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Molecules and Cells

Fine Mapping and Candidate Gene Analysis of the Floury Endosperm Gene, *FLO(a)*, in Rice

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In addition to its role as an energy source for plants, animals and humans, starch is also an environmentally friendly alternative to fossil fuels. In rice, the eating and cooking quality of the grain is determined by its starch properties. The floury endosperm of rice has been explored as an agronomical trait in breeding and genetics studies. In the present study, we characterized a floury endosperm mutant, *flo(a)*, derived from treatment of *Oryza sativa* ssp. *japonica* cultivar Hwacheong with MNU. The innermost endosperm of the *flo(a)* mutant exhibited floury characteristics while the outer layer of the endosperm appeared normal. Starch granules in the *flo(a)* mutant formed a loosely-packed crystalline structure and X-ray diffraction revealed that the overall crystallinity of the starch was decreased compared to wild-type. The *FLO(a)* gene was isolated via a map-based cloning approach and predicted to encode the tetratricopeptide repeat domain-containing protein, *OsTPR*. Three mutant alleles contain a nucleotide substitution that generated one stop codon or one splice site, respectively, which presumably disrupts the interaction of the functionally conserved TPR motifs. Taken together, our map-based cloning approach pinpointed an OsTPR as a strong candidate of *FLO(a)*, and the proteins that contain TPR motifs might play a significant role in rice starch biosynthetic pathways.

INTRODUCTION

Cereal crops accumulate starch in the seed endosperm as an energy reserve, and these grains comprise the primary carbohydrate component in the diets of humans and livestock. In addition, starch has numerous important industrial applications. The current paradigm of starch biosynthesis in higher plants begins with the synthesis of amylopectin, the major component of starch, which is produced by the action of ADP glucose pyrophosphorylase (AGPase), soluble starch synthases (SS), starch-branching enzymes (BE), and starch-debranching enzymes (DBE) (Smith et al., 1997). There is also evidence that disproportionating enzyme (Ball and Morell, 2003; Colleoni et al., 1999) and α-glucan phosphorylase (Dauvillee et al., 2006; Schupp and Ziegler, 2004) are involved in this process. In rice, starch comprises ~90% of the dry weight of the grains and provides up to 80% of the calories. Starch properties determine various aspects of rice quality, particularly with regard to eating and cooking quality (Bao et al., 2004; Hannah et al., 2008).

The endosperm, in which large quantities of starch are deposited during grain filling, nourishes the embryo during the early stages of development. Endosperm starch consists of a linear fraction (amylose) and a branched fraction (amylopectin). Based on the appearance and physiochemical characteristics of the endosperm, mutant strains have been identified and categorized as *amylase extender (ae), brittle (bt), dull (du), floury (flo), glutinous (glu), shrunken (sh), sugary 1 (su1),* and *white-core (wc)* varieties (Kaushik and Khush, 1991; Nelson and Pan, 1995; Satoh and Omura, 1981). These mutant strains provide valuable genetic material for elucidating the metabolic processes related to starch storage during grain filling (Nelson and Pan, 1995). They also facilitate the identification of genes that encode starch biosynthetic enzymes (Hunter et al., 2002). Some of these mutations can produce traits that make the grains attractive for use in the food industry. For example, opaque endosperms with poorly packed, compound starch granules in the central cells of the endosperm are useful in the production of rice wine (Hoshikawa, 1989).

Because of its economical importance, some of the genes responsible for maize opaque endosperm mutants have been identified. These include *waxy (wx),* which encodes granule-bound starch synthase I (GBSSI) (Klosgen et al., 1986; Shure et al., 1983), *amylase extender* (*ae*), which encodes starch-branching enzyme IIb (Fisher et al., 1993; Stinard et al., 1993), *shrunken1* (*sh1*), which encodes sucrose synthase1, *shrunken2 (sh2)* and *brittle2* (*bt2*), which encode the large and small subunits of ADP-glucose pyrophosphorylase, respectively (Bae et al., 1990; Bhave et al., 1990), *brittle1 (bt1),* which encodes an adenylate translocator (Cao and Shannon, 1997; Shannon et

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al., 1996; Sullivan et al., 1991), *sugary1 (su1)*, which encodes a starch-debranching enzyme (James et al., 1995), and *dull1 (du1)*, which encodes a starch synthase (Gao et al., 1998).

