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Original Paper

A novel FLOURY ENDOSPERM2 (FLO2)-interacting protein, is involved in maintaining fertility and seed quality in rice

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Abstract Crop plants accumulate a large amount of storage starch and storage proteins in the endosperm. Genes involved in the biosynthesis of these substances work in concert during development of the rice endosperm. The rice *flo2* mutant produces aberrant seeds with reduced grain quality. *FLOURRY ENDOSPERM 2 (FLO2)*, the causative gene of the *flo2* mutant, is considered to be a regulatory protein that controls the biosynthesis of seed storage substances. FLO2 contains tetratricopeptide repeat (TPR) motifs that may mediate protein–protein interactions. In this study, we identified the protein that interacts with the TPR motif of FLO2. We generated a transformant that produced the FLAG-tagged fusion FLO2 protein in the *flo2* mutant and used this in the shotgun proteomic analysis. A protein, which we named FLOC1, interacted with FLO2. In vitro pull-down assays indicated that the TPR motif was involved in this interaction. A knock-down transformant of FLOC1 showed significantly reducted fertility and generation of seeds with abnormal features. These findings suggest that FLOC1 is involved not only in seed fertility but also in seed quality. These phenotypes were also observed on the RNAi transformants of the *flo2* mutant although the effect of the *flo2* mutation remained. these findings imply that there is a difference in the functions of FLO2 and FLOC1 although both of appear to be involved in the control of seed quality during seed formation.

Key words: developing seed, fertility, protein–protein interaction, rice, seed formation.

Introduction

Rice is an important crop that accounts for more than 20% of the world's grain production. The rice endosperm forms within a few weeks after flowering, and accumulates a large amount of storage substances, such as starch and storage proteins (Zhou et al. 2013). The quality of the rice endosperm affects the appearance, taste, and yield of crop production, both of which are one of the goals of rice breeding. Elucidating the mechanism that determines endosperm quality provides important knowledge for creating crops with high-quality, high-yield grains (Zhang 2007).

In rice, several key enzymes are involved in starch biosynthesis, such as AGPase, granule-bound starch synthase, soluble starch synthase, and starch branching enzyme (Caballero et al. 2008; Hirose and Terao 2004; Mizuno et al. 1993; Müller-Röber et al. 1992). Storage proteins, such as glutelin, globulin, and prolamin, are also produced during seed development and accumulate in the endosperm (Krishnan and White 1995; Tanaka et al. 1980). These proteins work in concert during a short period during development stage. The rice floury mutants, *flo1* to *flo5*, exhibit the aberrant features in the endosperm. The causative genes for *flo3*, *flo4*, and *flo5* have been identified as the 16-kDa globulin gene, OsPPDKB and OsSSSIIIa, respectively (Kang et al. 2005; Nishio and Iida 1993; Ryoo et al. 2007). Other floury mutants have been reported recently. The causative gene for *flo6* encodes a CBM-containing protein, which may influence the starch content and its physicochemical features (Peng et al. 2014).

The rice *flo2* mutant produces the aberrant endosperm that shows cloudy, dwarf, and pulverized features, and results in a significantly reduced grain quality (Kawasaki et al. 1996). In this mutant, the expression levels of many genes involved in starch biosynthesis and storage protein biosynthesis are significantly decreased. FLOURY

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ENDOSPERM 2 (FLO2), product of the causative gene of the *flo2* mutant, the *FLO2* gene, is considered to be a regulatory protein that controls the biosynthesis of seed substance storage (She et al. 2010). However, it is unclear how FLO2 regulates the genes involved in the biosynthesis of the storage substances in developing seeds.

FLO2 is a large protein comprising 1720 amino acid residues. The middle region of this protein contains three repeats of the tetratricopeptide repeat (TPR) motif. In other part, no homology to any other protein with known function has beem found (She et al. 2010). The TPR motif is a degenerate 34-amino acid tandem repeat that forms scaffolds that mediate protein–protein interactions and can assemble multiple protein complexes (Chadli et al. 2008). We have found a LEA protein and bHLH, a potential transcription factor, as candidate proteins that interact with FLO2 (She et al. 2010). We have predicted that FLO2 builds a complex of multiple proteins. In this study, we sought to identify another regulatory factor that interacts with FLO2 and which may be involved in the regulatory mechanism controlling the quality of rice seed development.

Materials and methods

Plant materials and growth conditions

Japonica rice (*Oryza sativa* L. cv. Nipponbare) was used as the wild-type plant. The *flo2* mutant, EM37, which was generated by MNU treatment, was used (Satoh and Omura 1981). Seeds were germinated at 30°C in a dark chamber and were grown in a greenhouse. After flowering, the rice plants were cultivated in the growth chamber under 12-h-light (28°C) and 12-h-dark (25°C) conditions. The gene for the fusion FLO2 protein was created as follows. We chemically synthesized a 1.5-kb DNA fragment that encodes the C-terminal portion of the FLO2 protein followed by the 3xFLAG tag sequence, and replaced it with the BanIII–XhoI region of the DNA fragment containing the full-length *FLO2* gene, which was inserted in the pGWB1 vector (She et al. 2010). The resultant gene was introduced into the *flo2* mutant. The transformants were generated and grown in the greenhouse.

