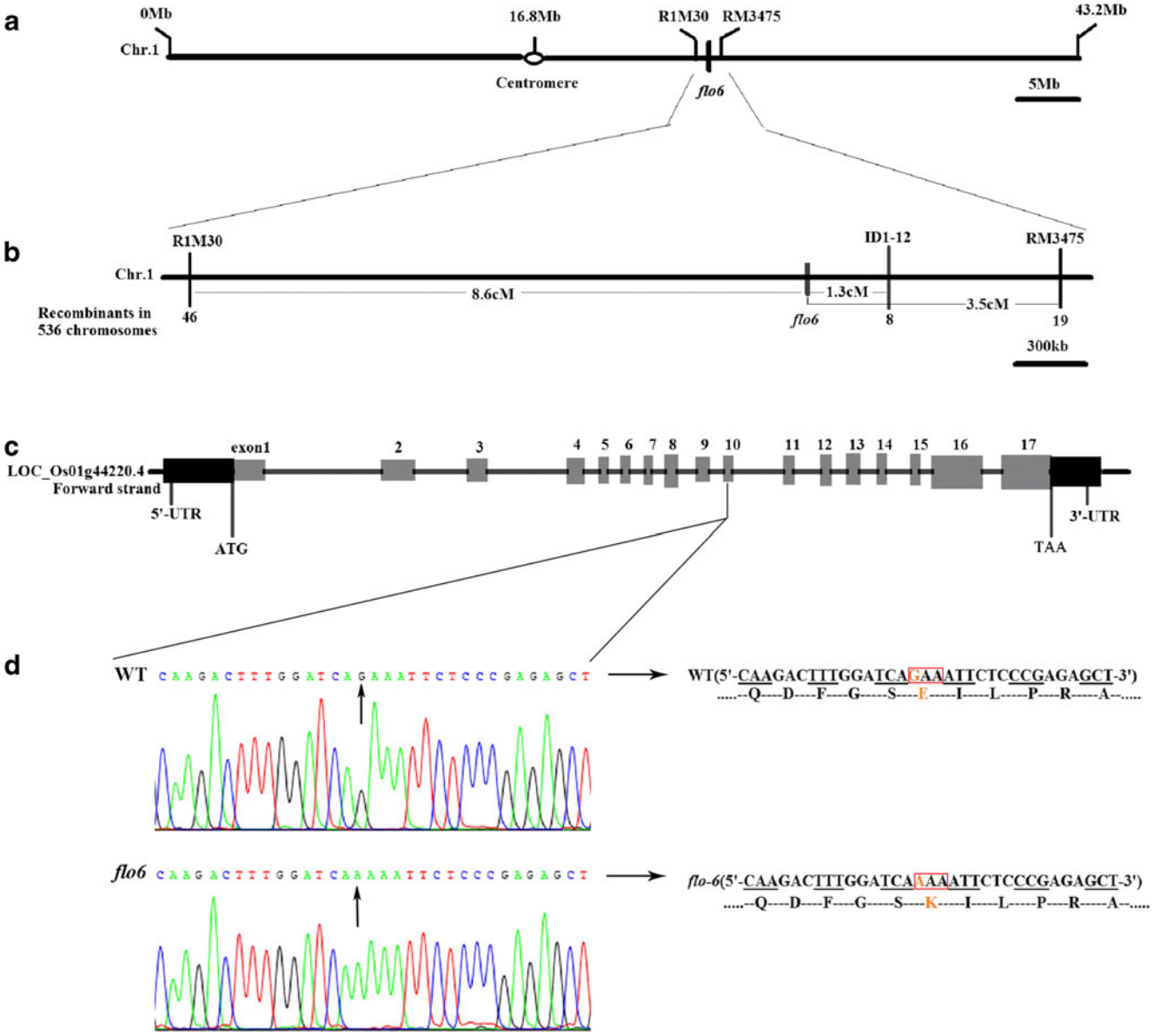

Fig. 4 Map-based cloning of the *flo6* gene. **a** Primary gene mapping of the *flo6* locus. **b** Genetic linkage map derived from F_2 population with 286 individual mutants. *Above the vertical line* are the names of the marks and *below* are the numbers of recombinants. *Numbers between vertical lines* are genetic distances of the *flo6* locus with marks. **c** The construction of candidate gene LOC_Os01g44220 in MSU database (IRGSP locus: Os01g0633100, NCBI GeneBank: AK071497) that encodes glucose-1-phosphate adenylyltransferase large subunit is suspected to be the *flo6* gene. *Black boxes* and *gray boxes* indicate untranslated region (UTR) and open reading frame (ORF), respectively. **d** Mutation site of DNA and protein sequence

