Du13 encodes a C_2H_2 zinc-finger protein that regulates Wx\(\beta\) pre-mRNA splicing and microRNA biogenesis in rice endosperm

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Summary

Amylose content is a crucial physicochemical property responsible for the eating and cooking quality of rice (Oryza sativa L.) grain and is mainly controlled by the Waxy (Wx) gene. Previous studies have identified several Dull genes that modulate the expression of the Wx\(\beta\) allele in japonica rice by affecting the splicing efficiency of the Wx\(\beta\) pre-mRNA. Here, we uncover dual roles for a novel Dull gene in pre-mRNA splicing and microRNA processing. We isolated the dull mutant, du13, with a dull endosperm and low amylose content. Map-based cloning showed that Du13 encodes a C_2H_2 zinc-finger protein. Du13 coordinates with the nuclear cap-binding complex to regulate the splicing of Wx\(\beta\) transcripts in rice endosperm. Moreover, Du13 also regulates alternative splicing of other protein-coding transcripts and affects the biogenesis of a subset of microRNAs. Our results reveal an evolutionarily conserved link between pre-mRNA splicing and microRNA biogenesis in rice endosperm. Our findings also provide new insights into the functions of Dull genes in rice and expand our knowledge of microRNA biogenesis in monocots.

Keywords: rice, dull endosperm, OsGBSSI/Wx, C_2H_2 zinc-finger protein, microRNA, pre-mRNA splicing.

Introduction

Starch accounts for approximately 90% of the dry weight of rice grains, which constitute the primary food source consumed by over half of the world’s population (Zeng et al., 2007). Two different kinds of starch, amylose and amylopectin, are deposited in the rice endosperm. Moreover, amylose content (AC) is the major determinant of the eating and cooking quality of rice. Thus, modifying AC is a major strategy for quality improvement in rice breeding (Kiswara et al., 2014). AC in rice endosperm is determined by the major Waxy (Wx) locus and many minor loci, including more than a dozen dull endosperm loci, named du1 to du12(t) (Aluko et al., 2004; Kiswara et al., 2014). The rice Wx locus encodes granule-bound starch synthase I (OsGBSSI), a key enzyme involved in amylose biosynthesis in rice endosperm (Wang et al., 1990). Rice cultivars carry two wild-type alleles at the Wx locus, Wx\(\alpha\) and Wx\(\beta\). Wx\(\alpha\) predominates in O. sativa spp. indica species with high AC and produces 10 times more OsGBSSI protein than Wx\(\beta\), which is mainly distributed in japonica cultivars with low or intermediate AC (Zeng et al., 2007). The lower abundance of OsGBSSI in japonica cultivars is due to decreased transcript levels of Wx\(\beta\) caused by a G-to-T mutation at the splicing donor site of Wx\(\beta\) intron 1, which diminishes its splicing efficiency and activates two cryptic splice donor sites within exon 1 (Cai et al., 1998; Frances et al., 1998; Hirano et al., 1998; Ishikii et al., 1998). The two splice sites show a weaker match to the 5′ consensus splice site sequence, leading to the lower splicing efficiency of Wx\(\beta\) transcripts. However, dull mutations do not affect the splicing of the Wx\(\beta\) transcripts with the normal splice donor site of intron 1 (Ishikii et al., 2000).

dull mutants exhibit an endosperm with a dull appearance and low AC that contrasts with the typical translucent endosperm of wild-type rice. Several Dull genes have been shown to modulate the splicing efficiency of Wx\(\beta\) intron 1 (Ishikii et al., 2000, 2008). Although rice breeders have employed dull mutants to improve the eating and cooking quality of rice, only two Dull genes have been cloned to date. Du1 encodes a member of the pre-mRNA processing protein (PRP) family and affects the splicing of Wx\(\beta\) and starch biosynthesis (Zeng et al., 2007), while Du3 encodes the mRNA cap-binding protein OsCBP20 that forms a hetero-dimeric cap-binding complex (OsCBC) with OsCBP80 (Ishikii et al., 2008). OsCBP20 might affect the transcription, splicing, and stability of Wx\(\beta\) in rice endosperm (Ishikii et al., 2008).

The CBC can bind to the cap structure of transcripts produced by RNA polymerase II. In Arabidopsis (Arabidopsis thaliana), the CBC participates in the biogenesis of microRNAs (miRNAs) and splicing of cap-proximal introns in pre-mRNAs (Kim et al., 2008; Laubinger et al., 2008; Raczyńska et al., 2009). The CBC coordinates its various roles with the C_2H_2 zinc-finger protein SERRATE (SE; Grigg et al., 2005; Laubinger et al., 2008; Raczyńska et al., 2014). However, the exact functional relationship
between SE and CBC in regulating miRNA biogenesis and RNA splicing has remained elusive.

In this study, we describe and characterize the novel rice dull mutant, du13. Map-based cloning revealed that the Du13 gene encodes a C_{2}H_{2} zinc-finger protein homologous to Arabidopsis SE. We determined that Du13 and OsCBC act together to regulate the splicing of Wx. We observed significant changes in the alternative splicing (AS) patterns of some genes in the du13 mutant background. Du13 also affects the accumulation of a subset of miRNAs in rice endosperm. We thus documented here that the rice Du13 gene, Du13, may be involved in the regulation of miRNA biosynthesis.

Results
Identification of du13
We identified the du13 mutant from a mutant population of japonica cv. Koshihikari (Figure S1a) for its endosperm appearance between waxy (as in the wx mutant w53 in the Koshihikari background) and translucent (wild-type Koshihikari; Figure 1a, b). Transverse sections of du13 grains took on a reddish-brown colour upon iodine staining that is distinct from the dark-blue staining of wild-type grains and the light-brown staining of w53 grains (Figure 1c). The AC of du13 grains was 7.05%, compared to 16.77% in the wild-type (Figure 1d). Scanning electron microscopy (SEM) analysis showed that the du13 endosperm consisted of tightly packed and polyhedron-shaped starch granules (SGs), as in the wild type (Figure S1b–g). We obtained similar results using semi-thin sections of developing endosperm (Figure S1h, i), but noticed small cavities within SGs in du13 (Figure S1g), resembling those of previously reported dull mutants (Kaushik and Khush, 1991).

Compared to the wild type, the du13 mutant showed a slower grain-filling rate, in accordance with smaller and lighter grains (Figure S1j–m; Table S1). du13 plants also exhibited an increased plant height and panicle length, but a reduced tilling number (Figure S1a; Table S1). Collectively, these data showed that the du13 mutation has pleiotropic effects on multiple plant phenotypes.

Altered starch physicochemical characteristics of the du13 mutant
Total starch and protein contents were comparable between du13 and wild-type grains (Figure S2a, b), whereas the lipid content of du13 was almost twice that of the wild type (Figure S2c). In du13 developing endosperm, AC was substantially lower at the onset of amylose biosynthesis, and this tendency continued until seed maturation (Figure S2d, e). We also determined the amylopectin chain-length distribution of du13 endosperm starch. When compared to wild-type levels, the du13 mutant had a lower proportion of short chains with a degree of polymerization (DP) in the range of 9–15, and a higher proportion of amylopectin chains with DP in the ranges of 6–8 and 16–33 (Figure S2f).

We also analyzed the pasting properties of du13 endosperm starch with a rapid visco analyzer (RVA). The viscosity profiles of du13 and wild-type pasting starch followed a similar pattern, although that of du13 maintained a lower level relative to the wildtype (Figure S2g). In addition, du13 grains displayed higher values of peak viscosity and breakdown viscosity, but lower values for other RVA parameters when compared to the wild type (Table S2), indicating a relatively soft and elastic texture. We concluded that the du13 mutant alters the physicochemical characteristics of the starch.

Map-based cloning of Du13 and complementation of the du13 mutant
For genetic analysis, we crossed the du13 mutant to the parental cultivar Koshihikari. In the F2 population, translucent to dull endosperm segregated with a ratio of about 3:1 (n = 778, \( \chi^{2} = 0.829 < \chi_{0.05}^{2} = 3.84 \)), indicating that du13 is a single-gene recessive mutation.