Generation of transformants containing the RNAi construct for the Os03g0663800 gene

For the RNAi construct, two parts of the *Os03g0663800* gene were PCR amplified from its cDNA using KOD-plus-NEO (Toyobo, Osaka, Japan) and cloned using pENTER/D-TOPO kits (Invitrogen, Waltham, MA, USA). These fragments corresponded to the positions 1 to 305 and 1564 to 1689 of the cDNA, and were connected and introduced into the pANDA vector, which was driven by the CaMV 35S promoter (Miki and Shimamoto 2004). The resultant plasmid was used for the transformation of the wild-type and *flo2* mutant using the Agrobacterium-mediated method (Hiei et al. 1994). The transformants were grown on Murashige–Skoog plates (Murashige and Skoog 1962) supplemented with 50 mg l^{-1} hygromycin B (Fuji-Film Wako Pure Chemical Industries, Ltd. Corp., Tokyo, Japan) to screen for hygromycin-resistant lines before their cultivation in soil.

The plants were grown in a growth cabinet or greenhouse. The gene introduced into the transformants was confirmed by PCR using the primer set corresponding to the GUS linker region in the pANDA vector, 5′-CAT GAA GAT GCG GAC TTA CG and 5′-ATC CAC GCC GTA TTC GG for the GUS linker. PCR was performed with an initial denaturation at 94°C for 2 min followed by 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s using a GeneAmp PCR System 2720 (Applied Biosystems, Foster City, CA, USA).

RNA isolation and reverse transcription (RT)-PCR

Total RNA was isolated from rice using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). The first-strand cDNA was synthesized from 1 μg of total RNA using a ReverTra Ace cDNA synthesis kit (Toyobo) with an oligo-dT(20) primer. RT-PCR was performed using the GeneAmp PCR System 2720 mentioned above. To detect the transcript for the RNAi construct, the region of the GUS liner lying in the regions of the RNAi transcript was used. The procedure for amplification comprised initial denaturation at 94°C for 2 min followed by 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s. The *Actin I* transcript (acc no. AK100267) was used as the control and was amplified by an initial denaturation at 94°C for 2 min followed by 30 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s using the primer set of 5′-CCC TCC TGA AAG GAA GTA CAG TGT and 5′-GTC CGA AGA ATT AGA AGC ATT TCC.

RNA blot analysis

For the RNA-blot analysis, 20 μg of total RNA was applied in each lane of a 1% agarose gel containing 1xMops running buffer (20 mM Mops, pH 7.0, 8 mM acetate, and 1 mM EDTA) supplemented with 38% formaldehyde and electrophoresed, and the bands were then transferred onto Hybond-N^+ nylon membranes (GE Healthcare, Buckinghamshire, UK). The membranes were subjected to detection of the transcript. Hybridization was performed according to Ausubel et al. (1987) using the DIG-labeled probe corresponding to the 3′ region of the *Os03g0663800* gene, which was PCR-amplified using the primers, 5′-ATC CAG TTC TTC TTC GCC CC and 5′-CCC CTT GTC CTG CTT CCC, in which the region between positions of 685 and 819 in the cDNA for the *Os03g0663800* gene (acc. no. AK105347) was amplified. DIG-labeled probe was synthesized using PCR DIG Labeling Mix (Roche, Basel, Switzerland). Fragments hybridized to the probe were detected by the interaction with anti-digoxigenin-AP Fab fragments (Roche) using an ImageQuant LAS 4000 imager (GE Healthcare).

Fractionation, digestion, and identification of proteins interacting with FLO2

Developing rice seeds (15 days after flowering) were ground

in TBS buffer (200 mM Tris-HCl, pH 7.5, 10% glycerol, 5 M NaCl, and 0.01% Protease Inhibitor Cocktail (Sigma-Aldrich, St Louis, MO, USA)). The cell lysate was centrifuged at 1000 g to remove the cell debris and insoluble materials (P1 fraction). The resulting supernatant (S1) was fractionated by centrifugation at 11,400 g into the fractions comprising precipitates (P2) and supernatant (S2) of the cell lysates. The P2 fraction was suspended in the same amount of TBS buffer. The P1, P2, and S2 fractions were used for further analysis. A 10-ml aliquot of the S2 fraction was incubated with the agarose beads with 5 μl of monoclonal anti-FLAG antibody (Sigma-Aldrich) at 4°C for 2 h. After incubation, the reaction mixture was applied to a Micro Bio-Spin chromatography column (Bio-Rad, Hercules, CA, USA), and the agarose beads were collected by centrifugation. After washing with TBS buffer, the proteins bound to the agarose beads were extracted with 150 μl of extraction buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 1.5% DTT) with heating.