AGPase enhances seed weight and starch content (Li et al. 2011). Expression of a cytoplasmic-localized AGPase mutant gene from *E. coli* in rice endosperm resulted in enhanced starch synthesis and, in turn, higher seed weights (Nagai et al. 2009). Furthermore, the lesion of *OsAGPL2* causes a shrunken endosperm due to a remarkable reduction in starch synthesis (Lee et al. 2007) and the direct effect is the formation of floury endosperm in rice. The increased ratio of chalk in rice grains could be linked with the rising global temperatures (Yamakawa et al. 2008; 2007). A common unfavorable effect of chalky or floury endosperm on grains is the decrease of 1,000-grain weight, and thus reducing its yield. Finding the gene related to chalk or floury endosperm was an effective approach towards solving this problem.

In the present study, a *floury endosperm* mutant (*flo6*) was isolated from the M_2 population of the *japonica* cultivar Nipponbare. The morphology, cooking, and nutrition quality

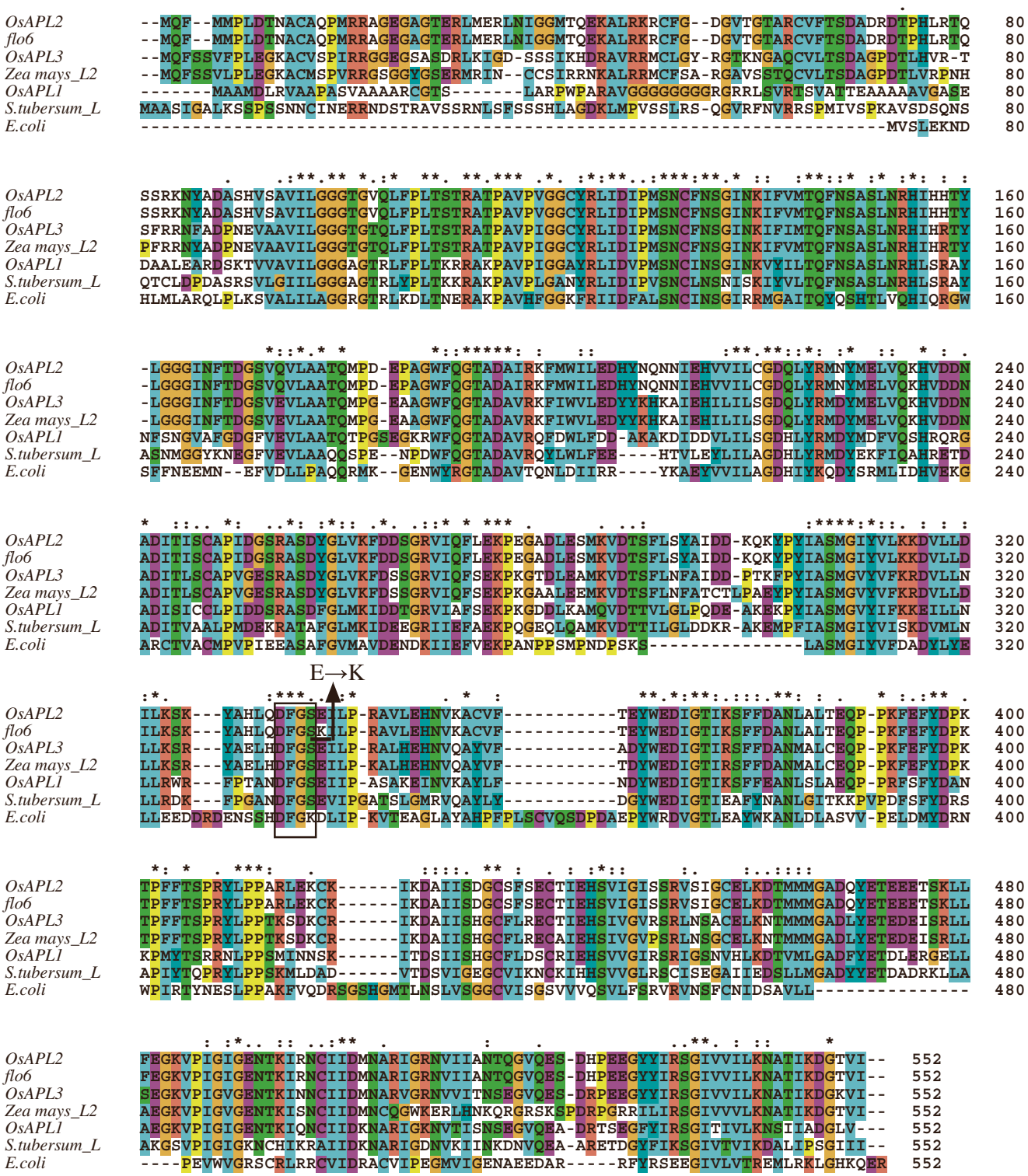

Fig. 5 Comparison of the predicted amino acid sequences of AGPases large submits. Multiple sequence alignment of AGPase large subunits was data-mined using the ClustalW2 program. The sequences were obtained from the NCBI database: *OsAPL1* (AK069296), *OsAPL2* (AK071497), *OsAPL3* (AK100910), *E. coli* (AAY18580.1), *S. tuberosum_L* (P23509.2), and *Zea mays_L2* (NP_001105717.1). The residues interacting with ADP-Glc were represented by *black box* (Jin et al. 2005). The mutant site was represented by *black arrow*

of the *flo6* mutant grains were analyzed. A map-based cloning strategy was used to identify the *flo6* gene. The results showed that the *flo6* gene was located within a genetic distance of 1.3 cM away from the InDel marker ID1-12 on the long

