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Natural alleles of a uridine 5′-diphospho-glucosyltransferase gene responsible for differential endosperm development between upland rice and paddy rice

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ABSTRACT

Traditional upland rice generally exhibits insufficient grains resulting from abnormal endosperm development compared to paddy rice. However, the underlying molecular mechanism of this trait is poorly understood. Here, we cloned the uridine 5′-diphospho (UDP)-glucosyltransferase gene *EDR1* (*Endosperm Development in Rice*) responsible for differential endosperm development between upland rice and paddy rice by performing quantitative trait loci analysis and map-based cloning. *EDR1* was highly expressed in developing seeds during grain filling. Natural variations in *EDR1* significantly reduced the UDP-glucosyltransferase activity of EDR1^{YZN} compared to EDR1^{YD1}, resulting in abnormal endosperm development in the near-isogenic line, accompanied by insufficient grains and changes in grain quality. By analyzing the distribution of the two alleles EDR1^{YD1} and EDR1^{YZN} among diverse paddy rice and upland rice varieties, we discovered that EDR1 was conserved in upland rice, but segregated in paddy rice. Further analyses of grain chalkiness in the alleles of EDR1^{YD1} and EDR1^{YZN} varieties indicated that rice varieties harboring EDR1^{YZN} and EDR1^{YD1} preferentially showed high chalkiness, and low chalkiness, respectively. Taken together, these results suggest that the UDP-glucosyltransferase gene *EDR1* is an important determinant controlling differential endosperm development between upland rice and paddy rice.

Keywords: *EDR1*, endosperm development, grain quality, UDP-glucosyltransferase, upland rice

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INTRODUCTION

Rice is one of the most important crops worldwide, and half of the world's population regards it as a major staple every day (Kusano et al., 2012; Zhang et al., 2021). As the human population continues to grow, crop production in 2050 will need to double (Godfray et al., 2010; Tilman et al., 2011; Ray et al., 2013). Therefore, continuously improving rice yield is necessary. Since irrigatable cropland area is limited, improving paddy rice yield seems to be more difficult, and popularizing upland rice cultivation may be a more effective way to improve global rice yield (Belder et al., 2007; Zhao et al., 2010).

Upland rice is an ecotype of cultivated rice and is predominantly cultivated in Asia, Africa, and Latin America (Garrity et al., 1992; Kikuta et al., 2016; Cavite et al., 2020). The cultivation area of upland rice is approximately 4% of global rice. Compared to paddy rice, upland rice can be cultivated not only in paddy fields but also on slopes, land terraces, or tropical to temperate uplands with less rain, areas in which paddy rice cannot survive (Kikuta et al., 2016). As natural rainfall can meet the water demand for upland rice growth, no additional manual irrigation is required. The water requirement of upland rice across the whole life cycle is only 20% of that of paddy rice (Zhang et al., 2020). On the other hand, upland rice shows higher tolerance to biotic and abiotic stresses than paddy rice (Narenoot et al., 2017; Phapumma et al., 2020). Although upland rice has many advantages, upland rice exhibits abnormal endosperm development and causes a sharp decrease in yield, and poor rice quality has become a problem for traditional upland rice (Fageria et al., 1982; Cavite et al., 2020).

Due to severe water scarcity in rice cultivation areas, upland rice has formed many drought-resistance mechanisms during evolutionary processes. The drought-resistance mechanisms of upland rice are mainly realized through the morphological adaptation of leaves and roots. Compared with paddy rice, leaves of upland rice have a stronger water retention capacity, leaf cells are smaller, and their stomatal resistance is greater (Cutler et al., 1980). On the other hand, the root depth of upland rice can even reach the 70–80 cm soil layer, which is conducive to optimizing upland rice water use efficiency under drought stress, while that of paddy rice is generally distributed in the 15 cm soil layer (Kang et al., 1994; Mishra et al., 1997; Araki et al., 2000). It was previously reported that upland rice has a faster grain-filling rate than paddy rice, and 60% of its fixed carbon was allocated for root growth, which is another mechanism by which upland rice escapes drought stress (Yang, 2002). Abnormal endosperm development in upland rice may also be a manifestation of upland rice adaptation to drought stress.