The fractions obtained were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and protein blot analysis using the anti-FLAG antibody. SDS-PAGE was performed according to Ausubel et al. (1987). In parallel, the protein samples were loaded onto an SDS-polyacrylamide gel and electrophoresed for a short time to remove the impurities in the fraction. The gel containing the protein fraction was excised and subjected to protein digestion by trypsin using an In-Gel Tryptic Digestion Kit (Thermo Scientific, Vantaa, Finland). Digested peptides were applied onto a Magic C18 AQ nano-column (Michrom Bioresources, Auburn, CA, USA) in an Advance UHPLC system (Michrom Bioresources) equilibrated with 0.1% formic acid in acetonitrile, and the peptides were eluted using a linear gradient from 5 to 45% acetonitrile at a flow rate of 500 nl min^{-1}. The degested peptides were analyzed using an LTQ Orbitrap XL (Thermo Scientific) mass spectrometer operated with Xcalibur software (version 2.0.7, Thermo Scientific). Peptides were identified using an in-house Mascot server (MS/MS ion search, Mascot version 2.5, Matrix Science Inc.).

Phylogenetic analysis

Alignment of the proteins was performed using Clustal X program from the Clustal website (http://www.clustal.org/). The phylogenetic tree was constructed using the Phylip neighbor-joining method with bootstrap values from 1,000 neighbor-joining bootstrap replicates (Felsenstein 2005). The tree was visualized using the TreeView program (Page 1996).

In vitro pull-down assay for detecting the protein–protein interactions

3xFLAG-tagged proteins, corresponding to the entire and the portions of FLO2 proteins, and the 6xHis-tagged target protein were used for the in vitro pull-down experiment. For the 3xFLAG-tagged FLO2 proteins, the fragments encoding the entire and part of FLO2, corresponding to the positions 1–1720, 568–1720, and 933–1050 in the amino acid sequence, were prepared by PCR amplification from *FLO2* cDNA, and connected with the fragment for 3xFLAG tag at their 3′ ends.

The 6xHis-tagged target proteins were constructed as follows. A fragment encoding the protein encoded by the *Os03g0663800* gene was chemically synthesized. The nucleotide sequence for this protein was modified without any alteration of its amino acid sequence for efficient in vitro translation (Supplementary Figure S1). Using this artificial gene, the fragments for the truncated proteins, corresponding to the positions 1–314 and 265–562 of the amino acid sequence, were PCR amplified. They were connected with the fragment for the 6xHis tag and then inserted into the vector, pEU-dMac3, for cell-free protein synthesis. pEU-dMac3 was constructed by replacing the E01 sequence with the dMac3 translational enhancer in pEU-E01. dMac3 is a portion of the 5′ untranslated region of OsMac3 mRNA, comprising 161 nucleotides, which exhibits sufficient activity as a translational enhancer (Aoki et al. 2014).

Using the resultant plasmids, mRNAs were synthesized using an in vitro transcription system. They were used in the preparation of the corresponding proteins using a wheat germ protein synthesis kit (CellFree Science, Yokohama, Japan). The general procedure followed the manufacture's protocol. The generated proteins were evaluated by SDS-PAGE and protein blot analysis. The 3xFLAG-tagged FLO2 protein was applied to ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich), and the 6xHis-tagged target proteins were added to the resin. The resin was washed with a buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM $MgCl_2$, 1% Triton X-100, and 2% glycerol), and the proteins interacting with the resin were eluted with boiling buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 1.5% DTT). The eluted fraction was subjected to SDS-PAGE, and the His-tagged proteins were detected using protein blot analysis wth the anti-6xHis tag antibody (Clontech-takara, Kusatsu, Japan). As a control, reactions were performed in the absence of FLO2 protein.

Results

Generation of a rice transformant containing the fusion gene encoding FLO2 fused with the FLAG tag

FLO2 is strongly expressed in developing seeds, coincident with the expresssion of other genes that encode storage starch and storage proteins (She et al. 2010). To examine the behavior of FLO2 protein, we created a transformant containing the fusion gene encoding the FLO2 fused to the FLAG tag, which was driven by the native *FLO2* promoter (Figure 1A).

This fusion gene was introduced into the rice *flo2* mutant, and three independent transformants were obtained. These transformants, designated as FLO2-MF, produced normal-shaped seeds whose features were similar with those of the wild-type plants. It has been reported that the *flo2* mutant produces seeds with floury endosperm and that their size and weight are