We generated an F2 segregating population derived from a cross between du13 (japonica) and Nanjing 11 (indica) to map the Du13 locus between simple sequence repeat (SSR) markers W26 and W6 on the long arm of chromosome 6 (Figure 2a). Subsequent fine-mapping narrowed down the candidate interval to a 187-kb region with 26 putative open reading frames (ORFs; Figure 2a). Sequence analysis revealed a C-to-T substitution at position 4482 in exon 8 of ORF 26 (LOC_Os06g48530) in the du13 mutant (Figure 2b), introducing a premature stop codon in place of the wild-type Arg-333 residue (Figure S3).

To assess whether LOC_Os06g48530 corresponds to the Du13 gene, we introduced a construct consisting of the LOC_Os06g48530 coding region, driven by the UBIQUITIN promoter, into the du13 mutant background. Seeds harvested from three independent transgenic T2 lines recovered their vitreous appearance (Figure 2c). Iodine staining of transverse-cut grains from these lines was also similar to that of the wild type (Figure 2c), as reflected by their similar amylose contents (Figure S4a). We failed to detect the protein encoded by LOC_Os06g48530 in the du13 mutant, while a protein of the appropriate molecular weight reappeared in the transgenic lines, using a specific antibody raised in rabbits (Figure 2d). For an independent confirmation, we introduced a CRISPR-Cas9 construct expressing a single guide RNA (sgRNA) targeting exon 8 of LOC_Os06g48530 into Nipponbare to generate additional mutants in this gene. The sequencing of PCR amplicons of LOC_Os06g48530 from the genomic DNA of transgenic plants identified knockout mutant Y6 with a 1-bp insertion in exon 8 (Figures 2e and S3b). Relative to Nipponbare, Y6 produced visibly smaller seeds with dull endosperms, and their transverse sections stained reddish-brown with iodine, as observed in du13 (Figure 2f). The AC of Y6 seeds also dramatically decreased from 16.26% in the wildtype to 5.00% (Figure 2g). We failed to detect the protein-encoded LOC_Os06g48530 in the Y6 mutant by immunoblot analysis (Figure 2h). We obtained similar results with three other knockout mutants in the japonica cv. Ningjing 7 background (Figure S4b–d). Given that these knockout mutants shared the same phenotypes as Y6, we focused on the Y6 mutant chosen for further study. Together, these data demonstrated that LOC_Os06g48530 is the Du13 gene.

Du13 is a zinc-finger protein homologous to SE
The Du13 gene contains 12 exons and encodes a protein with 723 amino acids that is predicted to harbour a putative nuclear localization signal at its N terminus, a domain of unknown function 3546 (DUF3546), and an Arsenite-resistance protein-2 (ARS2) domain near its C terminus (Figure 2b, e; http://smart.embl-heidelberg.de/; https://www.ncbi.nlm.nih.gov/protein?with=F). The ARS2 domain included a single C_{2}H_{2}-type zinc finger, which
is a general feature of SE orthologues (Wilson et al., 2008; Figure 2e). In the du13 and Y6 mutants, the truncated Du13 proteins would completely or partially lack the ARS2 domain, if they indeed accumulate in these mutants (Figure 2e).

To understand the phylogenetic relationship of Du13 and SE homologs in plants, we extracted the predicted protein sequences from phylogenetically different organisms (Figure S5a). Our phylogenetic analysis revealed that the rice genome encodes three distinct SE homologs; Du13, ZOS2-03 (LOC_Os02g05610) and ZOS8-11 (LOC_Os08g40560). The three rice SE orthologs clustered with SE-related proteins from purple false brome (Brachypodium distachyon). Du13 and ZOS2-03 share about 78% and 53% amino acid identity with SE, respectively, while ZOS8-11 shares the highest identity with SE at 85%. Interestingly, the monocots rice, purple false brome, and sorghum (Sorghum bicolor), have at least two SE-related genes, whereas there is only a single SE homolog in dicots such as Arabidopsis, soybean (Glycine max), and barrel clover (Medicago truncatula), as in mammals (Wilson et al., 2008). These putative SE orthologues harbour similar domains in the same relative order, suggesting a high degree of conservation (Figure S5b, c).

We determined the expression pattern of Du13 using quantitative RT-PCR (qRT-PCR). We detected Du13 expression in seedlings, young roots, leaves, leaf sheaths, culms, panicles, and mature roots, with the highest level being seen in leaves (Figure S6a). In developing seeds, Du13 expression peaked at an early stage (approximately 3 days after fertilization [DAF]) and then gradually declined (Figure S6b), thus echoing the results of immunoblot analysis (Figure S6c). In the du13 mutant, Du13 expression was remarkably lower than in the wild type (Figures 2d and S6d). These results indicate that Du13 is constitutionally expressed in all tested tissues, and that the largest increase in Du13 expression in the grain coincides with the initiation stage of grain development (Wang et al., 2013).

We transiently transfected a 35S-Du13-GFP construct in rice protoplasts, expressing a fusion between Du13 and the green fluorescent protein (GFP) under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The Du13-GFP fusion protein localized to the nucleus, while the free GFP control was widely distributed in the cytoplasm (Figure S6e). The mutation in du13 had no effect on Du13 subcellular localization (Figure S7).

Du13 affects splicing of the Wxb pre-mRNA in rice endosperm

As the Wx gene is predominantly expressed in endosperm and pollen (Isshiki et al., 2000), we investigated whether the du13 mutant also affected amylose biosynthesis in pollen. Iodine staining showed that wild-type and du13 pollens are comparable in their appearance (Figure 3a, b). We conclude that the du13 mutant differentially influences the expression of Wx in endosperm and pollen.

Compared to the wild type, OsGBSSI accumulated to remarkably lower levels in the endosperms of du13 and the knockout mutant Y6 (Figures 2h and 3c). In agreement, OsGBSSI activity also decreased dramatically in du13 and Y6 developing endosperms, compared to their respective wild type and a du13 complemented line (Figure 3d). qRT-PCR analysis showed no significant changes in the levels of Wx transcript between du13 and the wild type or the complemented line, or between Nipponbare and Y6 (Figure 3e). We then examined the splicing efficiency of Wx pre-mRNA, with one site (site 2) located 93 nucleotides upstream of the normal splicing site and the second site (site 1) one base upstream of the normal site. As Wx pre-mRNA is activated in the Wx pre-mRNA, with one site (site 2) located 93 nucleotides upstream of the normal splicing site and the second site (site 1) one base upstream of the normal site. As Wx transcripts spliced from the normal site or the second cryptic site are indistinguishable by RT-PCR, these two sites are collectively called site 1 (Isshiki et al., 2008). qRT-PCR analysis showed that...
Figure 2  Map-based cloning and complementation of *Du13*. (a) Fine-mapping of the *Du13* locus. The molecular markers and the numbers of recombinants are indicated. CEN, centromere. (b) Structure of the *Du13* locus and positions of the mutations in *du13* and the *Du13*-knockout mutant Y6. A single nucleotide substitution in *du13* generated a premature stop codon, while an additional nucleotide A (4795) was inserted in Y6. (c) Functional complementation of the *du13* mutant with LOC_Os06g48530 restored a normal grain appearance in three independent transgenic lines (T1371-1, -2, and -3, upper panel). Lower panel, transverse seed sections stained with iodine. (d) Immunoblot analysis of *Du13* protein abundance in mature seeds of the wild type (WT), *du13*, and three transgenic lines. (e) Schematic diagram of the functional domains present in *Du13*. Nuclear localization signal, NLS, green line; DUF3546 domain, blue box; ARS2 domain, orange box, containing a C2H2 zinc finger (green box). Double red slashes indicate the approximate location of the premature stop codon in *du13*. a.a., amino acids. (f) Mature seeds of the WT, *du13*, Nip, and Y6 (upper panel). Lower panel, transverse seed sections stained with iodine. (g) Amylose contents of Nip and Y6 seeds. (h) Immunoblot analysis of *Du13* and OsGBSSI in mature seeds of the WT, *du13*, Nip, and Y6. Anti-HSP82 antibodies were used as a loading control in (d) and (h). Nip, Nipponbare; IB, immunoblotting.
the abundance of \( Wxb \) transcripts spliced at site 1 was much lower in \( du13 \) and Y6 developing endosperms at 10 and 12 DAF compared to the wild type, concomitantly with a stark increase in unspliced \( Wxb \) levels (Figure 3g, h). Indeed, the splicing efficiency of \( Wxb \) transcripts in \( du13 \) dropped to approximately one-third and one-tenth of the wild type in developing endosperm at 10 and 12 DAF, respectively, similar to that in Y6 (Figure 3i, j). Therefore, loss of Du13 function impaired splicing of \( Wxb \) transcripts in rice endosperm.