Endosperm development is a complex process regulated by intrinsic and environmental factors. The endosperm of rice is derived from fertilized central cells following double fertilization (Sabelli and Larkins, 2009; Wu et al., 2019). After cellularization of the endosperm, as well as differentiation and maturation, the inner starchy endosperm and an outer aleurone layer are subsequently formed (Krishnan and Dayanandan, 2003; Wu et al., 2016). Moreover, sugars from maternal tissues are transported to the endosperm for starch biosynthesis and accumulation, and a series of enzymatic reactions are involved in these processes (Weschke et al., 2000; Patrick and Offler, 2001; James et al., 2003; Yu et al., 2021). Many genes encoding starch biosynthesis enzymes in rice influence endosperm development, and mutations in these genes can lead to abnormal endosperm development (James et al., 2003). In addition to the genes encoding starch biosynthesis enzymes, many other regulatory factors involved in endosperm development have been reported, such as the pentatricopeptide repeat (PPR) protein FLO10 (Wu et al., 2019), tetratricopeptide repeat (TPR) protein FLO2 (She et al., 2010), starch binding protein FLO6 (Peng et al., 2014), cell-wall invertase GIF1 (Wang et al., 2008), transcription factors OsMADS29, OsbZIP58 and OsNF-YB1 (Yin and Xue, 2012; Wang et al., 2013; Bai, 2016; Xu et al., 2016), and sugar transporters SWEET11 and SWEET15 (Ma et al., 2017; Yang et al., 2018). Although many studies have been conducted on rice endosperm development, little is known about the genes responsible for this trait in upland rice.

Glycosylation is an important defense mechanism for abiotic and biotic stresses in plants, and uridine 5′-diphospho (UDP)-glucosyltransferase is a critical enzyme in glycosylation (Michlmayr et al., 2015). Many genes have been reported to be involved in regulating abiotic and biotic stress tolerance in plants. In *Arabidopsis*, the UDP-glucosyltransferase UGT74E2 is involved in regulating *Arabidopsis* water stress tolerance by perturbing indole-3-butyric acid homeostasis (Tognetti et al., 2010). On the other hand, UGT84A2 from *Arabidopsis* and UGT13248 from barley confer resistance to *Phakopsora pachyrhizi* and *Mycotoxin deoxynivalenol*, respectively (Sanghyun et al., 2012; Langenbach et al., 2013). However, according to the latest reports, the UDP-glucosyltransferase gene *GSA1* not only regulates rice abiotic stress resistance but also plays an important role in controlling grain size in rice (Dong et al., 2020). These results indicate that UDP-glucosyltransferase genes may be involved in regulating endosperm development in rice.

In this study, we identified and cloned a UDP-glucosyltransferase gene, *EDR1*, which was responsible for differential endosperm development between upland rice and paddy rice. The natural allele of *EDR1* from the upland rice Yanzhinuo (YZN) caused abnormal endosperm development in near-isogenic line (NIL)-*qEDR5.1*^{YZN} grains, as evidenced by insufficient grains. Grain quality analysis showed a significant increase in grain chalkiness but a decrease in the transparency and protein content of NIL-*qEDR5.1*^{YZN} seeds. These results indicated that *EDR1* plays an important role in regulating endosperm development, thereby influencing grain quality in rice.

RESULTS

Construction and phenotypic analysis of introgression lines for endosperm development

Yanzhinuo, a landrace upland rice variety, exhibits insufficient grains and poor grain quality resulting from abnormal endosperm development. Yundao1 (YD1) is a paddy rice variety with normal endosperm. To identify quantitative trait loci (QTL) influencing endosperm development in rice, we constructed a set of introgression lines (ILs, BC2F8) with YZN as the donor parent and YD1 as the recurrent parent. IL J77 showed abnormal plumpness of the endosperm compared with YD1. By determining grain size, we found that J77 exhibited a significant decrease in 1,000-grain weight, grain width, grain thickness, and grain length compared with YD1 (Figure 1A–E). On the other hand, J77 showed an increase in grain chalkiness compared with YD1 (Figure 1A). In the following study, we used the 1,000-grain weight and grain chalkiness to represent the endosperm development state of rice.