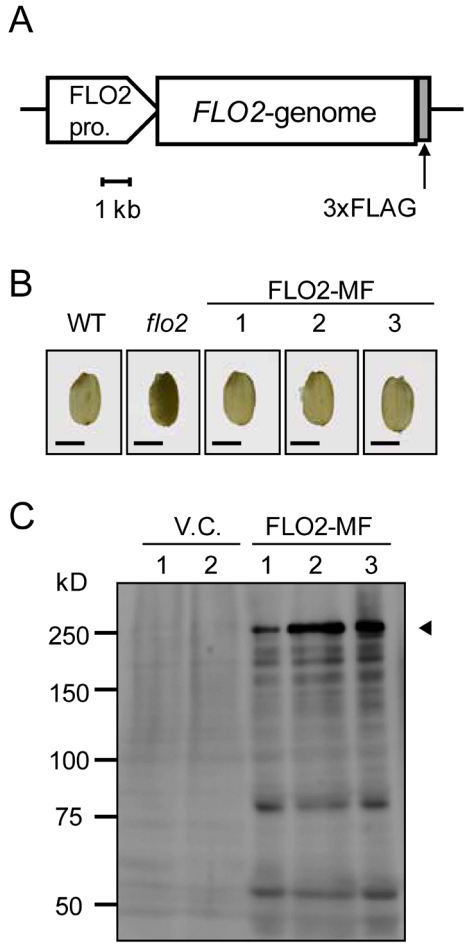

Figure 1. The fusion *FLO2* gene can complement the *flo2* mutation. (A) Structure of the gene for the fusion protein containing the FLO2-3xFLAG tag. FLO2-pro: promoter of the *FLO2* gene, 3xFLAG: 3xFLAG tag. Nucleotide sequence of the 3′ region of the fusion *FLO2* gene was shown in Supplementary Figure S3. (B) Photographs of the seeds of FLO2-MF transformants. WT, Nipponbare; *flo2*, untransformed *flo2* mutant; FLO2-MF, FLO2-MF transformants (1–3). (C) Detection of the fusion FLO2 protein. Proteins were separated by SDS-PAGE and detected using protein blot analysis with anti-FLAG antibody. VC, vector control plants; FLO-MF, FLO2-MF transformants (1–3). The arrowhead indicates the band corresponding to the full-length FLO2-FLAG protein. Scale bars indicate 5 mm.

reduced (Kawasaki et al. 1996). Our result indicated that introduction of the fusion *FLO2* gene resulted in restoration of the phenotype of the *flo2* mutant (Figure 1B). This obsevation suggests that the fusion *FLO2* gene was expressed sufficiently to induce the expression of functional FLO2 in the developing seeds of FLO2-MF.

Next, we attempted to detect the fusion FLO2 protein in the developing seeds. A crude extract was prepared from the developing seeds of FLO2-MF, and was subjected to protein blot analysis using anti-FLAG antibody. A band corresponding to the 250 kDa protein was detected in the developing seeds of FLO2-MF (Figure 1C). This size was considered to represent the full-length protein transcribed from the fusion *FLO2* gene.

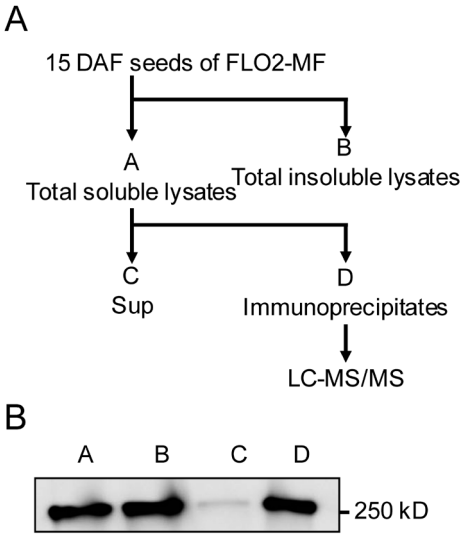

Figure 2. (A) Scheme of the shotgun proteomic analysis. (B) Detection of the fusion FLO2 proteins in each fraction indicated in panel A. Bands were detected using protein blot analysis with anti-FLAG antibody.

Search for the factor that interacts with FLO2

Using the developing seeds of FLO2-MF, we sought to identify the protein that interacts with FLO2. A cell lysate was prepared from the developing seeds harvested at 15 days after flowering (Figure 2A). The resultant solution was combined with an anti-FLAG antibody (FLAG-IP), and the immunoprecipitated fraction, which was expected to contain the fusion FLO2 protein, was collected (Figure 2B). In parallel, to obtain a control fraction, a similar procedure was performed using the developing seeds of the wild-type plants. All of these fractions were then subjected to shotgun proteomic analysis.

The shotgun proteomic analysis produced many peptide fragments, which were candidate proteins that interacted with FLO2 (Supplementary Tables S1 to S3). We screened for the unique protein in both FLO2-MF and control by first removing the proteins bound nonspecifically to the antibody. We obtained two proteins that we considered to be unique in the FLO2-MF transformant. One was a novel protein whose function is unclear, and the other was FLO2.