The \( du13 \) mutation also affected the expression of several starch biosynthesis-related genes (Figure S8), which might explain the altered fine structure of amylopectin in \( du13 \) (Figure S2f). We

Figure 3  Loss of \( Du13 \) function affects the splicing of \( Wxb \) in rice endosperm. (a, b) Pollen grains of the wild type (WT, a) and \( du13 \) (b) stained with iodine. (c) SDS-PAGE analysis of OsGBSSI abundance in WT and \( du13 \) developing endosperm at 10 days after fertilization (DAF) or in mature endosperm. OsGBSSI is inside the red frame. The protein was extracted from developing or mature endosperms as described previously (Liu et al., 2009). (d, e) OsGBSSI activity (d) and OsGBSSI expression levels (e) in developing endosperms from the WT, \( du13 \), the representative complemented line T1371-1, Nip, and Y6 at 10 DAF. The levels of OsGBSSI in the WT and Nip were set to 1 in (e). (f) Schematic diagram of the possible splicing patterns of the \( Wxb \) pre-mRNA. (g, h) qRT-PCR analysis of \( Wxb \) transcripts spliced at site 1 in developing endosperms of the WT and \( du13 \) (g), or Nip and Y6 (h) at 10 and 12 DAF. The levels of \( Wxb \) transcripts in the WT and Nip were set to 1. (i, j) qRT-PCR analysis of \( Wxb \) splicing efficiency in developing endosperms of the WT and \( du13 \) (i), or Nip and Y6 (j) at 10 and 12 DAF. The splicing efficiency of \( Wxb \) was calculated as previously described (Zhang et al., 2014a); WT or Nip values were set to 1. Values are means ± SD (n = 3). *P < 0.05, **P < 0.01, as determined by Student’s t-test between the WT and mutant. Actin I was used as an internal control in (e, g–j). Nip, Nipponbare. (k) \( Du13 \) does not have transcription activity.

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tested whether Du13 exerted direct transactivation activity in yeast cells by fusing Du13 to the DNA binding domain of the transcription factor GAL4, but observed no transactivation activity (Figure 3k). Therefore, the altered expression levels of starch biosynthetic genes in du13, might result from indirect effects of the du13 mutation.

Functional analysis of Du13 in transient assays using rice protoplasts

To explore the effects of Du13 on the splicing of Wxb, we used a transient assay system in rice protoplasts and the Wxb-gus (β-glucuronidase) reporter construct for the analysis of splicing (Figure 4a). Co-transfection of rice protoplasts with the Wxb-gus reporter and the 35S-Du13 cDNA construct elevated GUS activity approximately three-fold relative to the control (Figure 4b). By contrast, GUS activity was much lower in du13 approximately three-fold relative to the control (Figure 4b). Co-transfection of rice protoplasts with the 35S-Du13 reporter gene. The cDNAs of Wxb were co-transfected into protoplasts with the Wxb-gus reporter (Figure 4c).

We also examined the effects of Du13 on splicing efficiency and splice site selection of Wxb-gus. In protoplasts transfected with the Wxb-gus reporter, site 1 was preferentially used (Figure 4d). However, when Wxb-gus was co-transfected with 35S-Du13, Wxb-gus transcripts showed a strong increase in preference for splicing at site 1 together with a slight increase at site 2, indicating enhanced splicing efficiency (Figure 4d). Co-transfection of Wxb-gus with 35S-du13 largely abolished Wxb-gus splicing though slightly increased the GUS activity (Figure 4b, d). Moreover, only site 2 appeared to be selected at the 5′ splice site by du13 (Figure 4d).

In developing endosperms from the du13 and Y6 mutants and their respective wild-type cultivars, site 1 was preferentially used (Figure 4e, f). The du13 mutant also exhibited a lower splicing efficiency of Wxb, which progressively decreased over the course of endosperm development (Figure 4e), consistent with the results of Figure 3i. We observed a similar trend in the Y6 mutant (Figures 3h, j, 4f). Notably, unspliced transcripts of Wxb-gus accumulated to lower levels in protoplasts co-transfected with 35S-Du13, but highly accumulated when co-transfected with 35S-du13 (Figure 4d). We noticed similar effects in developing endosperms (Figure 4e, f), in agreement with the qRT-PCR results (Figure 3g, h).

Du13 interacts with OsCBC and splicing factors

The du13 mutant had a similar dull endosperm phenotype as du3, carrying a mutation in OsCBP20 (Ishiki et al., 2008), prompting us to hypothesize that Du13 and OsCBC might work together to regulate Wxb splicing. We thus examined the potential for physical interactions between Du13 and OsCBC subunits.
We analysed the subcellular localization of OsCBP20 and OsCBP80 in *Nicotiana benthamiana* leaves. OsCBP20 showed a clear nuclear localization, while OsCBP80 localized to the cytoplasm and the nucleus (Figure S7). OsCBP20 accumulated in the nucleoplasm as well as in nuclear speckles. These two rice proteins, therefore, displayed the same localization pattern as their *Arabidopsis* counterparts (Kierzkowski et al., 2009). A bimolecular fluorescence complementation (BiFC) assay indicated that OsCBP20 interacted with OsCBP80 in nuclear speckles (Figure S9a). BiFC assays also demonstrated physical interaction between Du13 and the OsCBC subunits OsCBP20 and OsCBP80 (Figure 5a). We observed a strong and homogeneous fluorescence signal from reconstituted enhanced yellow fluorescent protein (eYFP) in the nuclei of cells co-infiltrated with Du13 and OsCBP20 constructs, but predominantly in nuclear speckles of cells co-infiltrated with Du13 and OsCBP20 constructs (Figure 5a). An in vivo co-immunoprecipitation (Co-IP) assay also confirmed that OsCBP20 and OsCBP80 could be co-immunoprecipitated by Du13 in total *N. benthamiana* leaf extracts (Figure 5b). Together, our results indicated that both OsCBC subunits form a complex with Du13 in the nucleus. Interestingly, like OsGBSSI, OsCBP20 and OsCBP80 expression peaked during the middle stage of grain development (Figure S9b–d), when a rapid increase in starch accumulation and seed weight occurs (Wang et al., 2013). We observed no significant changes in the expression of OsCBP20 or OsCBP80 in *du13* endosperm (Figure S9e, f).

We detected no interactions between Du1 and Du13 or between Du1 and OsCBC using yeast two-hybrid (Y2H) and BiFC assays (Figure S10). By contrast, Du13 interacted with the four Ser/Arg-rich (SR) splicing factors RSp29, RSp23, and two homologs of RSZp23 (Figures S7 and S11). RSp29 and RSZp23 were previously shown to affect AS of gene transcripts in rice (Isshiki et al., 2006). The N terminus of Du13 mediated these interactions (Figure S11c).

Du13 interacts with OsCBC. (a) BiFC assays showing that Du13 interacts with OsCBC in the nucleus of *N. benthamiana* leaf cells. The insets are magnified views of a representative nucleus in the dotted rectangles. OsMADS3-mCherry was used as a nuclear marker. Bars, 20 μm. (b) Co-IP assays showing that OsCBP20-GFP, OsCBP80-GFP, and ZOS2-03-GFP co-immunoprecipitate with Du13-Flag in total *N. benthamiana* leaf extracts with anti-Flag antibodies. IB, immunoblotting.