Many studies also have been performed in order to characterize and isolate the genes that control abnormal endosperm mutants in rice. For example, *Du1* encodes a member of the pre-mRNA processing (Prp1) family and functions as a regulator of starch biosynthesis by facilitating the splicing of *Wxb* and the expression of other genes involved in the rice starch biosynthetic pathway (Zeng et al., 2007). *Du3* encodes the rice homolog of the cap-binding protein 20 kD subunit (CBP20), a component of the heterodimeric nuclear cap-binding complex (CBC) that plays a role in pre-mRNA splicing, RNA nuclear export, and nonsense-mediated decay (Isshiki et al., 2008). PHO1 plays a crucial role in starch biosynthesis in rice endosperm, and the phosphorylase mutant (*Pho1*) exhibited small starch granules that resulted in the accumulation of a modified amylopectin structure (Satoh et al., 2008). *OGR1* encodes a pentatricopeptide repeat-DYW protein and is essential for RNA editing in mitochondria (Kim et al., 2009). Furthermore, the loci of six floury endosperm mutants (*flo(a), flo1-flo5*) were identified on chromosomes 4, 5, 4, 4, 3, and 8, respectively, by morphological marker and T-DNA tagging pool analysis (http://www.gramene.org/). The *flo(a)* locus was linked with one morphological marker, *lg (liguleless)*, on chromosome 4 (Kim et al., 1993). The *flo1* mutant is known to generate a floury-white endosperm that contains round and loosely packed starch granules (Satoh and Omura, 1981). The *flo2* mutant possesses an endosperm that has high lysine content and an increased level of histidine (Kumamaru et al., 1997). The *flo3* strain is a floury endosperm mutant with a low level of 16-kDa globulin, the primary allergen in rice (Nishio and Iida, 1993). Recently, *flo4* and *flo5*, two T-DNA insertion mutants with an abnormal endosperm consisting of loosely packed starch granules, were characterized. The *flo4* strain appears to harbor a mutation in the gene that encodes pyruvate orthophosphate dikinase (PPDK) (Kang et al., 2005). The *flo5* mutant expresses a defective starch synthase III (SSIII) gene that plays an important role in generating long starch chains and coordinating the activity of other SS isoforms during starch synthesis in the endosperm (Ryoo et al., 2007).

Some of the physicochemical properties of the *flo(a*) mutant have been reported (Kim et al., 1993). The *flo(a)* mutant contains a much lower amylose content, crude protein content, gel consistency, viscosity, grain hardness and density than wild-type strains. The *flo(a)* mutant also produced a larger volume of steamed rice-cakes, a greater degree of Brix decrease and an increased amount of alcohol compared with the wild-type. Currently, our understanding of starch biosynthesis in the *flo(a)* endosperm mutant is incomplete. In this study, we further characterized the phenotype of the floury endosperm mutant and isolated the *FLO(a)* gene using map-based cloning strategy.

MATERIALS AND METHODS

Plant materials and phenotypic evaluation

The *flo(a)* mutant was induced by N-methyl-N-nitrosourea (MNU) treatment of rice *japonica* cultivar (cv.) Hwacheong (an elite Korean *japonica* cultivar) and maintained at the Rice Gene Bank of the Department of Plant Science, Seoul National University, Seoul, Korea. The parental plants and their F_1, F_2 progenies were grown by conventional methods at the Experimental Farm of Seoul National University. To detect the floury endosperm phenotype, all seeds of F_1 or F_2 plants were husked and observed on an illuminator or by visual inspection. In addition, we used another two independent *flo(a)* mutants, which were induced separately through chemical mutagenesis and had the same phenotype, for allelism test and sequence analysis of the *flo(a)* gene.

Preparation of starch from rice endosperm

The whole dried seeds of *flo(a)* mutants were dehulled, and 9.1% of the outer seed layer was removed using a rice polisher (Kett, Japan). The polished rice was powdered in a mortar and filtered through 150 μm mesh. The filtrated powder (~1.0 g) was then suspended in 10 ml of methanol and boiled for 10 min. The homogenate was centrifuged at 2,500 × *g* for 10 min at 20°C, and the precipitate was washed twice with 5 ml of 90% (v/v) methanol. The dried precipitate was resuspended in 15 ml of distilled water and stirred gently for 20 min at room temperature. The suspension was centrifuged at 600 × *g* for 20 min at 20°C. The precipitate was dissolved in 15 ml of water and centrifuged. The precipitate was washed again with 15 ml of distilled water and stirred again for 20 min at room temperature, and then centrifuged. The resulting precipitate, defined as starch, was washed with methanol and dried under reduced pressure. The starch preparation was used for scanning electron microscopy (SEM) observation and X-ray diffraction analysis.

Scanning electron microscopy analysis

Scanning electron microscope (SEM) analysis was carried out according to the methods described by Ryoo et al. (2007). Starch samples were coated with gold and the mounted specimens were observed through a Stereoscan Leica Model 440 Scanning Electron Microscope (Leica Cambridge, UK; http://www.leica-microsystems.com) at an accelerating voltage of 10-20 kV.