This protein composed 562 amino acid residues and was encoded by the *Os03g46100* gene. The corresponding cDNA to this protein was registered as AK105347. This gene had a nucleotide sequence with extremely high GC content (Supplementary Figure S1). The deduced amino acid sequence contained multiple repeats of some amino acid residues, such as glutamine and glutamic acid (Figure 3A). This protein was predicted to be a member of the cupin super family that had two cupin domains with a molecular weight of 63 kDa (acc. no. Q75GX9) (Figure 3B). However, this protein has not been reported and its function is unclear. We named this

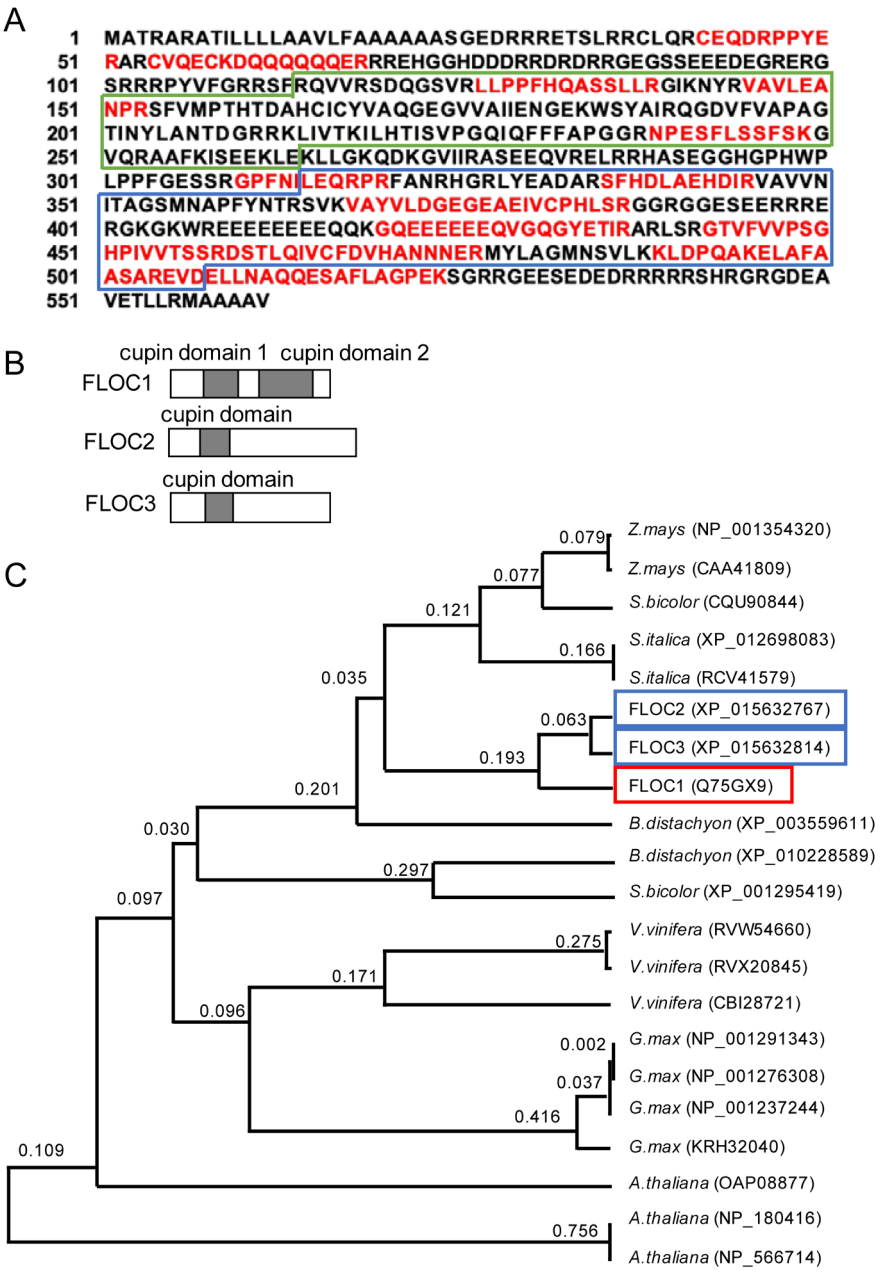

Figure 3. Properties of FLOC1. (A) The deduced amino acid sequence of FLOC1. Red letters indicate the peptide fragments detected by LS-MS/MS. Cupin domains are enclosed in boxes. (B) Structures of FLOC1, FLOC2, and FLOC3. Cupin domains are shaded. (C) Phylogenetic tree of FLOC1 and its homologues. Proteins are shown as the name of species with the protein accession number. Rice FLOC1, FLOC2, and FLOC3 are enclosed in boxes.

protein FLOC1 (FLO2-interacting cupin domain protein 1). The RiceXPro database (http://ricexpro.dna.affrc.go.jp/) showed that this gene was expressed specifically in immature seeds (Supplementary Figure S2A).

FLOC1 had two homologue genes in the rice genome, designated as *FLOC2* and *FLOC3*, whose cDNAs were registered as AK102651 and AK105307. The predicted FLOC2 and FLOC3 proteins had 28% and 35% similarity to FLOC1, respectively. FLOC2 and FLOC3 were found to contain a single cupin domain, whereas FLOC1 had two cupin domains (Figure 3B). These genes were also specifically expressed in the developing seeds (Supplementary Figures S2B and S2C). Both FLOC2 and FLOC3 were not included in the list of the proteins interacted with FLO2.