arm of chromosome 1. It is believed that, in the region of approximately 500 kb, locus LOC_Os01g44220 which encodes ADP-glucose pyrophosphrylase large subunit might be the mutant gene. Sequencing results revealed that a single nucleotide mutation (G→A) at the tenth exon of locus LOC_Os01g44220.4 lead to an amino acid substitution (E→K) in the protein sequence. The interior of the endosperm in the *flo* phenotype was floury-white while the outer portion was normal, suggesting that starch accumulation was abnormal in the early stages of ripening but recovered later on in development (Kang et al. 2005). *OsAGPL2* functions mainly during the middle and late stages of endosperm development and accompanies a huge increase in the demand for starch synthesis (Akihiro et al. 2005; Ohdan et al. 2005; Lee et al. 2007). We analyzed the effects of amino acid substitutions on protein function of *OsAGPL2* by the methods of Ng and Henikoff (Ng and Henikoff 2003, 2006). The results indicate that the single amino acid substitution of E→K on the site of E_{326} of the protein of *OsAGPL2* will affect the protein function. A possible explanation was that the mutant effect was stronger than the normal catalytic effect of *OsAGPL2* at the early stages of endosperm development, but this phenomenon reversed due to the huge increase the amount of *OsAGPL2* at the middle and late stages, finally resulting in the mutant phenotype of floury endosperm. Based on the above results, we consider that the *flo6* is a new mutant of *OsAGPL2*, and it was renamed *osagpl2-3*.

Acknowledgments This work was supported by Zhejiang Provincial Natural Science Foundation of China (no. Y3080217), the Science and Technology Office of Zhejiang Province (no. 2010C32002 and no. 2007C12902), the Program for Innovative Research Team in University(IRT1185), the Fundamental Research Funds for the Central Universities" after 2007C12902), and 151 Foundation for Talents of Zhejiang Province of China. We thank Mr. Bin Zhang and Kemin Wang for the assistance of getting some data during the project. We also thank Dr. Wenqiang Li and Alfred Quampah in the English revision of the manuscript.