Identification of a QTL for endosperm development in rice

By backcrossing J77 and YD1, we obtained a BC_3F_2 mapping population of 472 individuals. The 1 000-grain weight of the 472 F_2 plants varied from 18.36 to 32.30 g and showed a normal distribution but multiple peaks, suggesting that the endosperm development state was a quantitative trait that was regulated by multiple genes (Figure 2). Using over 500 published simple sequence repeat (SSR) markers covering the whole genome, a major QTL on chromosome 5 was identified, named *qEDR5.1* (*Endosperm Development in Rice*), in the interval between two molecular markers, RM18829 and RM19121, and explained 31.2% of the phenotypic variation for the 1,000-grain weight (Table 1). Next, the NIL for *qEDR5.1*, NIL-*qEDR5.1*^{YZN}, which harbored a YZN-derived *qEDR5.1* allele in the YD1 genetic background, was constructed to further investigate the effects of the *qEDR5.1* locus on endosperm development.

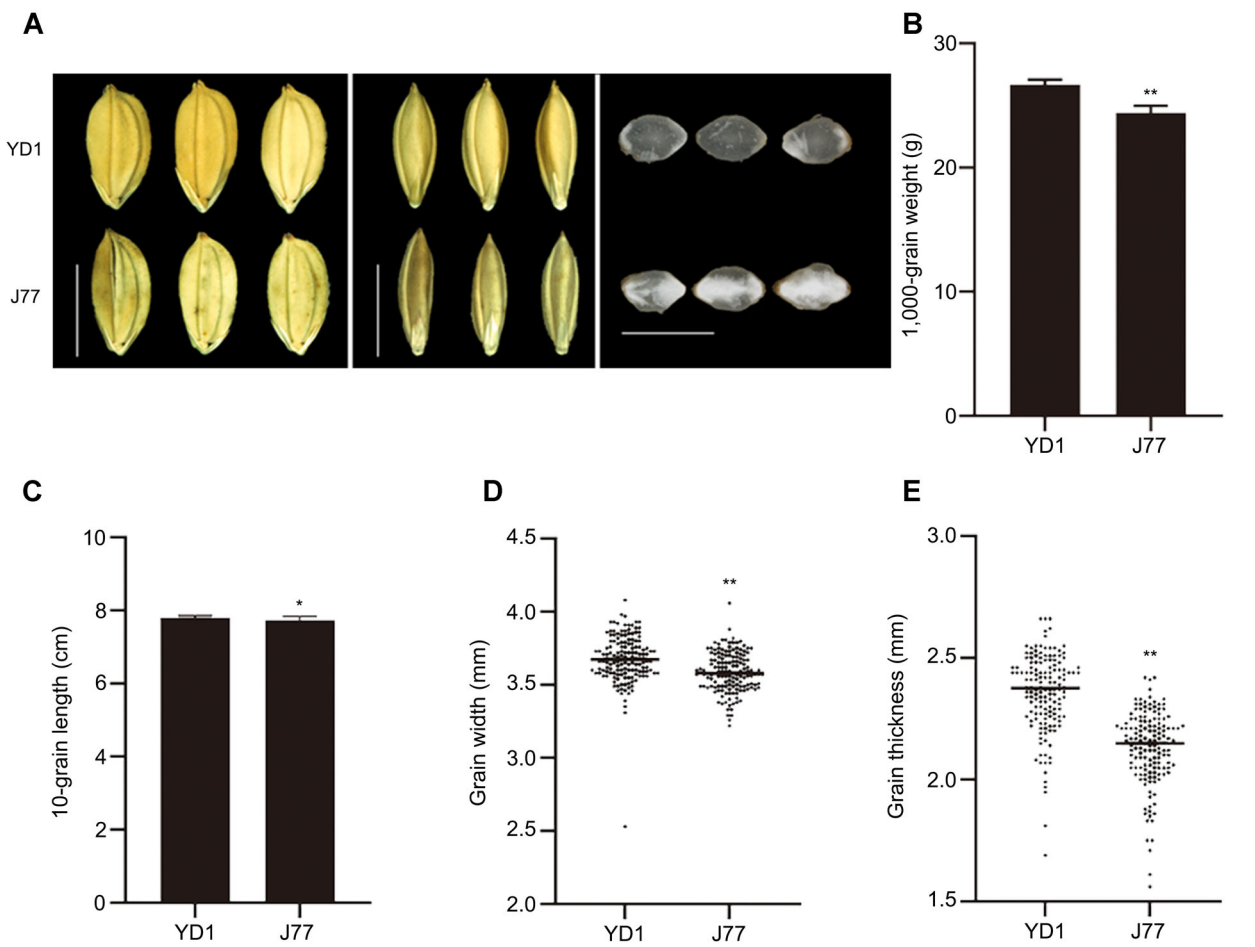