The orthologues of FLOC1 were widely distributed in various plants, such as wheat, sorghum (*Sorghum bicolor*), grape (*Vitis vinifera*), and *Arabidopsis thaliana* (Figure 3C). However, no orthologues with FLOC1 were found in yeast and animals. These results suggest that the FLOC1, FLOC2, and FLOC3 proteins are unique and are conserved in higher plants.

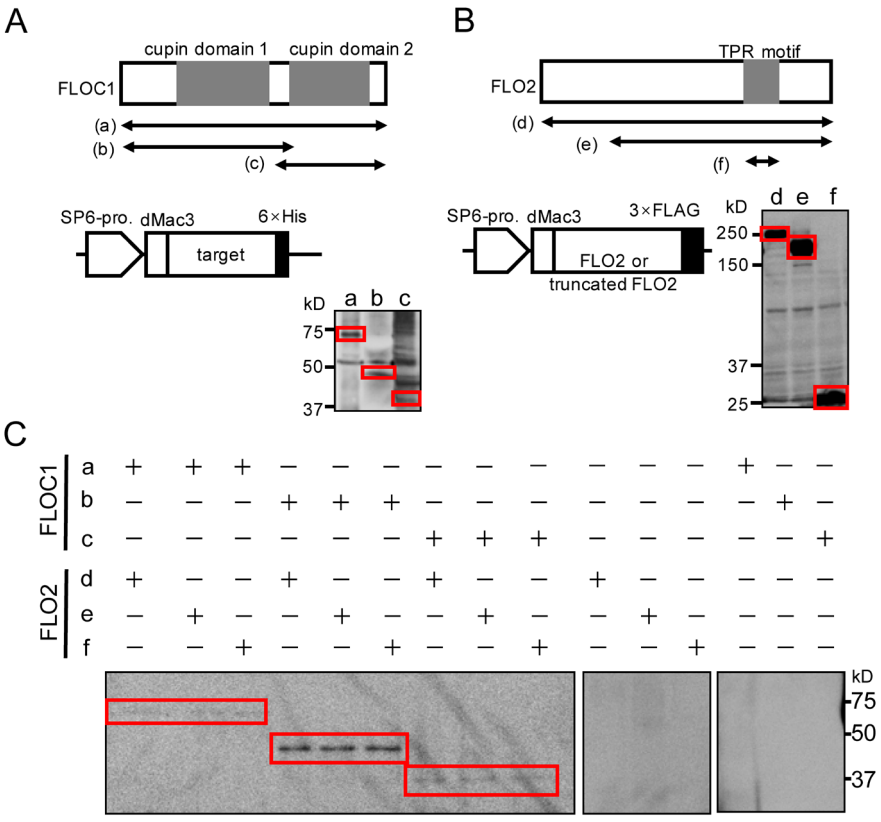

Figure 4. In vitro pull-down assay of FLOC1 with FLO2. (A) Structure of truncated FLOC1 and protein production by the wheat germ cell-free protein synthesis system. Cupin domains are shaded. Regions of truncated FLOC1 are indicated by the double-headed arrows (a–c). Lower panels indicate the structure of the gene used for protein synthesis; the corresponding proteins (a–c) were separated on SDS-PAGE and detected in the protein blot analysis using anti-6xHis tag antibody. The proteins synthesized are enclosed in boxes. (B) Structure of truncated FLO2. The TPR motif is shaded. Regions used in the assay are indicated by double-headed arrows (d–f). Lower panels indicate the structure of the gene used for protein synthesis; the corresponding proteins (d–f) were separated on SDS-PAGE and detected in the protein blot analysis using anti-FLAG tag antibody. The proteins synthesized are enclosed in boxes. (C) Results of the pull-down assay. The upper panel indicates the presence (+) and absence (−) of proteins in the reaction cocktail, and the lower panel shows the results of the interactions, which were detected by protein blot analysis using anti-6xHis tag antibody. Proteins detected are enclosed in boxes.

Detection of the interaction between FLO2 and FLOC1

To examine the interactions between FLO2 and FLOC1, we performed an in vitro pull-down assay to identify the protein–protein interactions. As the target, the genes encoding a truncated FLOC1 protein containing cupin domain 1 (N-region FLOC1), a truncated FLOC1 protein containing cupin domain 2 (C-region FLOC1), and the entire FLOC1, were constructed (Figure 4A). Similarly, the genes for a series of FLAG-tagged FLO2 proteins, the entire FLO2, a truncated FLO2 protein containing the C-terminal region, and a small protein containing the region for TPR motif, were constructed (Figure 4B). These proteins were produced by the wheat germ cell-free protein production system using the assembled vectors to the translational enhancer dMac3. Sufficient amounts of the proteins were obtained (Figure 4C), and the proteins were subjected to the following pull-down assay.

The pull-down assay detected the interaction between FLO2 and FLOC1. All of the FLOC1 proteins, the entire FLOC1, the N-region FLOC1, and the C-region FLOC1 interacted with the proteins corresponding to the entire FLO2, truncated FLO2, and the region containing the TPR motif (Figure 4C). No protein was detected on any reaction in absence of FLO2 protein. These results suggested that both the cupin domains are involved in interactions with the TPR motif of FLO2.