X-ray diffraction of starch

The X-ray diffraction patterns produced by the starches were analyzed as described by Kubo et al. (2005). The insoluble glucans isolated using the above method were equilibrated in a 100% relative humidity chamber for 24 h at room temperature. The X-ray diffraction patterns of the starches were obtained with copper, nickel foil-filtered, Kα radiation using a RINT2000 x-ray diffractometer (Rigaku, Japan) at 40 kV and 40 mA. The scanning region of the two-theta angle (2Θ) ranged from 4.0 to 40.0° with a scan speed of 1 deg/min.

DNA extraction and PCR analysis

Total rice genomic DNA was extracted from the fresh leaf tissue of the field-grown F_2 and parental plants using a previously described procedure (Causse et al., 1994). PCR conditions mainly adhered to the method previously described by Qiao et al. (2008), with the exception of the annealing temperature of primers.

Mapping

The F_2 mapping population was obtained from a cross between *flo(a)* and Milyang23 (M.23), which produced a total of 1571 F_2 plants. For the initial mapping of the *flo(a)* gene, we performed recessive class analysis (RCA) (Zhang et al., 1994) using STS markers designed by the Crop Molecular Breeding Lab, Seoul National University (unpublished). Linkage analysis was conducted using MAPMAKER version 3.0 software (Lander et al., 1987). Map distances were estimated by the Kosambi equation (Kosambi, 1944). For construction of the physical map of the *flo(a*) locus region, new STS markers were developed by designing primers based on differences in the DNA sequences of *indica* and *japonica* rice, which are available at http://www.ncbi.

Table 1. The PCR-based molecular markers designed and utilized for fine mapping.

Marker	Marker type	Size (bp)	Forward primer sequence	Reverse primer sequence
S04107	STS	211	5′-TGTTCAGACCTGGGTCACTTT-3′	5′-ATCAACTCGCCTGCTGTTCT-3′
P07	STS	257	5′-CCTGACCAAAAATGGGCTAA-3′	5′-GCAAGAAACGGAAACGAAAC-3′
S04109	STS	167	5′-GGCTGAAGCATTTGGAGAAG-3′	5′-AAGTATACCTTGGCTTCAAGTGG-3′
P05	STS	289	5′-TCTCCAGATTTTCCTTCGTGA-3′	5′-GAATTCTGAAGGGGTTGTGG-3′
P12	STS	231	5′-ATCGAGGGACGACGTTGTAG-3′	5′-GCGTAGGAGCGTTTTTAAGG-3′
P14	STS	239	5′-TATCCGCAGCAATGCTTTAG-3′	5′-TAGCTCATATTGGCGTGGTG-3′
P18	STS	159	5′-GGTCGAATGGTGGTGATAGG-3′	5′-GCGTATCGATCTGGGTTAGC-3′
RM8217	SSR	178	5′- ACTAGCGATGTCTGAGTTGAC-3′	5′-TATTCACATGCTTGCTCATC-3′
P22	STS	183	5′-AGCCCAACATATGGCAGAAG-3′	5′-TCCTTTCGAGGGAGGAATTT-3′
RM1153	SSR	126	5′-ACCAACGCCAAAAGCTACTG-3′	5′-TACTCGCCCTGCATGAGC-3′
P02	STS	284	5′-GGATGGTGAGGTGAGGTGTT-3′	5′-CGCCGTCAGAGAGGTAAAAG-3′
S04114	STS	169	5′-TGCATGCAAAATCTATCTACGTG-3′	5′-AGTCCGTTTCGCATGTGTTT-3′

nlm.nih.gov/ (for *indica*) and at http://www.rgp.dna.affrc.go.jp/ (for *japonica*). Primer sequences and the amplified lengths of the DNA markers used in this study are listed in Table 1. These STS markers were anchored on corresponding bacterial artificial chromosome (BAC) or P1-derived artificial chromosome (PAC) clones of the Nipponbare cultivar, released by the IRGSP through the software tool, BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Finally, these BAC or PAC clones were assembled into a contig by alignment analysis using Pairwise BLAST (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html).

Analysis of candidate genes

Based on the physical map of the *flo(a)* gene, corresponding BAC or PAC sequences of the Nipponbare cv. in the *flo(a)* gene region were downloaded from the TIGR database (http://www.tigr.org). Open reading frames (ORFs) and potential exon/intron boundaries were predicted for the sequences described above using Rice GAAS (Rice Genome Automated Annotation System; http://ricegaas.dna.affrc.go.jp/rgadb/). The ORF sequence of each predicted gene was used to search for its full length cDNA and EST (expressed sequence tag) in KOME (http://cdna01.dna.affrc.go.jp/cDNA/) and Genbank. The translated amino acid sequences of candidate genes were analyzed through BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) for functional prediction. The full-length genomic DNA sequence of candidate genes was divided into several segments in order to design specific PCR primers that were used to amplify genomic DNA from Hwacheong and *flo(a)* mutants. PCR products were purified using a PCR purification kit for TA cloning (iNtRON Biotechnology, INC., Korea). Purified PCR product was introduced into the pGEM-T Easy Vector (Promega, USA), and transformed into *E. coli* strain DH5α. The recombinant plasmid was sequenced with an ABI Prism 3730 XL DNA Analyzer (PE Applied Biosystems, USA), and sequence alignment was performed with the BLAST network service (National Center for Biotechnology Information, NCBI).