Figure 1. Phenotypic characterization of introgression line (IL) J77

(A) Representative grains of the recurrent parent Yundao1 (YD1) and IL J77. Grain chalkiness was shown in transverse sections (bar, 5 mm). **(B)** 1,000-grain weight, **(C)** 10-grain length, **(D)** grain width, and **(E)** grain thickness of YD1 and J77 ($n = 150$, five plants, 30 grains per plant). *Significantly different values (Student's *t*-test, $P \leq 0.05$), and **highly significantly different values (Student's *t*-test, $P \leq 0.01$).

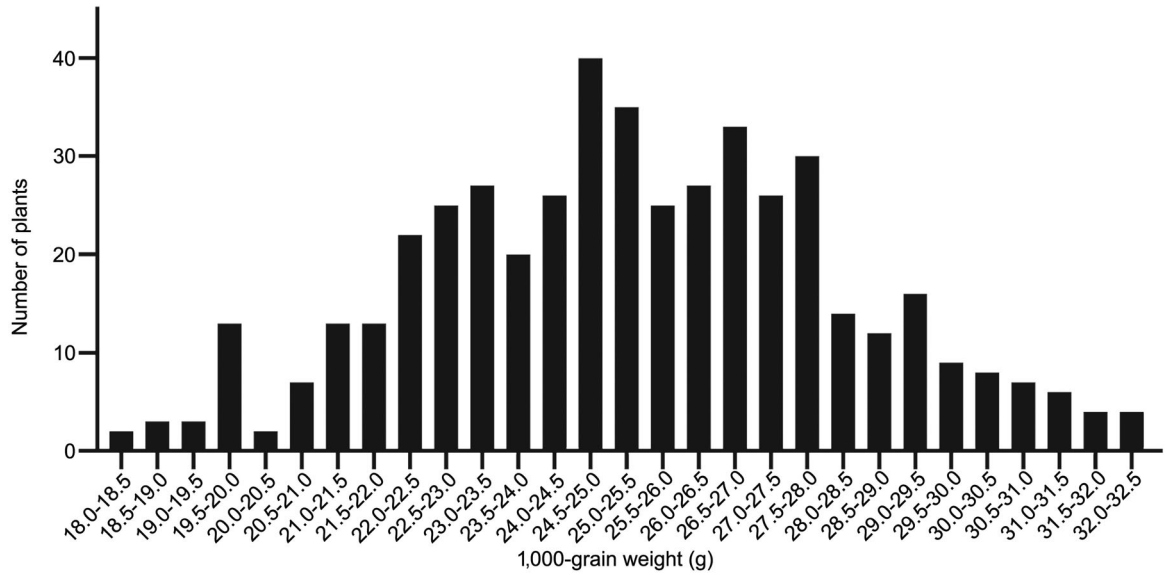


Figure 2. Frequency distribution of the 1,000-grain weight of an BC_3F2 population of 472 plants from a backcross between Yundao1 (YD1) and J77

Table 1. QTL influencing endosperm development in rice

Trait	QTL	Chr	LOD	PVE (%)	Additive effect	Left marker	Right marker
1,000-grain weight (g)	*qEDR5.1*	5	12.63	31.189	1.8231	RM6841	RM6339
	qEDR9.1	9	2.99	27.309	2.0905	RM7175	RM5535
	qEDR12.1	12	4.92	30.158	2.0797	RM3331	RM3331