Analysis of the phenotype of the transformant containing the RNAi for FLOC1

To examine the physiological roles of FLOC1, we examined the phenotype caused by the lack of FLOC1. Performing this analysis in the presence or absence of FLO2 would indicate whether FLOC1 and FLO2 have independent functions, complement those functions, or a synergistic function. An RNAi construct for *FLOC1* was constructed using the sequences in the 5′ and 3′ regions of *FLOC1* (Figure 5A). These were introduced into wild-type and the *flo2* mutant to generate transformants (Figure 5B), in which the expression of the *FLOC1* gene was suppressed (Figure 5C). These transformants

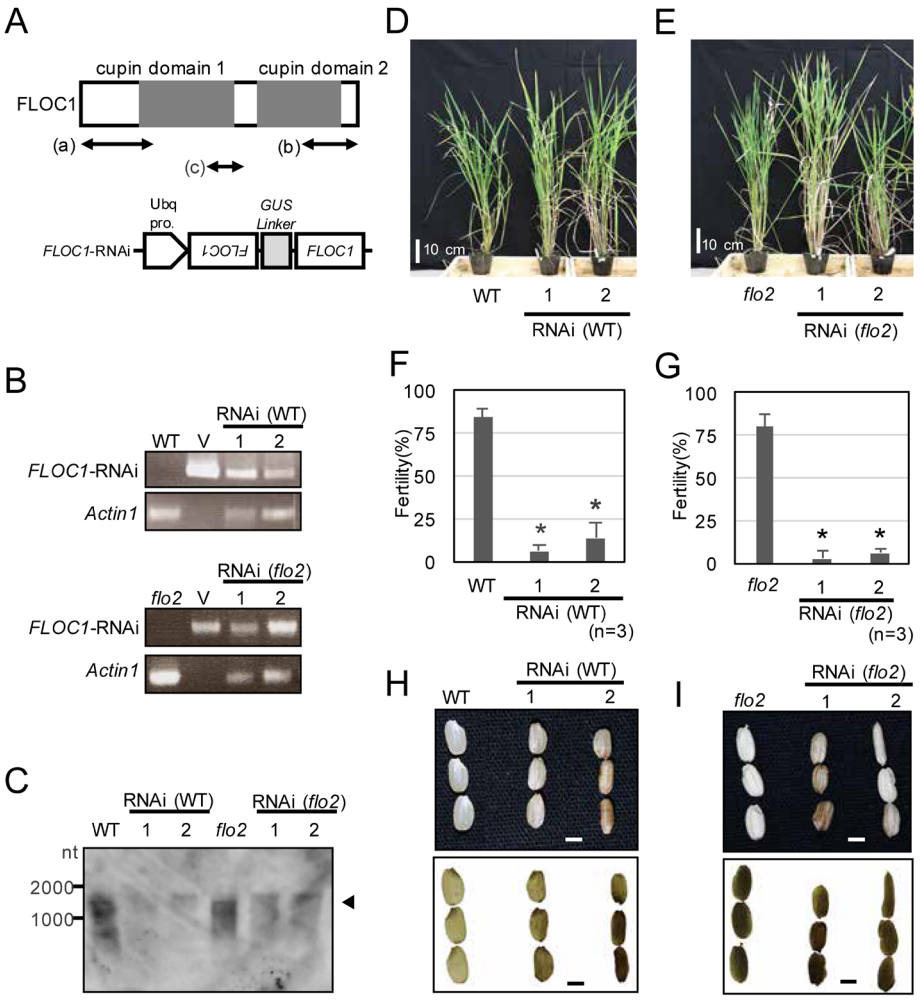

Figure 5. Phenotypes of the RNAi transformants. (A) Structure of the RNAi construct. Upper panel indicates the structure of *FLOC1*. Cupin domains are shaded. (a) and (b) indicate the regions used to construct the RNAi construct. (c) indicates the region of the probe used for RNA-blot analysis. The lower panel indicates the structure of the RNAi construct. Ubq pro, rice ubiqutin promoter; FLOC1, fragments comprising (a) and (b) fragments in the RNAi construct. (B) Detection of the RNAi construct and its expression in the transformants of the Nipponbare (RNAi (WT)) and the *flo2* mutant (RNAi (*flo2*)). The transcript of the RNAi construct was detected by RT-PCR. *Actin1* transcript was detected as the control. (C) RNA blot analysis of the *FLOC1* transcript. The arrowhead indicates the position of the band in the *FLOC1* transcript. (D) Features of the RNAi transformants of Nipponbare. (E) Features of the RNAi transformants of *flo2*. (F) Fertility of the RNAi transformants of Nipponbare. (G) Fertility of the RNAi transformants of *flo2*. Error bars show the SD ($n=3$). One-way ANOVA with Dunnet post tests were performed for RNAi lines to the wild type ($p<0.05$). Asterisks indicate the significance level. (H) Feature of seeds of RNAi transformants of Nipponbare. (I) Feature of seeds of RNAi transformants of *flo2*. WT, non-transformed host plant; V, DNA fragment of the plasmid for the RNAi construct; RNAi (WT) and RNAi (*flo2*), RNAi transformants of Nipponbare and *flo2* mutant, respectively. Numbers indicate the lines of transformants. The upper panels of (H) and (I) show the grain shapes of seeds, and the lower panels show the seeds illuminated with backlight. Scale bars indicate 5 mm.