dCAPS analysis

For dCAPS analysis of the *flo(a)* allele, primers based on two base mismatches in the 5′ direction from the SNP of interest (mismatched forward, 5′-CGGCGGCAAAGGCGACAAGAGGATT-3′; reverse, 5′-CAGCAGCAGCGAATTCAATGTTCGC-3′) were designed using the dCAPS Finder 2.0 program (http://helix.wustl.edu/dcaps/dcaps.html). These primers produced a 179-bp PCR amplicon with an *Mse*I site in the wild-type allele of *flo(a)*. PCR amplicons were detected after digestion with *Mse*I, and separated on horizontal 3% agarose gels that were then visualized and photographed under UV light.

RESULTS

Phenotypic characteristics of the *flo(a)* mutant

The *flo(a)* mutants did not exhibit a visibly abnormal phenotype during the vegetative stage of plant growth and development compared to wild-type plants. The mature kernel phenotype was similar to that of wild-type rice cv. Hwacheong (Figs. 1A and 1D). Husked rice grains of the *flo(a)* mutant exhibited an opaque endosperm phenotype, which resulted in an easily broken endosperm and floury appearance, whereas visual inspection of the wild-type cultivar revealed a normal transparent endosperm (Figs. 1B and 1E). Cross section of mature mutant rice grains further revealed that it is the inner part of the endosperm that is floury, whereas the exterior portion maintains a normal appearance (Figs. 1C and 1F).

In addition, we examined the effects of *FLO(a)* mutations on grain shape and yield traits (Table 2). The grain size of brown rice of the *flo(a)* mutant was marginally smaller than that of the wild-type (95.4% in grain length; 96.0% in grain width). The panicle number per plant in the *flo(a)* strain was also slightly less than that of the wild-type (94.6%). The 1000-grain weight in *flo(a)* rice was significantly lighter than that of the wild-type (81.9%); however, the grain number per panicle was distinctly increased in *flo(a)* compared to the wild-type (112.4%). The grain yield of the mutant was lower than that of the wild-type. With respect to agronomic traits, such as plant architecture and heading date, no major differences were observed.

The *flo(a)* mutation alters the morphological properties of starch granules in rice endosperm

SEM observation of cross sections of polished rice grains revealed that the *flo(a)* mutant had a loosely packed endosperm (Figs. 2A and 2C), probably due to an abnormal starch granule morphology. In contrast, the wild-type endosperm was found to be occupied by densely packed starch granules (Figs. 2B and 2D). Starch granules in the wild-type cultivars were found to form an irregular polyhedron-shape with sharp edges, while starch granules in the *flo(a)* mutant were slightly larger than those of wild-type and exhibited both size variation and an irregular spherical shape (Figs. 2E and 2F).

Starch is a semi-crystalline biopolymer containing both

Table 2. Comparison of grain and yield traits in wild-type and *flo(a)*

	Length (mm)	Width (mm)	1000-Grain weight (g)	No. of panicles/ plant	No. of grains/ panicle	Grain yield (g/10 plants)
Wild-type	4.79 ± 0.18	2.75 ± 0.25	18.4 ± 0.8	10.6 ± 0.97	135.4 ± 7.88	353.9 ± 3.58
flo(a)	4.60 ± 0.13	2.64 ± 0.16	14.8 ± 1.1	11.2 ± 0.78	150.2 ± 5.68	324.5 ± 2.42

Fig. 1. Morphology of the grain and seed endosperm in the *flo(a)* mutant and wild-type plants. (A, D) Grains from *flo(a)* (A) and wild-type (D) plants at full maturation. (B, E) Dehusked seeds from *flo(a)* (B) and wild-type (E) plants. (C, F) Longitudinal and horizontal sections of polished seed endosperms of *flo(a)* mutants (C) and wild-type (F). The floury inner region of the endosperm is evident in the *flo(a)* mutant.

amorphous and crystalline regions that exist in two primary different crystal structures, A-type and B-type. It has been shown that the endosperm starch in rice grains exhibits an A-type crystal pattern (Ryoo et al., 2007). We performed X-ray diffraction analysis using endosperm starch from both wild-type and *flo(a)* mutant plants and confirmed that all strains exhibited a typical A-type crystal pattern (Fig. 3). The starch granules in the *flo(a)* mutant, however, showed a lower peak intensity than those from their respective wild-type counterpart, Hwacheong (Fig. 3). Thus, X-ray diffraction analysis demonstrated that the loss of *FLO(a)* gene function results in impaired starch crystal structure and a lower amount of crystallization, but no change in the crystal type.