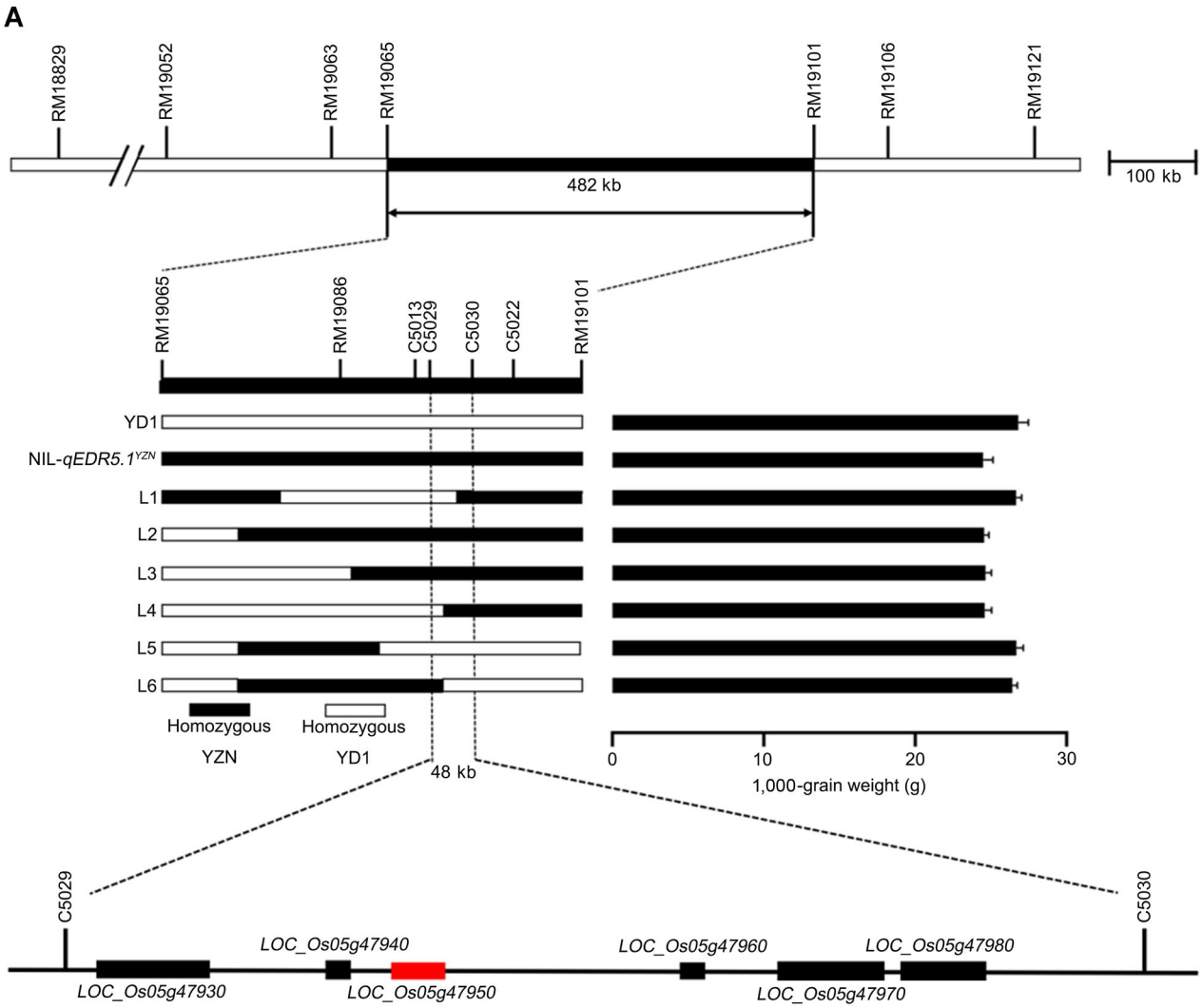
LOD, logarithm of odds; PVE, phenotypic variance explained; QTL, quantitative trait loci.