Du13 is involved in alternative splicing of genome-wide rice genes

We examined whether alternative splicing (AS) was affected in *du13* through transcriptome deep sequencing (miRNA-seq). In wild-type endosperm, we detected a high frequency of the alternative 3′ splice site (40.44%), followed by the alternative 5′ splice site (22.98%), exon skipping (21.33%), intron retention (13.10%), and mutually exclusive exon choice (2.15%; Figure 6a). In *du13* endosperm, the frequency of AS events changed, for alternative 3′ or 5′ splice sites (35.97% and 19.71%, respectively), exon skipping (23.42%), intron retention (17.80%), and mutually exclusive exon (3.10%; Figure 6a; Table S3). These results thus suggested that AS at the alternative 3′ splice site occurs more frequently than at the 5′ splice site in plants, in agreement with a previous study (Raczynska et al., 2014). When looking at the distribution of different AS events within introns, we noticed that intron retention AS events increased in the first and last introns or genes with a single intron in the *du13* mutant (Figure 6b), while AS at alternative 5′ and 3′ splice sites increased greatly in the first intron (Figure 6c, d).

To confirm a regulatory role for Du13 in AS of rice gene transcripts, we characterized AS of several mRNAs by RT-PCR with intron-flanking primers. We detected higher levels of unspliced transcripts accumulating in *du13* compared to the wild type (Figure 6e), potentially reflecting an effect on general splicing efficiency in the *du13* mutant and confirming a role for Du13 as a regulator of AS of gene transcripts in rice.

Du13 participates in miRNA processing

We tested whether Du13 might also affect the accumulation of mature miRNAs. A subset of miRNAs accumulated to lower levels in *du13* compared to the wild type, as determined by miRNA-seq analysis (Table S4). In agreement, the target genes of these miRNAs largely exhibited higher transcript levels (Table S4). We
used qRT-PCR analyses to validate the altered expression of these miRNAs in du13. The levels of osa-miR435, osa-miR444e, osa-miR1866-3p, osa-miR5144-5p, and osa-miR5794 were dramatically reduced in du13 and Y6 compared to their respective wild-type cultivars (Figures 7a and S12a). The levels of osa-miR1850.2 were slightly reduced in du13 (Figure 7a), but markedly reduced in Y6 (Figure S12a). These results were largely in agreement with the miRNA-seq analysis (Table S4). Intriguingly, only a subset of intron-less pri-miRNAs were affected in du13 (http://structuralbiology.cau.edu.cn/cgi-bin/hgGateway?hgsid=3390&clade=plant&org=Nipponbare_v7&db=0). We further examined the expression levels of miRNA biogenesis components in du13 but observed no substantial differences in their expression between du13 and the wild type (Figure S9g).

We examined pri-miRNA transcript levels in du13 and Y6 endosperms by semi-quantitative RT-PCR to test whether the du13 and Y6 mutants lowered primary miRNA (pri-miRNA) levels or the processing efficiency of the pri-miRNA transcripts. The pri-miRNA transcripts of less abundant miRNAs in du13 and Y6 accumulated to higher levels compared to their respective wild types (Figures 7b and S12b), indicating less effective processing of pri-miRNAs. By contrast, pri-miRNA transcripts of upregulated miRNAs in du13 and Y6 displayed a decreased abundance, while we saw no obvious changes for the pri-miRNA transcripts of a slightly reduced miRNA in du13 (Figures 7b and S12b). As miRNAs negatively regulate the abundance of their target mRNAs, we determined the expression levels of target mRNAs by qRT-PCR. Indeed, the abundance of most target mRNAs was elevated in the du13 and Y6 mutants compared to their wild types (Figures 7c and S12c; Table S4). Collectively, these findings indicate that Du13 is involved in the proper processing of pri-miRNAs.

To investigate potential mechanisms by which Du13 might affect miRNA processing, we performed a Y2H assay and determined that Du13 interacted with the key component of the miRNA processing complex, Hyponastic Leaves 1 (OsHYL1, LOC_Os11g01869; Figure S10a). Further BiFC and Co-IP assays are under the way to verify the interaction between Du13 and OsHYL1.

**Discussion**

Du13 is a novel splicing factor affecting amylose biosynthesis in rice endosperm

In this study, we report the isolation and characterization of the novel dull mutant, du13. In developing seeds, du13 accumulated amylose in a temporal pattern similar to that of the wildtype but at a dramatically slower rate (Figure S2d), which correlated with the decrease in seed AC, OsGBSSI abundance, and activity (Figures 1d, 2h, 3c, d). Lower AC in du13 endosperm was apparent at 5 DAF (Figure S2d, e), indicating that Du13 may function from the very early stages of endosperm development, when starch biosynthesis is initiated, in agreement with the high expression of Du13 at this stage (Figure S6b, c).
Therefore, Du13 may have distinct effects on Wxb splicing. In addition, Du13 enhanced the splicing efficiency of Wxb at both cryptic sites, though preferentially at site 1 (Figure 4d), whereas Wxb transcripts spliced at both sites were equally affected in du1 or du2 (Isshiki et al., 2000). Moreover, like du2 (Isshiki et al., 2000), du13 had minimal effects on AC in pollen grains (Figure 3a, b), reflecting its tissue-specific effects.

The splicing of transcripts for genes encoding starch biosynthetic enzymes, such as starch branching enzyme I (BEI), was affected in du13 (Figure 6e; Table S3), in contrast to du1, du2, and du3 (Isshiki et al., 2000; Zeng et al., 2007). Moreover, transcripts of many important splicing factor genes, such as U2 auxiliary factor (U2AF), and some miRNA biogenesis component genes, such as DRB-2 (double-stranded RNA-binding motif containing protein-2), also showed altered AS profiles in du13 (Figure 6e; Table S3). However, we observed no significant changes in BEI abundance and the levels of DRB-2 transcripts (Figures S8c and S9g), which was consistent with a previous study.

**Figure 7** Analysis of expression levels for selected miRNAs, pri-miRNA transcripts, and miRNA target genes in the wild type and du13. (a) qRT-PCR analysis of differentially expressed miRNAs identified from miRNA-seq analysis. U6 snRNA was used as an internal control. The levels of these miRNAs in the wildtype (WT) were set to 1. Values are means ± SD (n = 3). (b) Abundance of pri-miRNAs in developing WT and du13 endosperm at 10 DAF. The arrowhead indicates the specific band. (c) Relative expression levels of the target genes of osa-miR1866-3p (LOC_Os09g21110 and LOC_Os02g44360), osa-miR435 (LOC_Os02g44360), osa-miR444e (LOC_Os02g36924, LOC_Os05g47560, LOC_Os08g06510, LOC_Os07g45120, and LOC_Os09g06970), osa-miR5144-5p (LOC_Os07g37400, LOC_Os05g47560, LOC_Os02g44360, LOC_Os07g06470, and LOC_Os12g40440), and osa-miR5794 (LOC_Os09g06970). Transcript levels in the WT were set to 1. Actin I was used as an internal control in (b) and (c). Values are means ± SD (n = 3).

*P < 0.05, **P < 0.01, as determined by Student’s t-test. LOC_Os09g21110 encodes leucyl-tRNA synthetase; LOC_Os02g44360 encodes scarecrow transcription factor family; LOC_Os02g36924 encodes OsMADS27; LOC_Os05g47560 encodes serine/threonine-protein kinase; LOC_Os08g06510 encodes C3HC4-type zinc-finger protein; LOC_Os07g45120 encodes expressed protein; LOC_Os09g06970 encodes transport protein particle component (TRAPP) domain containing protein; LOC_Os07g23520 encodes expressed protein; LOC_Os07g37400 encodes F-box domain containing protein OsFBX257; LOC_Os05g47560 encodes nuclear transcription factor Y subunit (NF-Y); LOC_Os12g40440 encodes lipoate-protein ligase 8.
(Wang et al., 2019), implicating that the effects of altered splicing on the expression of protein-coding genes might be relatively weak. In summary, we saw more genes with intron retention in the du13 mutant (Figure 5a). The first and last introns appeared to be more sensitive to loss of Du1 activity than internal introns (Figure 6b). In addition, alternative 5’ and 3’ splice site selection by du13 greatly increased within the first intron (Figure 6c, d), suggesting that Du13 might affect splice site choice mostly within the first intron, similar to SE and Arabidopsis CBPs (Raczynska et al., 2009, 2014).