Fine mapping of the *FLO(a)* gene

The genetic locus defined by the *flo(a)* mutant was previously linked with a single morphological marker, *lg (liguleless)* on chromosome 4 (Kim et al., 1993). To more precisely map the *FLO(a)* gene using a map-based cloning approach, we generated an F_2 population of 1571 individuals derived from a cross between *flo(a)* and M.23, in which 371 segregants expressed the *flo(a)* mutant phenotype and 1200 segregants exhibited the wild-type phenotype. Genetic analysis of 40 *flo(a)* mutant segregants initially placed the *FLO(a)* gene on the genome segment between the two STS markers, S04107 and S04114 (Fig. 4A). However, no recombinant was detected by the STS marker S04109, indicating that this marker had co-segregated with the mutated *flo(a)*. To further define the position of the *FLO(a)* gene, we utilized 12 PCR-based molecular markers within this region (Table 1) and performed PCR using the remaining plants from the F_2 population. Finally, the *flo(a)* locus was delimited to an ~81 kb region defined by markers P18 and P22 and covered by two bacterial artificial chromosome (BAC) clones, AL606454 and AL606445 (Fig. 4B). No recombinant was identified for RM8217 in the F_2 mapping population (Fig. 5A), indicating that this marker were tightly co-segregated with the *FLO(a)* gene.

The tetratricopeptide repeat (TRP) domain containing protein is a candidate *FLO(a)* gene

Based on the data obtained from fine mapping and available rice genome annotation databases (http://www.tigr.org, http://www.gramene.org), a total of 15 putative open reading frames (ORFs) were identified in this 81 kb genomic region. To determine which one carried the *flo(a)* mutation, the genomic DNA sequences from 15 hypothetical genes were amplified by PCR and sequenced. Comparison of the sequences of Hwacheong wild-type and the *flo(a)* mutant revealed that the *flo(a)* allele carried a single nucleotide substitution in nucleotide 73 (AAG to TAG) in the 1st exon of Os04g55230. This substitution converted lysine into a stop codon and disrupted protein expression. This point mutation was also confirmed through direct sequencing of the RT-PCR products (Figs. 4C and 4D). Further, we performed genome sequencing of other putative ORFs. Because no other differences were detected in the DNA sequences of any other ORF regions, Os04g55230 was strongly implicated as a candidate for *FLO(a)* (Fig. 4C). The gene consists of 23 exons and 22 introns. The complementary DNA for this gene is 5163 bp long, and the deduced protein sequence is comprised of 1720 amino acids, including three TPR motifs that function in protein-protein interactions (Blatch and Lassle, 1999).

To examine whether the A-to-T mutation in the 1st exon of *FLO(a)* was present as a natural variant in other cultivars, we performed dCAPS analysis of 22 typical *indica* and *japonica* rice cultivars. All 22 strains revealed digested fragments that

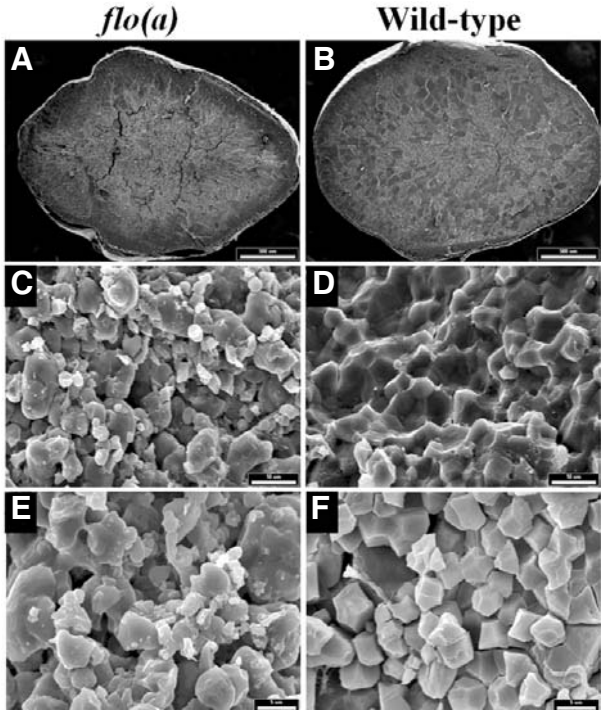

Fig. 2. Scanning electron microscope analysis of cut endosperms and purified starch granules of the *flo(a)* mutant and wild-type control. The cutting planes of seed endosperms of the *flo(a)* mutant (A, C) and wild-type (B, D). The *flo(a)* mutant endosperms are loosely packed with irregularly shaped starch granules compared to wild-type. Starch granules in the *flo(a)* mutant (E) vary in size and are rounder compared to the wild-type strain (F). Bars represent 500 μm (top), 10 μm (middle) or 5 μm (bottom)