References

Akihiro T, Mizuno K, Fujimura T (2005) Gene expression of ADP-glucose pyrophosphorylase and starch contents in rice cultured cells are cooperatively regulated by sucrose and ABA. Plant Cell Physiol 46(6):937–946. doi:10.1093/pcp/pci101

Bao JS, Kong XL, Xie JK, Xu LJ (2004) Analysis of genotypic and environmental effects on rice starch. 1. Apparent amylose content, pasting viscosity, and gel texture. J Agric Food Chem 52 (19):6010–6016

Chen M, Presting G, Barbazuk WB, Goicoechea JL, Blackmon B, Fang FC, Kim H, Frisch D, Yu YS, Sun SH, Higingbottom S, Phimphilai J, Phimphilai D, Thurmond S, Gaudette B, Li P, Liu JD, Hatfield J, Main D, Farrar K, Henderson C, Barnett L, Costa R, Williams B, Walser S, Atkins M, Hall C, Budiman MA, Tomkins JP, Luo MZ, Bancroft I, Salse J, Regad F, Mohapatra T, Singh NK, Tyagi AK, Soderlund C, Dean RA, Wing RA (2002) An integrated physical and genetic map of the rice genome. Plant Cell 14(3):537–545. doi:10.1105/tpc.010485

Comparot-Moss S, Denyer K (2009) The evolution of the starch biosynthetic pathway in cereals and other grasses. J Exp Bot 60 (9):2481–2492. doi:10.1093/jxb/erp141

Denyer K, Patron NJ, Greber B, Fahy BE, Laurie DA, Parker ML (2004) The lys5 mutations of barley reveal the nature and importance of plastidial ADP-Glc transporters for starch synthesis in cereal endosperm. Plant Physiol 135(4):2088–2097. doi:10.1104/pp.104.045203

Dickinso DB, Preiss J (1969) Presence of Adp-glucose pyrophosphorylase in shrunken-2 and brittle-2 mutants of maize endosperm. Plant Physiol 44(7):1058–1062

Dickinson DB, Preiss J (1969) Presence of ADP-glucose pyrophosphorylase in shrunken-2 and brittle-2 mutants of maize endosperm. Plant Physiol 44(7):1058–1062

Fitzgerald MA, Lisle AJ, Martin M (2000) Chalky and translucent rice grains differ in starch composition and structure and cooking properties. Cereal Chem 77(5):627–632

Fitzgerald MA, McCouch SR, Hall RD (2009) Not just a grain of rice: the quest for quality. Trends Plant Sci 14(3):133–139. doi:10.1016/j.tplants.2008.12.004

Furlong CE, Preiss J (1969) Biosynthesis of bacterial glycogen. VII. Purification and properties of the adenosine diphosphoglucose pyrophosphorylase of Rhodospirillium rubrum. J Biol Chem 244(10):2539–2548

Giroux MJ, Smidansky ED, Martin JM, Hannah LC, Fischer AM (2003) Seed yield and plant biomass increases in rice are conferred by deregulation of endosperm ADP-glucose pyrophosphorylase. Planta 216(4):656–664. doi:10.1007/s00425-002-0897-z

Han X, Wang Y, Liu X, Jiang L, Ren Y, Liu F, Peng C, Li J, Jin X, Wu F, Wang J, Guo X, Zhang X, Cheng Z, Wan J (2011) The failure to express a protein disulphide isomerase-like protein results in a floury endosperm and an endoplasmic reticulum stress response in rice. J Exp Bot. doi:10.1093/jxb/err262

Hannah LC, Greene TW (1998) Maize seed weight is dependent on the amount of endosperm ADP-glucose pyrophosphorylase. J Plant Physiol 152(6):649–652

Haugen TH, Ishaque A, Preiss J (1976) Biosynthesis of bacterial glycogen. Characterization of the subunit structure of *Escherichia coli* B glucose-1-phosphate adenylyltransferase (EC 2.7.7.27). J Biol Chem 251(24):7880–7885

James MG, Denyer K, Myers AM (2003) Starch synthesis in the cereal endosperm. Curr Opin Plant Biol 6(3):215–222. doi:10.1016/S1369-5266(03)00042-6

Jeon JS, Ryoo N, Hahn TR, Walia H, Nakamura Y (2010) Starch biosynthesis in cereal endosperm. Plant Physiol Biochem 48 (6):383–392. doi:10.1016/j.plaphy.2010.03.006