Isshiki et al. (2000) proposed that Du1 (OsCBP20) and Du2 may be essential for recognizing the two weak splice sites activated in Wxα exon 1 and stabilizing the spliceosomes assembled at both sites. Here, our data suggest that Du13 and OsCBC (OsCBP20 and OsCBCPD) may coordinate influence splicing of Wxα intron 1 (Figure 5), although the mechanistic details have not been resolved. In mammals, the CBC is essential for co-transcriptional spliceosome assembly via interaction with the U4/U6.U5 tri-snRNP (small nuclear ribonucleoprotein; Pabis et al., 2013). In Arabidopsis, SE colocalizes and interacts with U1 snRNP auxiliary proteins (Knop et al., 2017). We also determined that Du13 interacted with the splicing factor RSP29 in nuclear speckles (Figure S11d) where splicing factors are enriched (Fang and Spector, 2007). As we detected no interaction between Du1 and Du13 or OsCBC (Figure S10), further investigations into the detailed mechanism of Wxα transcript splicing are still required. Notably, du13 grains displayed a relatively soft and elastic texture compared to the wild-type Koshikihari, an elite japonica variety from Japan (Figure S2g, Table S2). The eating quality of du13 was also better than that of Wuyujing 3, a well-known high-quality rice in China (Table S5). Therefore, the du13 mutant may contribute to the improvement of rice quality.

**Du13 is involved in miRNA biogenesis**

The abundance of several mature miRNAs was reduced in du13 endosperm (Figure 7a; Table S4). Of them, osa-miR444 was reported to be differentially expressed during rice grain filling (Yi et al., 2013). Many miRNAs including osa-miR443 are significantly upregulated from 5 to 7 DAF in rice grains, when a rapid increase in grain weight occurs (Wang et al., 2013; Yi et al., 2013). Moreover, the levels of osa-miR443, osa-miR444e, osa-miR1850.2 and osa-miR1866-3p are higher in superior spikelets compared to inferior spikelets, which show a slower grain-filling rate and a lower grain weight relative to superior spikelets (Peng et al., 2011). Hence, these results indicated the positive regulatory roles played by these miRNAs in grain filling. Many of their predicted target genes encoded transcription factors (Table S4). For instance, osa-miR444 was predicted to target MADS-box genes (Yi et al., 2013) that are considered necessary for rice ovule and seed development (Duan et al., 2005). The MADS gene (LOC_Os02g36924) displayed a higher expression level due to the lower abundance of osa-miR444 in du13 endosperm (Figure 7a, c; Table S4). The constitutive co-expression of the two Arabidopsis MADS-box genes SHATTERPROOF1 (SHP1) and SHP2 resulted in plants with smaller fruits (Liljegren et al., 2000). Hence the higher expression of the MADS gene might contribute to the smaller and lighter grains of du13 (Figure S1j-m; Table S1). Collectively, these results might at least in part explain the slow grain-filling rate and reduced grain weight in the du13 mutant (Figure S1j-m; Table S1).

F-box genes, targeted by osa-miR1850.2, osa-miR1866-3p and osa-miR5144-5p (Table S4), have been shown to regulate grain size and plant architecture, particularly panicle architecture (Chen et al., 2013b; Duan et al., 2012; Li et al., 2011; Piao et al., 2009). Knockdown of the F-box Kelch repeat protein (FBK) gene OsFBK12 produced smaller and lighter grains. OsFBK12 expression levels also influenced plant height and panicle architecture including panicle length (Chen et al., 2013b). Moreover, mutations in the F-box gene LARGER PANICLE (LP) resulted in altered grain size, higher plant height, increased grain number per panicle and decreased tiller number (Li et al., 2011). We noticed altered expression levels for F-box genes (LOC_Os07g37400, LOC_Os07g37400, and LOC_Os07g48250) in du13 (Figure 7c; Table S4), which might contribute to the pleiotropic phenotypes seen in du13 plants, including reduced grain size and increased plant height (Figure S1a, j–l; Table S1). Additionally, osa-miR5144-5p is predicted to target transcripts for a nuclear transcription factor Y gene (NF-Y, LOC_Os07g06470), while both osa-miR444e and osa-miR5794 target a gene encoding a transport protein particle component (TRAPP, LOC_Os09g06970; Figure 7c; Table S4), which are required for shoot growth (Garcia et al., 2020; Sun et al., 2016). The higher expression of NF-Y and TRAPP in du13 might contribute to the increased plant height of du13 (Figure S1a, Table S1). Therefore, Du13 is required for the accumulation of important miRNAs critical to rice plant and grain development. We noted here that Du13 interacted with OsHYL1, a key component of the miRNA processing complex (Figure S10a), which might affect the cleavage of pri-miRNAs (Dong et al., 2008).

Previous studies have shown that many mutants of genes required for pre-mRNA splicing also exhibit defects in miRNA production (Chaabane et al., 2013; Kim et al., 2008; Laubinger et al., 2008). Indeed, pre-mRNA splicing and miRNA processing share many factors, including SE, CBPs, Stabilized 1 (STA1) and the Modifier of snc1,4 (MOS4)-associated complex (MAC; Chaabane et al., 2013; Jia et al., 2017; Kim et al., 2008; Laubinger et al., 2008; Li et al., 2018a; Zhang et al., 2013, 2014b). Moreover, important factors linked to spliceosomal function also appear to regulate both alternative pre-mRNA splicing and miRNA biogenesis, including SE, CBPs, STA1, MAC, Glycine-rich RNA-binding Protein 7 (ATGRP7), Increased Level of Polypeptide-1D (LP1), NTC-Related Protein 1 (NTR1), The Complex Subunit2 (THOC2), Sickle (SIC), Small1 (SMA1), High Osmotic Stress Gene Expression 5 (HOSS), FIERY2/RNAP II CTD phosphatase-like 1 (CPL1), RS40 and RS41 (Chaabane et al., 2013; Chen et al., 2013a, 2015; Francisco-Mangili et al., 2015; Jia et al., 2017; Kim et al., 2008; Köster et al., 2014; Laubinger et al., 2008; Li et al., 2018a, 2018b; Manavella et al., 2012; Wang et al., 2019; Zhan et al., 2012; Zhang et al., 2013, 2014b). The significance of direct communication between the spliceosome and the miRNA biogenesis machinery in miRNA production has also been verified (Bielewicz et al., 2013; Knop et al., 2017; Schwab et al., 2013; Yan et al., 2012). Our results showed that Du13 also plays dual roles in alternative pre-mRNA splicing and miRNA biogenesis, confirming an evolutionarily conserved link between pre-mRNA splicing and microRNA biogenesis in land plants. Notably, Du1 is homologous to the pre-mRNA splicing factor STA1 (Chaabane et al., 2013; Zeng et al., 2007). It will thus be interesting to examine the abundance of miRNAs in the du1, du2 and du3 mutants.

**The complicated function of Du13 in rice**

SE homologs are highly conserved in yeast, plants, and animals (Wilson et al., 2008). They may have arisen early in evolution and...
served a basic cellular function. Null alleles of SE homologs confer embryonic lethality in Arabidopsis, fission yeast (Schizosaccharomyces pombe), fruit fly (Drosophila melanogaster), zebrafish (Danio rerio) and mouse (Mus musculus; Amsterdam et al., 2004; Golling et al., 2002; Kim et al., 2010; Lobbes et al., 2006; Oh et al., 2003; Wilson et al., 2008). Most eukaryotic genomes encode a single SE homolog, whereas monocot genomes harbour multiple SE homologs (Figure 5A). As ZOS2-03 interacted with Du13 and OsCBC, ZOS2-03 might play a role similar to that of Du13 (Figures 5b and S13).

Like SE, Du13 may exert dual roles in pre-mRNA splicing and pri-miRNA processing in rice (Figure 8; Laubinger et al., 2008). Increasing evidence also suggests that SE is a multifunctional protein, playing an important role in epigenetic regulation of pri-miRNA processing in rice (Figure 8; Laubinger et al., 2008). Most eukaryotic genomes encode a single SE homolog, whereas monocot genomes harbour multiple SE homologs (Figure 5A). As ZOS2-03 interacted with Du13 and OsCBC, ZOS2-03 might play a role similar to that of Du13 (Figures 5b and S13).

**Experimental procedures**

**Plant materials**

The du13 mutant was identified from an N-methyl-N-nitrosourea-induced M2 population of the japonica rice cv. Koshihikari. du13 was backcrossed to Koshihikari for genetic analysis. An F2 population was derived from a cross between du13 and the indica cv. Nanjing 11 for mapping. All plants were grown in an experimental field plot at Nanjing Agricultural University under natural conditions.