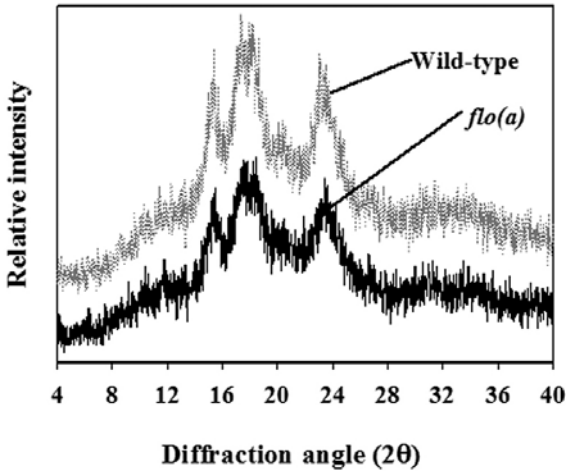

Fig. 3. X-ray diffraction patterns of purified starch granules from the mature endosperm of the *flo(a)* mutant and its wild-type cultivar, Hwacheong. The relative crystallinity of the *flo(a)* starch is decreased compared to wild-type.

correspond to the wild type allele (Fig. 5B; Table 3). In addition, the genotypes associated with this dCAPS marker co-segregated with the matching phenotypes in the F_2 population (Fig. 5C).

To further confirm that the *FLO(a)* gene is causally related to the floury endosperm, the complementation test of alleles were performed by crossing among three floury endosperm mutants, *flo(a), flo(a)-2, and flo(a)-3*. All of F_1 plants from three crosses exhibited "floury endosperm" phenotype, which indicated that the phenotype of three mutants is controlled by the same gene. Furthermore, we determined point mutations in another two alleles of the *flo(a)* mutants by sequence analysis (Figs. 4E and 4F). The *flo(a)-2* allele carried a single nucleotide polymorphism (SNP) that generated a premature stop codon at nucleotide 2490 (TGG to TGA) in the 14th exon of Os04g55230. The *flo(a)-3* allele occurred an altered splicing site between the 14th exon and 14th intron (GT to AT) (Figs. 4C-4F). These results strongly suggest that *FLO(a)* gene encodes the tetratricopeptide repeat domain-containing protein, *OsTPR*.

DISCUSSION

The floury trait in the *flo(a)* mutant was limited to the inner part of the endosperm while the outer parts maintained a normal appearance. Although floury seeds tend to be easily broken during the polishing process, the taste of cooked rice harvested from the *flo(a)* mutant was not noticeably inferior to that of the

Table 3. Rice varieties used in dCAPS analysis

No.	Variety	Origin	Amino acid*	No.	Variety	Origin	Amino acid
1	*flo(a)* mutant		Stop codon	13	Gopumbyeo	Korea	Lys
2	Hwacheongbyeo	Korea	Lys	14	Milyang 23	Korea	Lys
3	Dongjinbyeo	Korea	Lys	15	Dasanbyeo	Korea	Lys
4	Nipponbare	Japan	Lys	16	Habataki	Japan	Lys
5	Hapcheon 1	Korea	Lys	17	Cheongcheongbyeo	Korea	Lys
6	CP-SLO	Philippines	Lys	18	IR 36	Philippines	Lys
7	Ilpumbyeo	Korea	Lys	19	Tadukan	Philippines	Lys
8	Hwacheongbyeo	Korea	Lys	20	Chinsurah Boro II	India	Lys
9	Koshihikari	Japan	Lys	21	IR 64	Philippines	Lys
10	Shennong 27	China	Lys	22	Diantun 502	China	Lys
11	Kunmingxiaobaigu	China	Lys	23	Nampungbyeo	Korea	Lys
12	Dianxi 1	China	Lys				