Jin X, Ballicora MA, Preiss J, Geiger JH (2005) Crystal structure of potato tuber ADP-glucose pyrophosphorylase. EMBO J 24 (4):694–704. doi:10.1038/sj.emboj.7600551

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

Kato H, Xie GS, Sato Y, Imai R (2010) Isolation of anther-specific gene promoters suitable for transgene expression in rice. Plant Mol Biol Rep 28(3):381–387. doi:10.1007/s11105-009-0162-8

Kumamaru T, Sato H, Satoh H (1997) High-lysine mutants of rice, *Oryza sativa* L. Plant Breed 116(3):245–249

Lee SK, Hwang SK, Han M, Eom JS, Kang HG, Han Y, Choi SB, Cho MH, Bhoo SH, An G, Hahn TR, Okita TW, Jeon JS (2007) Identification of the ADP-glucose pyrophosphorylase isoforms essential for starch synthesis in the leaf and seed endosperm of rice (*Oryza sativa* L.). Plant Mol Biol 65(4):531–546. doi:10.1007/s11103-007-9153-z

Li W, Wu J, Weng S, Zhang D, Zhang Y, Shi C (2010) Characterization and fine mapping of the glabrous leaf and hull mutants (gl1) in rice (*Oryza sativa* L.). Plant Cell Rep 29(6):617–627. doi:10.1007/s00299-010-0848-2

Li N, Zhang S, Zhao Y, Li B, Zhang J (2011) Over-expression of AGPase genes enhances seed weight and starch content in transgenic maize. Planta 233(2):241–250. doi:10.1007/s00425-010-1296-5

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis—a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88(21):9828–9832

Ministry of Agriculture PRC (1988) Standard of Ministry of Agriculture, P. R. China.NY147-88. Method for rice quality measurement, vol NY147-88. Standard Press of China, Beijing

Nagai YS, Sakulsingharoj C, Edwards GE, Satoh H, Greene TW, Blakeslee B, Okita TW (2009) Control of starch synthesis in cereals: metabolite analysis of transgenic rice expressing an up-regulated cytoplasmic ADP-glucose pyrophosphorylase in developing seeds. Plant Cell Physiol 50(3):635–643. doi:10.1093/Pcp/Pcp021

Ng PC, Henikoff S (2003) SIFT: predicting amino acid changes that affect protein function. Nucleic Acids Res 31(13):3812–3814

Ng PC, Henikoff S (2006) Predicting the effects of amino acid substitutions on protein function. Annu Rev Genomics Hum Genet 7:61–80. doi:10.1146/annurev.genom.7.080505.115630

Nishio T, Iida S (1993) Mutants Having a Low Content of 16-Kda Allergenic Protein in Rice (Oryza-Sativa L). Theor Appl Genet 86 (2–3):317–321

Ohdan T, Francisco PB Jr, Sawada T, Hirose T, Terao T, Satoh H, Nakamura Y (2005) Expression profiling of genes involved in starch synthesis in sink and source organs of rice. J Exp Bot 56 (422):3229–3244. doi:10.1093/jxb/eri292

Okita TW, Nakata PA, Anderson JM, Sowokinos J, Morell M, Preiss J (1990) The subunit structure of potato tuber ADPglucose pyrophosphorylase. Plant Physiol 93(2):785–790

Preiss J, Greenberg E, Sabraw A (1975) Biosynthesis of bacterial glycogen—kinetic studies of a glucose-1-phosphate adenylyltransferase (Ec 2.7.7.27) from a glycogen-deficient mutant of *Escherichia coli* B. J Biol Chem 250(19):7631–7638

Qiao Y, Lee SI, Piao R, Jiang W, Ham TH, Chin JH, Piao Z, Han L, Kang SY, Koh HJ (2010) Fine mapping and candidate gene analysis of the floury endosperm gene, FLO(a), in rice. Mol Cells 29(2):167–174. doi:10.1007/s10059-010-0010-6

Ryoo N, Yu C, Park CS, Baik MY, Park IM, Cho MH, Bhoo SH, An G, Hahn TR, Jeon JS (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. doi:10.1007/s00299-007-0309-8

Satoh H, Omura T (1981) New endosperm mutations induced by chemical mutagens in rice, *Oryza sativa* L. Jpn J Breed 31 (3):316–326