**Microscopy**

Scanning electron microscopy (SEM) was performed according to a previous report (Kang et al., 2005). Samples were examined using a Hitachi S-3400N scanning electron microscope. For the observation of compound starch granules, semi-thin sections (1 μm) were prepared as previously described (Peng et al., 2014). Semi-thin sections were stained with I2-KI and examined with a Nikon ECLIPSE®80i light microscope.

**Physicochemical properties of rice grains**

Mature rice grains were dehulled and ground into fine flour. The contents of amylase, lipid, total starch and protein, together with amylpectin chain-length distribution, were measured as previously described (Han et al., 2012). The starch pasting properties were determined as previously (Peng et al., 2014). Comprehensive taste evaluations of cooked rice were performed as previously described (Champagne et al., 1999).

**Map-based cloning of the du13 mutation**

A total of 695 individuals with dull endosperm were selected from the F2 mapping population. Over 180 genome-wide polymorphic SSR markers were used in preliminary mapping. For the fine-mapping of Du13, new genetic markers were developed by comparing the genomic sequences of japonica cv. Nipponbare and indica cv. 93-11 (Table S6).

**Generation of transgenic plants**

To complement the du13 mutant, the wild-type Du13 cDNA sequence was inserted into the binary vector pCUbi1390 (Du13 is driven by its native promoter). A CRISPR-Cas9 construct expressing a single guide RNA (sgRNA) targeting exon 8 of Du13 was assembled to generate knock-out mutants in Du13. The two constructs were individually introduced into du13, Nipponbare or Ningjing 7 calli by Agrobacterium (Agrobacterium tumefaciens)-mediated transformation as described previously (Hiei et al., 1994).}

**Protein extraction and immunoblotting**

Total protein extraction and immunoblot analysis were performed as previously described (Crofts et al., 2015; Wang et al., 2010). Polyclonal antibodies against Du13 and starch biosynthetic enzymes were produced in rabbits by Yingji Biotech (http://www.immunogenen.com.cn/). Anti-HSPB2 antibodies were purchased from Beijing Protein Innovation (AbM51099-31-PU).

**Phylogenetic analysis**

The neighbour-joining tree was generated using MEGA 5.0 software (http://www.megasoftware.net) by the bootstrap method (1000 replicates; Tamura et al., 2011). The amino acid sequences of Du13 homologs were retrieved on the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and aligned using ClustalX (http://www.clustal.org).

**RNA extraction, RT-PCR, and qRT-PCR analysis**

Total RNA was extracted using an RNasep pure plant kit (Tiangen Co., Beijing, China). One microgram of total RNA was then reverse transcribed in a 20-μL reaction using PrimeScript II Reverse Transcriptase (TaKaRa) with oligo(dT)18 primers. Levels of pri-miRNAs were measured according to a previously described method (Juarez et al., 2004; Yang et al., 2006). qRT-PCR was performed with the SYBR Premix Ex Taq™ Kit (TaKaRa). Actin I was used as an internal control. Primers used are listed in Table S6.
Subcellular localization

The Du13 cDNA was cloned in-frame and upstream of GFP in the pAN580-GFP vector (Peng et al., 2014). The resulting construct was transfected into rice protoplasts according to previously described protocols (Chen et al., 2006). du13, ZOS2-03, ZOS8-11, OsCBP20, OsCBP80, and RSp29 cDNAs were similarly cloned into the binary vector, pCAMBIA1305-GFP (driven by the CaMV 35S promoter), before transient infiltration in N. benthamiana leaves (Waadt and Kudla, 2008). Confocal imaging analysis was performed using a Zeiss LSM710 confocal microscope.

Enzyme activity assays

Developing endosperm collected at 10 days after fertilization (DAF) was homogenized in 10-fold volume of a solution consisting of 50 mM HEPES-NaOH (pH 7.4), 50 mM 2-mercaptoethanol, 2 mM MgCl₂, and 12.5% (v/v) glycerol (Nishi et al., 2001). After centrifugation, the supernatant was used to assay the activity of soluble starch synthases, while the pellet was resuspended in 1 mL of the above solution for GBSSI activity assay (Nakamura et al., 1989).

Y2H and transcriptional activity assays

Full-length Du13 and ZOS2-03 cDNAs were cloned into both pGBKTK7 and pGADT7 vectors. Du1 and truncated Du13 cDNAs were cloned into the pGBKTK7 vector, and OsCBP20, OsCBP80, HYL1, ZOS8-11, RSp29, RSp23, and two homologs of RSp2p23 (RSp2p21a and RSp2p21b) cDNAs were cloned into the pGADT7 vector. Yeast strain AH109 was co-transformed with various combinations of plasmids, according to the manufacturer’s instructions (Clontech, Mountain View, CA). Interactions were assayed using synthetic define (SD) – Leu–Trp–His–Ade medium. The BD-Du13 construct was transformed into AH109 and transcriptional activity was assayed on SD – Trp–His–Ade medium.

Transient assays using rice protoplasts

Du13, du13, and ZOS2-03 cDNAs were cloned into the pAN580-GFP vector (with its GFP fragment removed) to produce constructs driven by the CaMV 35S promoter. These plasmids were mixed with rice protoplasts together with a Wx²-gus plasmid (Isshiki et al., 2006). The Wx²-gus plasmid was also transfected into wild-type and du13 protoplasts. GUS activity and RT-PCR analyses were performed as previously described (Isshiki et al., 2006).

BiFC assay

Full-length Du13, OsCBP20, and ZOS2-03 cDNAs were introduced into both the p2YN and p2YC (encoding each half of eYFP) vectors (Zheng et al., 2015). Du1 and Rsp29 were cloned into the p2YN vector, and OsCBP80 was cloned into the p2YC vector. BiFC assays were conducted in N. benthamiana leaf epidermal cells, as described previously (Peng et al., 2014).

Co-IP assay

Coding sequences of Du13 and ZOS2-03 were introduced into the binary vector pCAMBIA1300-221-Flag and transformed into Agrobacterium strain EHA105. The plasmid for the subcellular localization of ZOS2-03 and plasmids for BiFC assays of OsCBP20 and OsCBP80 (as HA fusions), were also used in the Co-IP assay. N. benthamiana leaves were co-infiltrated with different combinations of plasmids and collected at 48 h after infiltration (Qiao et al., 2017). Anti-GFP antibodies (Medical Biological Laboratories (MBL, M048-3), anti-HA antibodies (MBL, M180-7), anti-Flag antibodies (Sigma-Aldrich, AB592), and anti-Flag agarose (MBL, M185-10) were used in Co-IP experiments.

mRNA-seq and miRNA-seq

Total RNA was extracted from immature rice grains at 10 DAF removed of the hull, pericarp, and embryo. Illumina sequencing libraries for mRNA-seq and miRNA-seq were prepared following the manufacturer’s instructions and then sequenced on a HiSeq platform. The raw reads were processed with Hisat2 and StringTie, followed by the software rMATS (Shen et al., 2014) to classify alternative splicing profiles in each sample. For miRNA-seq, DESeq was employed to identify differentially expressed miRNAs. To validate differentially expressed miRNAs obtained from miRNA-seq, miRNA extraction, RT-PCR, and qRT-PCR analyses were performed according to the manufacturer’s instructions (Tiangen Biotech, Beijing, China). U6 snRNA was used as an internal reference.

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Conflict of interests

The authors declare no competing interests.

Author contributions

YC, WWZ and JM were designed the experiments. WWC, XGC, JM, and JHX constructed some vectors. WW, XGC, JM, and XGC performed the experiments. XMY, JC, XL and JHX constructed some vectors. YQ, LJ, HYW and JMW directed the project. YC, WWZ, YSF, and ZZS contributed equally to this work.

References

Aluko, G., Martinez, C., Tohme, J., Castano, C., Bergman, C. and Oard, J.H. (2004) QTL mapping of grain quality traits from the interspecific cross Oryza sativa x O. glaberrima. Theoret. Appl. Genet. 109, 630–639.