grew normally during the vegetative growth phase and headed similarly as did the wild-type plants (Figure 5D). However, the RNAi transformants showed a significantly reduced fertility rate (Figure 5F). In addition, seeds produced by these transformants showed morphological abnormalities, such as small size and severely wrinkled features (Figure 5H). These results suggest that knock-down of *FLOC1* affected both the seed quality and fertility. Phenotypes of low fertility and abnormal seed features were also observed in the RNAi transformants derived from the *flo2* plants, although these formed floury seeds (Figure 5E, G, I). These findings suggest that, as well as FLO2, FLOC1 plays important roles in seed development, but they have distinct functions.

Discussion

The rice endosperm accumulates a large amount of storage substances. Enzymes involved in the production of these substances are the key factors that determine the yield and grain quality. Rice FLO2 plays a crucial role in seed development, for example, by regulating the biosynthesis of storage starch and proteins in the endosperm (She et al. 2010). We produced transformants harboring the fusion FLO2 protein gene in the *flo2* mutant. Considering the observation that these

transformants produced normal-shaped seeds, we suggest that the fusion FLO2 protein worked efficiently as FLO2 in developing seeds and compensated for the *flo2* mutation (Figure 1). In this study, we performed shotgun proteomic analysis to identify a novel protein that interacts with the fusion FLO2, which we expected to be involved in endosperm formation during endosperm development.

FLO2 is predicted to be a protein that interacts with other proteins to exhibit its function. We have found that a LEA protein and a bHLH transcription factor can interact with FLO2 (She et al. 2010). However, we had no data to indicate that they interact directly with the TPR motif of FLO2, and in this study, we attempted to identify other proteins that interact with the TPR motif of FLO2. Given that the fusion FLO2 protein was detected in the developing seeds by the anti-FLAG antibody, in this study, we tried to identify the proteins that can interact with FLO2 and found the novel protein, FLOC1, as a candidate protein that can interact with FLO2.

FLOC1 had two cupin domains, whose functions were unknown. *FLOC1* was wildly conserved in the plant genomes. Rice FLOC1 had two homologues, *FLOC2* and *FLOC3* (Figure 3). *FLOC1*, *FLOC2*, and *FLOC3* were expressed specifically in the developing seeds. This expression pattern coincided with that of FLO2. FLOC2 and FLOC3 had a single cupin domain, whereas FLOC1 had two cupin domains. In addition, FLOC2 and FLOC3 were not included in the list of the candidates that can interact with FLO2, which suggests that FLOC2 and FLOC3 may be functionally divergent from FLOC1.

The pull-down assay identified the interaction between FLOC1 and FLO2 and that the TPR motif may be involved in this interaction (Figure 4). The TPR motif comprises tandem repeats of 34 amino acid residues, and adopts a right-handed helical helix–loop–helix structure with an amphipathic channel; such channels are involved in many protein–protein interactions (Allan and Ratajczak 2011). Proteins containing cupin domain are widely distributed. It has been reported that germin and germin-like proteins (GLPs) play an important role in plant development and defense (Lane et al. 1993). However, the function of the cupin domain remains unclear.

FLO2 is also expressed in leaves, although the function of FLO2 in leaves is unknown (She et al. 2010). It has been reported that *A. thaliana* FLO2 is involved in the regulation of translocation and transport of assimilates and in quality control of substance supply or transfer (Kihira et al. 2017). The TPR motif has been shown to exhibit great flexibility and variability in terms of its substrate specificity (Nojima et al. 2017). FLOC1 was absent in leaves, and, therefore, it was expected that FLO2 would interact with other factors.

The knock-down transformants harboring the RNAi construct for *FLOC1* exhibited significantly reduced fertility, and produced aberrant seeds with small and severely wrinkled features (Figure 5). This suggests that FLOC1 is involved in both seed fertility and seed quality. It has been reported that the *flo2* mutation results in the formation of grains with white and floury endosperm that contains loosely packed, small, and spherical starch granules with large air spaces (She et al. 2010). In the RNAi transformants derived from the wild-type plant, the seeds became small and formed abnormal features. This suggests that a lack of FLOC1 caused insufficient grain filling and resulted in aberrant seed formation. In the RNAi transformants derived from the *flo2* mutant, these phenotypes were also observed along with the formation of floury seeds (Figure 5). This observation suggests that the effect of the *flo2* mutation remained in these transformants, which implies difference in the functions of FLO2 and FLOC1 even though both are involved in the control of seed quality during seed formation.

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