*The amino acid at the 25th position of the FLO(a) protein

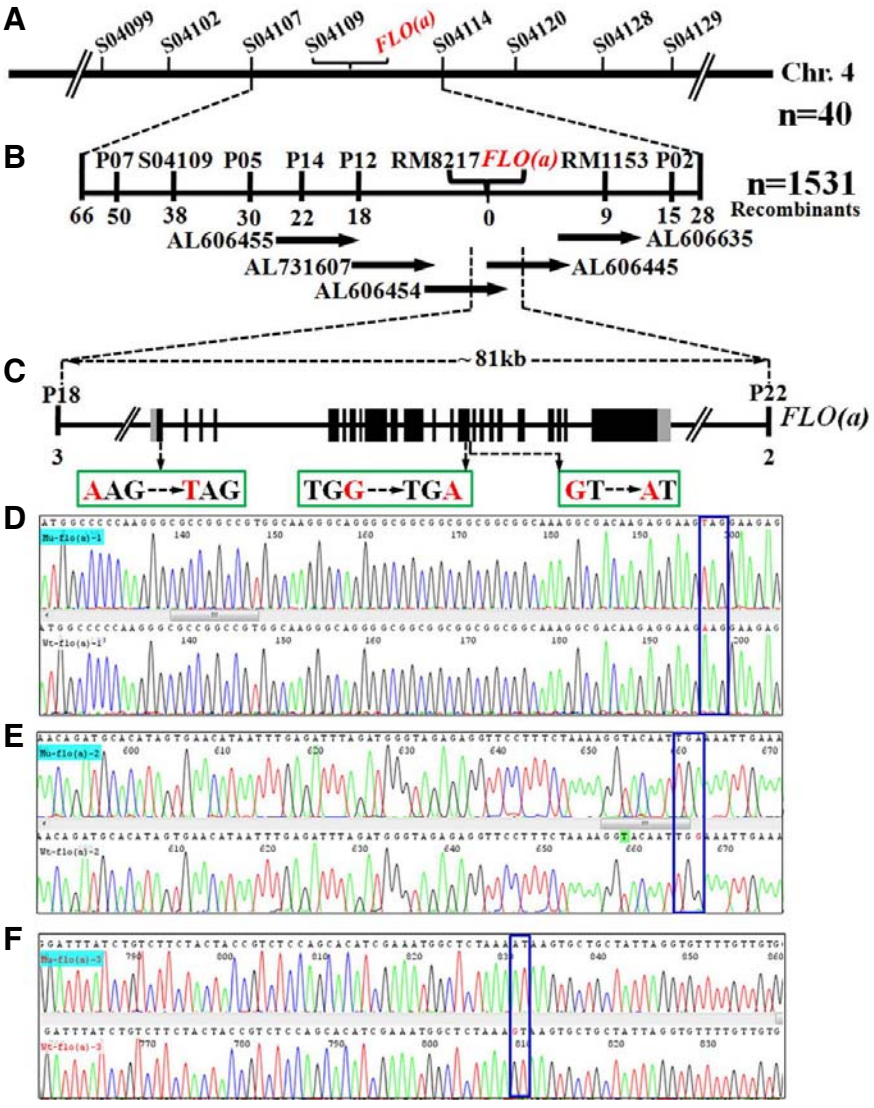

Fig. 4. Positional cloning of the *FLO(a)* gene. (A) The *FLO(a)* locus was mapped to chromosome 4 between markers S04107 and S04114. (B) Fine mapping of the *FLO(a)* gene with markers based on the genome sequence. The numerals indicate the number of recombinants identified in 1571 F_2 plants. The *FLO(a)* locus was narrowed to an 80-kb region of genomic DNA between markers P18 and P22. (C) The structure of *FLO(a),* showing the mutation site of *flo(a).* Closed boxes indicate the coding sequence, and the lines between the boxes represent introns. (D) Sequencing chromatograms were constructed by sequencing of RT-PCR products containing the mutation target site in *flo(a).* (E) Sequencing chromatograms corresponding to the missense mutationin in the 14th exon of *flo(a)-2* gene. (F) Sequencing chromatograms corresponding to the first nucleotide at 5′- splicing junction of the 14th intron of *flo(a)-3* gene. The PCR product was cloned and individual colonies were subjected to sequence analysis. Three mutant alleles of the *FLO(a)* gene included a nucleotide substitution that generated a premature stop codon (*flo(a)* and *flo(a)-2*) and an altered splicing site (*flo(a)-3*) (closed rectangle).

original cultivar, ‘Hwacheong’, and the estimated yield of rice from *flo(a)* plants was almost the same. In addition, fermentation of rice grains from *flo(a)* mutants produced a high yield of alcohol, which is both advantageous for the rice wine industry and represents a potential environmentally friendly alternative to petroleum-based fuels (Hannah and James, 2008; Hoshikawa, 1989; Kim et al., 1993). Therefore, the cloning and molecular characterization of the *flo(a)* mutant will facilitate further study of starch characteristics and allow for the development of rice strains that produce grains with ideal starch structure and storage capacity.

To date, three rice floury endosperm mutants, *flo2, flo3 and flo(a*), have been identified on chromosome 4. In addition to exhibiting a floury endosperm phenotype, they showed their own distinguishing characteristics (Kawasaki et al., 1996; Kumamaru et al., 1997; Kim et al., 1993; Nishio and Iida, 1993). Because there is no detailed information about the chromosomal position and allelic relationship of the three loci, it is unknown whether these three phenotypes are generated by the tight linkage of three genes or by the pleiotropism of a single mutated gene.