Amsterdam, A., Nissen, R.M., Sun, Z., Swindell, E.C., Farrington, S. and Hopkins, N. (2004) Identification of 315 genes essential for early zebrafish development. Proc. Natl Acad. Sci. USA, 101(35), 12792–12797.

Bielewicz, D., Kalak, M., Kalyna, M., Windels, D., Barta, A., Vasquez, F., Szweykowska-Kulinska, Z. et al. (2013) Intronals of plant pri-miRNAs enhance miRNA biogenesis. EMBO Rep. 14, 622–628.

Cai, X.L., Wang, Z.Y., Xing, Y.Y., Zhang, J.L. and Hong, M.M. (1998) Aberrant splicing of intron 1 leads to the heterogeneous 5′ UTR and decreased expression of waxy gene in rice cultivars of intermediate amylase content. Plant J. 14, 459–465.
Duan, Y.L., Li, S.P., Chen, Z.W., Zheng, L.L., Diao, Z.J., Zhou, Y.C., Lan, T., Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994) Efficient transformation of Arabidopsis cells, Plant Mol. Biol. 25, 483–487.

Ishiki, M., Matsuda, Y., Takasaki, A., Wong, H.L., Satoh, H. and Shimamoto, K. (2008) Du3, a mRNA cap-binding protein, regulates amylase content in japonica rice seeds. Plant Biotechnol. 25, 483–487.

Ishiki, M., Morino, K., Nakajima, M., Okagaki, R.J., Wessler, S.R., Iwaza, T. and Shimamoto, K. (1998) A naturally occurring functional allele of the rice waxy locus has a GT to TT mutation at the 5’ splice site of the first intron. Plant J. 15, 133–138.

Ishiki, M., Nakajima, M., Satoh, H. and Shimamoto, K. (2000) du3 rice mutants with tissue-specific effects on the splicing of the waxy pre-mRNA. Plant J. 23, 451–460.

Ishiki, M., Tsumoto, A. and Shimamoto, K. (2006) The Serine/Arginine-rich protein family in rice plays important roles in constitutive and alternative splicing of pre-mRNA. Plant Cell, 18, 146–158.

Jia, T.R., Zhang, B.L., You, C.J., Zhang, Y., Zeng, L.P., Li, S.J., Johnson, K.C.M. et al. (2017) The Arabidopsis MOS14-associated complex promotes microRNA biogenesis and precursor messenger RNA splicing. Plant Cell, 29, 2626–2634.}

Juez, M.T., Kui, J.S., Thomas, J., Heller, B.A. and Timmermans, M.C.P. (2004) microRNA-mediated repression of rolled leaf specifies maize leaf polarity. Nature, 428, 84–88.

Kang, H.G., Park, S., Matsuoka, M. and An, G. (2005) White-core endosperm floury endosperm-4 in rice is generated by knockout mutations in the C4-type pyruvate orthophosphate dikinase gene (OsPDK8). Plant J. 42, 901–911.

Kausik, R.P. and Khush, G.S. (1991) Endosperm mutants in rice: gene expression in japonica and indica backgrounds. Cereal Chem. 68, 487–491.

Kierzkowski, D., Kmečiak, M., Pontek, P., Wotajszek, P., Szwewowska-Kulinska, Z. and Jarmolowski, A. (2009) The Arabidopsis CBP20 targets the cap-binding complex to the nucleus, and is stabilized by CBP80. Plant J. 59, 813–823.

Kim, D.U., Hayles, J., Kim, D., Wood, V., Park, H.O., Won, M., Yoo, H.S. et al. (2010) Analysis of a genome-wide set of gene deletions in the fission yeast Schizosaccharomyces pombe. Nat. Biotechnol. 28, 617–623.

Kim, S., Yang, J.Y., Xu, J., Jiang, I.C., Prigge, M.J. and Chua, N.H. (2008) Two cap-binding proteins CBP20 and CBP80 are involved in processing primary microRNAs. Plant Cell Physiol. 49, 1634–1644.

Kiswara, G., Lee, J.H., Hur, Y.J., Cho, J.H., Lee, J.Y., Kim, S.Y., Sohn, Y.B. et al. (2014) Genetic analysis and molecular mapping of low amylose gene du12(1) in rice (Oryza sativa L.). Theor. Appl. Genet. 127, 51–57.

Knoop, K., Stepien, A., Barciszewska-Paick, M., Taube, M., Bielewicz, D., Michalak, M., Borst, J.W. et al. (2017) Active 5’ splice sites regulate the biogenesis efficiency of Arabidopsis microRNAs derived from intron-containing genes. Nucleic Acids Res. 45, 2757–2775.

Köster, T., Meyer, K., Weinholdt, C., Smith, L.M., Lummerm, M., Speth, C., Grosse, I. et al. (2014) Regulation of pri-miRNA processing by the hnrNPR-like protein ATGPR7 in Arabidopsis. Nucleic Acids Res. 42, 9925–9936.

Laubinger, S., Sachsenberg, T., Zeller, G., Busch, W., Lohmann, J.U., Rätsh, G. and Weigel, D. (2008) Dual roles of the nuclear cap binding complex and SERRATE in pre-mRNA splicing and microRNA processing in Arabidopsis thaliana. Proc. Natl Acad. Sci. USA, 105, 8795–8800.

Li, M., Tang, D., Wang, K.J., Wu, X.R., Lu, L.L., Yu, H.X., Gu, M.H. et al. (2011) Mutations in the F-box gene DU13 improve the panicle architecture and enhance the grain yield in rice. Plant Biotechnol. J. 9, 1002–1013.

Li, J.S., Liu, K., Zhou, B.J., Li, M., Zhang, S.X., Zeng, L.R., Zhang, C. et al. (2018a) Mac3a and Mac3b, two core subunits of the MOS14-associated complex, positively influence miRNA biogenesis. Plant Cell, 30, 481–494.

Li, J.S., Xu, R., Li, A.X., Liu, K., Gu, L.Q., Li, M., Zhang, H.R. et al. (2018b) SMA1, a homolog of the splicing factor Prp28b, has a multifaceted role in miRNA biogenesis in Arabidopsis. Nucleic Acids Res. 46, 9148–9159.

Lijegren, S.J., Ditta, G.S., Eshed, Y., Savidge, B., Bowman, J.L. and Yanofsky, M.F. (2000) SHATTERPROOF MADS-box genes control seed development in Arabidopsis. Nature, 404, 766–770.

Liu, L.L., Ma, X.D., Liu, S.J., Zhu, C.L., Jiang, L., Wang, Y.H., Shen, Y. et al. (2009) Identification and characterization of a novel Waxy allele from a Yunnan rice landrace. Plant Mol. Biol. 71, 609–626.
Lobbes, D., Rallapalli, G., Schmidt, D.D., Martin, C. and Clarke, J. (2006) SERRATE: a new player on the plant microRNA scene. EMBO Rep. 7, 1052–1058.

Ma, Z., Castillo-González, C., Wang, Z., Sun, D., Hu, X., Shen, X., Potok, M.E. et al. (2018) Arabidopsis srrate coordinates histone methyltransferases ATXR5/6 and RNA processing factor RDR8 to regulate transposon expression. Dev. Cell, 45, 769–784.

Manavella, P.A., Hagmann, J., Ott, F., Laubinger, S., Franz, M., Macek, B. and Weigel, D. (2012) Fast-forward genetics identifies plant CpL phosphatases as regulators of miRNA processing factor HYL1. Cell, 151, 859–870.

Nakamura, Y., Yuki, K., Park, S.Y. and Ohya, T. (1989) Carbohydrate metabolism in the developing endosperm of rice grains. Plant Cell Physiol. 30, 833–839.

Nishi, A., Nakamura, Y., Tanaka, N. and Satoh, H. (2001) Biochemical and molecular cloning of a rice nuclear regulator in primary microRNA processing factor HYL1. J. Mol. Biol. 310, 841–850.

Oh, S.W., Kingley, T., Shin, H.H., Zheng, Z., Chen, H.W., Chen, X., Wang, H. et al. (2003) A P-element insertion screen identified mutations in 455 novel essential genes in Drosophila. Genetics, 163, 195–201.

Pabis, M., Neufeld, N., Steiner, M.C., Bajtk, J., Shav-Tal, Y. and Neugebauer, K.M. (2011) The nuclear cap-binding complex interacts with the UAFS-us tri-snRNP and promotes spliceosome assembly in mammalian cells. RNA, 19, 1054–1063.