Fine mapping of the causal gene for *qEDR5.1*

To fine map the candidate gene for *qEDR5.1*, we crossed NIL-*qEDR5.1*^{YZN} and YD1, and obtained BC_4F_1 plants. Self-pollinating the BC_4F_1 plants produced BC_4F_2 mapping populations. By phenotype and genotype linkage analysis, *qEDR5.1* was preliminarily mapped to a 482-kb region between markers RM19065 and RM19101 (Figure 3A). The BC_4F_3 mapping populations were constructed by self-pollinating the heterozygous BC_4F_2 plants harboring the chromosome segment containing RM19065–RM19101 from YD1 and YZN. By developing 30 additional SSR and InDel markers within the region between markers RM19065 and RM19101, we finally narrowed the *qEDR5.1* locus to a 48-kb region between markers C5029 and C5030 (Figure 3A). In this region, six putative genes were annotated (Figure 3A). Through the sequence comparison of these six genes, we identified several sites that varied in the *LOC_Os05g47950* coding region between YD1 and NIL-*qEDR5.1*^{YZN} and resulted in amino acid sequence changes (Figures 3B, S1). However, the other five genes had no nucleotide variation causing the amino acid sequence to change between YD1 and NIL-*qEDR5.1*^{YZN}. Therefore, *LOC_Os05g47950* is the most likely candidate gene for the *qEDR5.1* locus.

LOC_Os05g47950 corresponds to *qEDR5.1*

A Basic Local Alignment Search Tool search (https://blast.ncbi.nlm.nih.gov) showed that *LOC_Os05g47950* is predicted to encode a UDP-glucosyltransferase comprising 490 amino acids. To further verify that *LOC_Os05g47950* corresponds to *qEDR5.1*, we performed a genetic complementation test in which a DNA fragment from YD1 containing the putative promoter region and coding DNA sequence (CDS) of *LOC_Os05g47950* was introduced into NIL-*qEDR5.1*^{YZN}, and we obtained two complementation transgenic lines, CL1 and CL2. Phenotypic analysis showed that the decrease in 1,000-grain weight and increase in grain chalkiness of NIL-*qEDR5.1*^{YZN} were rescued, and CL1 and CL2 showed similar YD1 phenotypes (Figure 4C, D). On the other hand, we knocked out *LOC_Os05g47950* using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system in the YD1 background and obtained two premature translation stop lines, *crisp1* and *crisp2* (Figure 4A). *crisp1* and *crisp2* exhibited a significant decrease in 1,000-grain weight and an increase in grain chalkiness compared with wild-type YD1 (Figure 4C, D). To test whether the grain endosperm development state depended on the expression level of *LOC_Os05g47950*, we



	Site	128	232-252	262	347	545	698	773	1002-1005	1239	1315-1317	1400	1439	1442
Nucleotide sequence	YD1	g	gagcggtgccagcagctggcg	g	t	t	c	g	c————gc	c	cga	g	t	t
	NIL-*qEDR5.1*^{YZN}	c	g-gcgacg—agctg-t—cg	a	a	a	t	a	cgcggcgccggc	t	cac	c	a	a
Amino acid sequence	YD1	G	ERCQQLA	V	F	V	A	G	R	L	R	G	I	M
	NIL-*qEDR5.1*^{YZN}	A	GDELS	M	Y	D	V	D	RGAG	F	H	A	N	K

Figure 3. Fine mapping of the *qEDR5.1* locus

(A) *qEDR5.1* was initially mapped to the interval between the markers RM19065 and RM19101, and then narrowed to a 48 kb region between the markers C5029 and C5030, which contained six predicted genes shown by the gene names registered in the database. The markers used for the mapping are shown in the figure and detailed in Table S1 online. **(B)** Natural variation causing amino acid sequence changes in *LOC_Os05g47950* between Yundao1 (YD1) and near-isogenic line (NIL)-*qEDR5.1*^{YZN} is shown in detail in the figure.

overexpressed *LOC_Os05g47950* in the YD1 background and obtained two overexpression transgenic lines, OX1 and OX2, but we found no obvious correlation between this trait and the expression of *LOC_Os05g47950* (Figure 4B–D). Taken together, the above results indicated that *LOC_Os05g47950* corresponds to *qEDR5.1* and regulates endosperm development in rice. In the remainder of the study, *LOC_Os05g47950* is named *EDR1*.