It was previously reported that the *flo(a)* locus was controlled by single recessive gene and linked with a specific morphological marker, *lg (liguleless)*, on chromosome 4 (Kim et al., 1993). Due to its economical importance, the *flo(a)* was developed as a cultivar Sunong 9 that has been widely utilized in agricultural production for improvement of the breeding process. To clone the *FLO(a)* gene, the key step was construction of a physical map that encompassed the target gene. In this study, the *FLO(a)* gene was mapped to within an ~81 kb interval bracketed by P18 and P22. A total of 15 ORFs were predicted by analysis of this region using a rice genome automated annotation system. Mapping and sequencing results revealed that a single A to T substitution in the first exon of the TPR repeat motif is most likely responsible for the floury endosperm phenotype. This was further confirmed by RT-PCR and dCAPS marker analysis of F_2 individuals and typical rice cultivars.

In the present study, the *FLO(a)* gene was predicted to encode a novel regulated factor, the tetratricopeptide repeat domain containing protein (*OsTPR*), which contains three TPR motifs in the region from amino acids 933 to 1050. The 34-amino acid TPR motif has been identified in all organisms studied and is present in proteins that are involved in a variety of cellular processes. Typically, TPR motifs are arranged in tandem repeats of 3 to 16, although individual TPRs can be dis-

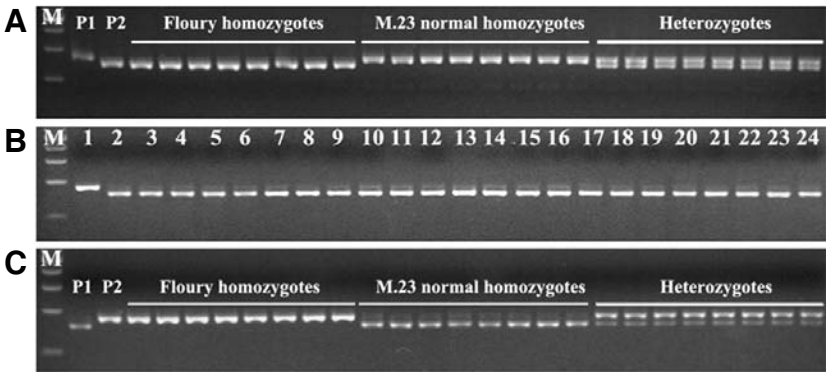

Fig. 5. Examples of co-segregation and point mutation using SSR and dCAPS markers. (A) Marker RM8217 did not detect any recombination and co-segregated with the floury phenotype in the F_2 population. (B) Confirmation of the splice variation in *flo(a)* mutants by analysis of PCR amplicon size in *flo(a)* mutant and 22 normal rice cultivars. Lane 1, *flo(a)* mutant; lanes 2-23, normal rice varieties (see Table 3). (C) Co-segregation of the splicing-error mutation with the *flo(a)* mutant phenotype was analyzed by size comparison of PCR amplicons from the F_2 population. P1, M.23; P2, *flo(a)* mutant; M, molecular marker.

persed throughout the protein. Alignment of TPR motifs reveals a consensus sequence defined by a pattern of small and large amino acids that form a helical secondary structure (Blatch and Lässle, 1999; D'Andrea and Regan, 2003). The basic function of TPR domains is to mediate protein-protein interactions that affect a wide variety of cellular functions, such as proper folding of proteins and mRNA stability, processing, and translation (Fedoroff, 2002; Gounalaki et al., 2000; Prodromou et al., 1999; Yang et al., 2005). Recently, proteins containing TPR motifs in Arabidopsis were implicated in abiotic stress responses, ethylene biosynthesis, cytokinin responses and regulation of hormones such as gibberellins and abscisic acid (Greenboim-Wainberg et al., 2005; Rosado et al., 2006; Tseng et al., 2001; Wang et al., 2004). In rice, the Prp1 protein, which contains 19 TPR motifs, was found to be involved in the splicing of Wx^b pre-mRNAs and regulation of the expression of starch biosynthesis genes (Zeng et al., 2007). However, the biochemical properties and the specific interacting partners of more than 87 additional TPR-containing proteins identified by Rice GAAS remain elusive. It is probable that the early transcription terminator generated by a single nucleotide substitution in the *flo(a)* mutant may affect its interaction with other proteins, leading to functional deficiency.

The *flo(a)* mutant displays loosely packed starch granules, as well as other physicochemical traits that distinguish it from the wild-type (Kim et al., 1993). These characteristics suggest that the *FLO(a)* gene is involved in starch synthesis. Because there is no other report of starch synthesis regulation by TPR-containing proteins, these findings are crucial for elucidating the factors that determine seed endosperm quality and the molecular function of this novel regulator in the rice starch synthesis pathway. Currently, functional studies of the *FLO(a)* gene are in progress, including analysis of the expression patterns of starch synthesis enzymes, identification of protein-protein interactions and evaluation of the mechanisms that produce floury endosperm related characteristics.

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