Peng, C., Wang, Y.H., Liu, F., Ren, Y.L., Zhou, K.N., Lv, J., Zheng, M. et al. (2014) FLOWERY ENDOSPERM6 encodes a CBM48 domain-containing protein involved in compound granule formation and starch synthesis in rice endosperm. Plant J. 77, 917–930.

Peng, T., Lv, Q., Zhang, J., Li, J.Z., Du, Y.X. and Zhao, Q.Z. (2011) Differential expression of the microRNAs in superior and inferior spikelets in rice (Oryza sativa). J. Exp. Bot. 62, 4943–4954.

Piao, R., Jiang, W., Ham, T.H., Choi, M.S., Qiao, Y., Chu, S.H., Park, J.H. et al. (2009) Map-based cloning of the ERECT PANICLE 3 gene in rice. Theor. Appl. Genet. 119, 1497–1506.

Qiao, S.L., Sun, S.Y., Wang, L.L., Wu, Z.H., Li, C.X., Li, X.M., Wang, T. et al. (2017) The RLAI1/MOS1 transcription factor functions with OsDZ1R1 to regulate brassinosteroid signaling and rice architecture. Plant Cell, 29, 292–309.

Raczynska, K.D., Simpson, C.G., Ciesiolka, A., Szewc, L., Lewandowska, D., McNicol, J., Szewkowska-Kulinska, O. et al. (2009) Involvement of the nuclear cap-binding protein complex in alternative splicing in Arabidopsis thaliana. Nucleic Acids Res. 38, 265–278.

Raczynska, K.D., Stepień, A., Kierekowski, D., Kalak, M., Bączczyk, M., McNicol, J., Simpson, C.G. et al. (2014) The SERRATE protein is involved in alternative splicing in Arabidopsis thaliana. Nucleic Acids Res. 42, 1224–1234.

Schwab, R., Speth, C., Laubinger, S. and Voinnet, O. (2013) Enhanced microRNA accumulation through stem-looop-adjoinnt introns. EMBO Rep. 14, 615–621.

Shen, S., Park, J.W., Lu, Z.X., Lin, L., Henry, M.D., Wu, Y.N., Zhou, Q. et al. (2014) MATS: robust and flexible detection of differential alternative splicing from replicate RNA-Seq data. Proc. Natl Acad. Sci. USA, 111, E5593-E5601.

Sun, X.D., Ren, Y.Q., Zhang, X.Z., Lian, H.F., Zhou, S.M. and Liu, S.Q. (2016) Overexpression of a garlic nuclear factor Y (NF-Y) B gene, AsNF-YB3, affects seed germination and plant growth in transgenic tobacco. Plant Cell, Tissue Organ Cult. 127, 513–523.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

Waadt, R. and Kudla, J. (2008) In planta visualization of protein interactions using bimolecular fluorescence complementation (BiFC). Cold Spring Harbor Protocols, 2008(6), pdb.prot4995.

Wang, J.L., Chen, S.S., Jiang, N., Lu, N., Wang, X.Y., Li, Z.P., Li, X. et al. (2011) Spliceosome disassembly factors L1P1 and NTR1 promote miRNA biogenesis in Arabidopsis thaliana. Nucleic Acids Res. 40, 7886–7900.

Wang, J.C., Xu, H., Zhu, Y., Liu, Q.Q. and Cai, X.L. (2013) OsDZIP5B, a basic leucine zipper transcription factor, regulates starch biosynthesis in rice endosperm. J. Exp. Bot. 64, 3453–3466.

Wang, Y.H., Ren, Y.L., Liu, X., Jiang, L., Chen, L.M., Han, X.H., Jin, M.N. et al. (2010) OsDub1a regulates endomembrane organization and storage protein trafficking in rice endosperm cells. Plant J. 64, 812–824.

Wang, Z.Y., Ma, Z.Y., Castillo-González, C., Sun, D., Li, Y.J., Yu, B., Zhao, B.Y. et al. (2018) SWI2/SNF2 ATPase CHR2 remodels pm-miRNAs via Serrate to impede miRNA production. Nature, 557, 516–521.

Wang, Z.Y., Wu, Z.L., Xing, Y.Y., Zheng, F.G., Guo, X.L., Zhang, W.G. and Hong, M.M. (1990) Nucleotide sequence of rice waxy gene. Nucleic Acids Res. 18, 5898.

Wills, M.D., Wang, D., Wagner, R., Breysse, H., Gerstein, M., Lobe, C., Lu, X. et al. (2008) ARF5 is a conserved eukaryotic gene essential for early mammalian development. Mol. Cell Biol. 28, 1503–1514.

Yan, K., Liu, P., Wu, C.A., Yang, G.D., Xu, R., Guo, Q.H., Huang, J.G. et al. (2012) Stress-induced alternative splicing provides a mechanism for the regulation of microRNA processing in Arabidopsis thaliana. Mol. Cell, 48, 521–531.

Yang, L., Liu, Z., Lu, F., Dong, A. and Huang, H. (2008) SERRATE is a novel nuclear regulator in primary microRNA processing in Arabidopsis. Plant J. 47, 841–850.

Yu, R., Zhu, Z.X., Hu, J.H., Qian, Q., Dai, J.C. and Ding, Y. (2013) Identification and expression analysis of microRNAs at the grain filling stage in rice (Oryza sativa L.) via deep sequencing. PLoS One, 8, e57683.

Zeng, D.L., Yan, M.X., Wang, Y.H., Liu, X.F., Qian, Q. and Li, J.Y. (2007) DU1, encoding a novel Prp1 protein, regulates starch biosynthesis through affecting the splicing of Wx® pre-miRNAs in rice (Oryza sativa L.). Plant Mol. Biol. 65, 501–509.

Zhan, Q.X., Wang, B.S., Li, H.J., Liu, R.Y., Kalia, R.K., Zhu, J.K. and Chinnusamy, V. (2012) Arabidopsis prolinc-rich protein important for development and abiotic stress tolerance is involved in microRNA biogenesis. Proc. Natl Acad. Sci. USA, 109, 18198–18203.

Zhang, H., Duan, L., Dai, J.S., Zhang, C.Q., Li, J., Gu, M.H., Liu, Q.Q. et al. (2014a) Major QTLs reduce the deleterious effects of high temperature on rice amylose content by increasing splicing efficiency of Wx pre-miRNA. Theor. Appl. Genet. 127, 273–282.

Zhang, S.X., Liu, Y.H. and Yu, B. (2014b) PRL1, an RNA-binding protein, positively regulates the accumulation of miRNAs and siRNAs in Arabidopsis. PLoS Genet. 10, e1004841.

Zhang, S.X., Xie, M., Ren, G.D. and Yu, B. (2013) CDC5, a DNA binding protein, positively regulates posttranscriptional processing and/or transcription of primary microRNA transcripts. Proc. Natl Acad. Sci. USA, 110(43), 17588–17593.

Zhang, M., Wang, Y.H., Wang, Y.L., Wang, C.M., Ren, Y.L., Li, J., Peng, C. et al. (2015) DEFORMED FLORAL ORGANI (DFO1) regulates floral organ identity by epigenetically repressing the expression of OsMAD558 in rice (Oryza sativa). New Phytol. 206, 1476–1490.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.
Figure S9 Relative expression levels of OsCBP20 and OsCBP80 and interaction between OsCBP20 and OsCBP80.

Figure S10 Du1 does not interact with Du13 or OsCBC.

Figure S11 Interactions among Du13 homologs and SR proteins.

Figure S12 Expression analysis of selected miRNAs, pri-miRNA transcripts, and miRNA target genes in the Y6 mutant.

Figure S13 ZOS2-03 interacts with Du13 and OsCBC.

Table S1 Agronomic traits of the wild type (WT) and du13.

Table S2 Pasting properties of wild-type (WT) and du13 endosperm starch.

Table S3 Changes in alternative splicing events in the du13 mutant.

Table S4 miRNAs of reduced abundance and their target genes of altered expression in du13 by miRNA-seq analysis.

Table S5 Eating quality of Wuyujing 3 and du13 grains.

Table S6 Primers used in this study.