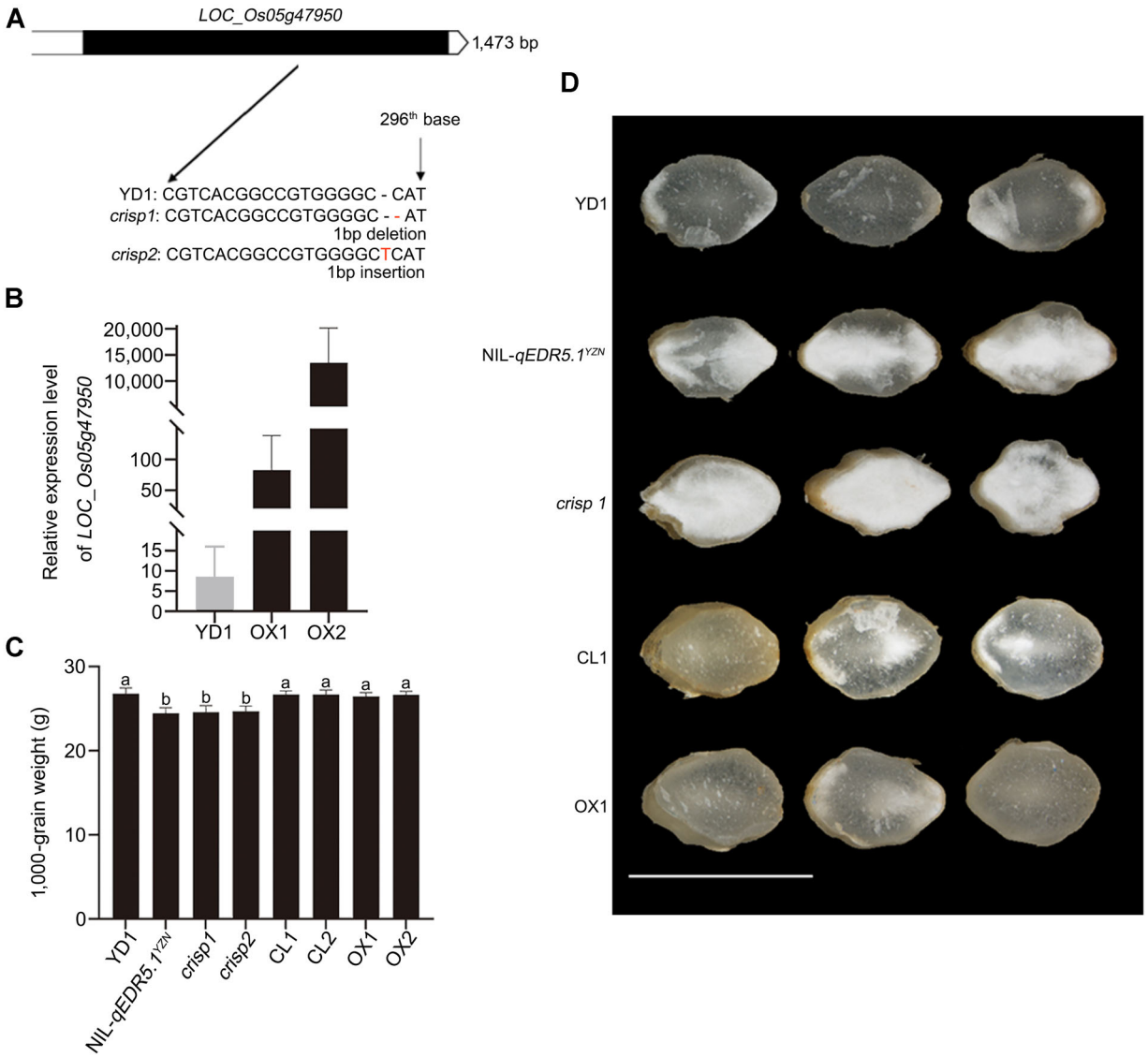


Figure 4. Identification of the *LOC_Os05g47950* transgenic plants and phenotypic analysis