She KC, Kusano H, Koizumi K, Yamakawa H, Hakata M, Imamura T, Fukuda M, Naito N, Tsurumaki Y, Yaeshima M, Tsuge T, Matsumoto K, Kudoh M, Itoh E, Kikuchi S, Kishimoto N, Yazaki J, Ando T, Yano M, Aoyama T, Sasaki T, Satoh H, Shimada H (2010) A novel factor FLOURY ENDOSPERM2 is involved in regulation of rice grain size and starch quality. Plant Cell 22(10):3280–3294. doi:10.1105/tpc.109.070821

Shen YJ, Jiang H, Jin JP, Zhang ZB, Xi B, He YY, Wang G, Wang C, Qian L, Li X, Yu QB, Liu HJ, Chen DH, Gao JH, Huang H, Shi TL, Yang ZN (2004) Development of genome-wide DNA polymorphism database for map-based cloning of rice genes. Plant Physiol 135(3):1198–1205. doi:10.1104/pp.103.038463135/3/1198

Singh R, Juliano BO (1977) Free sugars in relation to starch accumulation in developing rice grain. Plant Physiol 59(3):417–421

Sui JM, Guo BT, Wang JS, Qiao LX, Zhou Y, Zhang HG, Gu MH, Liang GH (2012) A new GA-insensitive semidwarf mutant of rice (*Oryza sativa* L.) with a missense mutation in the SDG gene. Plant Mol Biol Rep 30(1):187–194. doi:10.1007/s11105-011-0321-6

Supervision tSBoQaT (1999) The National Standard of the People's Republic of China. GB/T17891-1999. High Quality Paddy vol GB/T17891-1999. Beijing

Thang NB, Wu JG, Zhou WH, Shi CH (2010) The screening of mutants and construction of mutant library for *Oryza sativa* cv. Nipponbare via ethyl methane sulphonate inducing. Biologia 65 (4):660–669. doi:10.2478/s11756-010-0059-x

Wan JM, Wang JK, Wan XY, Li HH, Pfeiffer WH, Crouch J (2007) Application of identified QTL-marker associations in rice quality improvement through a design-breeding approach. Theor Appl Genet 115(1):87–100. doi:10.1007/s00122-007-0545-x

Wang E, Wang J, Zhu X, Hao W, Wang L, Li Q, Zhang L, He W, Lu B, Lin H, Ma H, Zhang G, He Z (2008) Control of rice grain-filling and yield by a gene with a potential signature of domestication. Nat Genet 40(11):1370–1374. doi:10.1038/ng.220

Wang BB, Zhu CX, Liu X, Wang WY, Ding HF, Jiang MS, Li GX, Liu W, Yao FY (2011) Fine mapping of qHD4-1, a QTL controlling the heading date, to a 20.7-kb DNA fragment in rice (*Oryza sativa* L.). Plant Mol Biol Rep 29(3):702–713. doi:10.1007/s11105-010-0278-x

Wu JG, Shi CH, Zhang XM (2002) Estimating the amino acid composition in milled rice by near-infrared reflectance spectroscopy. Field Crops Res 75(1):1–7

Xu LJ, Xie JK, Kong XL, Bao JS (2004) Analysis of genotypic and environmental effects on rice starch. 2. Thermal and retrogradation properties. J Agric Food Chem 52(19)):6017–6022. doi:10.1021/jf049235a

Yamakawa H, Hirose T, Kuroda M, Yamaguchi T (2007) Comprehensive expression profiling of rice grain filling-related genes under high temperature using DNA microarray. Plant Physiol 144 (1):258–277. doi:10.1104/pp.107.098665

Yamakawa H, Ebitani T, Terao T (2008) Comparison between locations of QTLs for grain chalkiness and genes responsive to high temperature during grain filling on the rice chromosome map. Breed Sci 58(3):337–343

Zhao F, Cai ZJ, Hu TZ, Yao HG, Wang L, Dong N, Wang B, Ru ZG, Zhai WX (2010) Genetic analysis and molecular mapping of a novel gene conferring resistance to rice stripe virus. Plant Mol Biol Rep 28(3):512–518. doi:10.1007/s11105-009-0178-0