(A) Diagram showing that single guide RNA (sgRNA) targets of *LOC_Os05g47950* were used in the clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system to generate *LOC_Os05g47950* mutants, and two different mutant transgenic lines, *crisp1* and *crisp2*, were obtained. **(B)** Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis in *LOC_Os05g47950*-overexpression transgenic plants. **(C)** 1,000-grain weight and **(D)** transverse sections of all genotype plant grains, including Yundao1 (YD1), near-isogenic line (NIL)-*qEDR5.1*^{YZN}, *crisp1*, *crisp2*, CL1, CL2, OX1, and OX2 (bar, 5 mm). Different letters above the columns indicate significant differences (Tukey's test, $P \leq 0.05$).

Expression pattern and subcellular localization of *EDR1*

To investigate the site of *EDR1* action, samples of YD1 from different growth stages and tissues were obtained to extract the total RNA. The results showed that *EDR1* was mainly expressed in the coleoptile and radicle during germination, indicating that *EDR1* may play a role in seed germination (Figure 5A). On the other hand, in the process of grain filling after flowering, the expression level of *EDR1* in the developing seeds gradually increased within a certain time frame (Figure 5A). This *EDR1* expression pattern is consistent with the NIL-*qEDR5.1*^{YZN} phenotypes, showing defects in endosperm development during the grain filling stage.

To perform subcellular localization of *EDR1 in vivo*, GFP (green fluorescent protein) was fused to the end of the CDS of *EDR1*^{YD1} without a stop codon and driven by the strong CaMV 35S promoter. Then, the full-length *EDR1*^{YD1} fusion construct *35S::EDR1*^{YD1}*-GFP* was transformed into epidermal cells of *Nicotiana benthamiana* leaves. GFP signals were observed in the nucleus and cytoplasm, indicating that *EDR1* was localized to the nucleus and cytoplasm (Figure 5B).

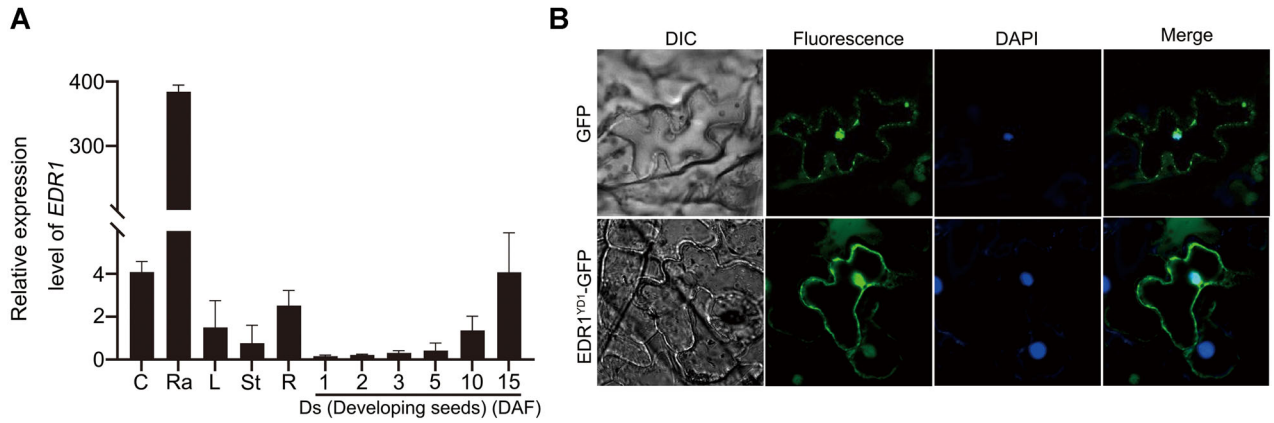


Figure 5. Expression profile and subcellular localization of *EDR1*

(A) Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis of *EDR1* in different growth stages and tissues. Tissues: C, coleoptile; Ra, radicle; L, leaf; St, stem; R, root; Ds, developing seeds at 1, 2, 3, 5, 10, 15 d after flowering. Values are the mean ± *SD* from three biological replicates. **(B)** The subcellular localization pattern of the EDR1^{YD1}-GFP fusion proteins.

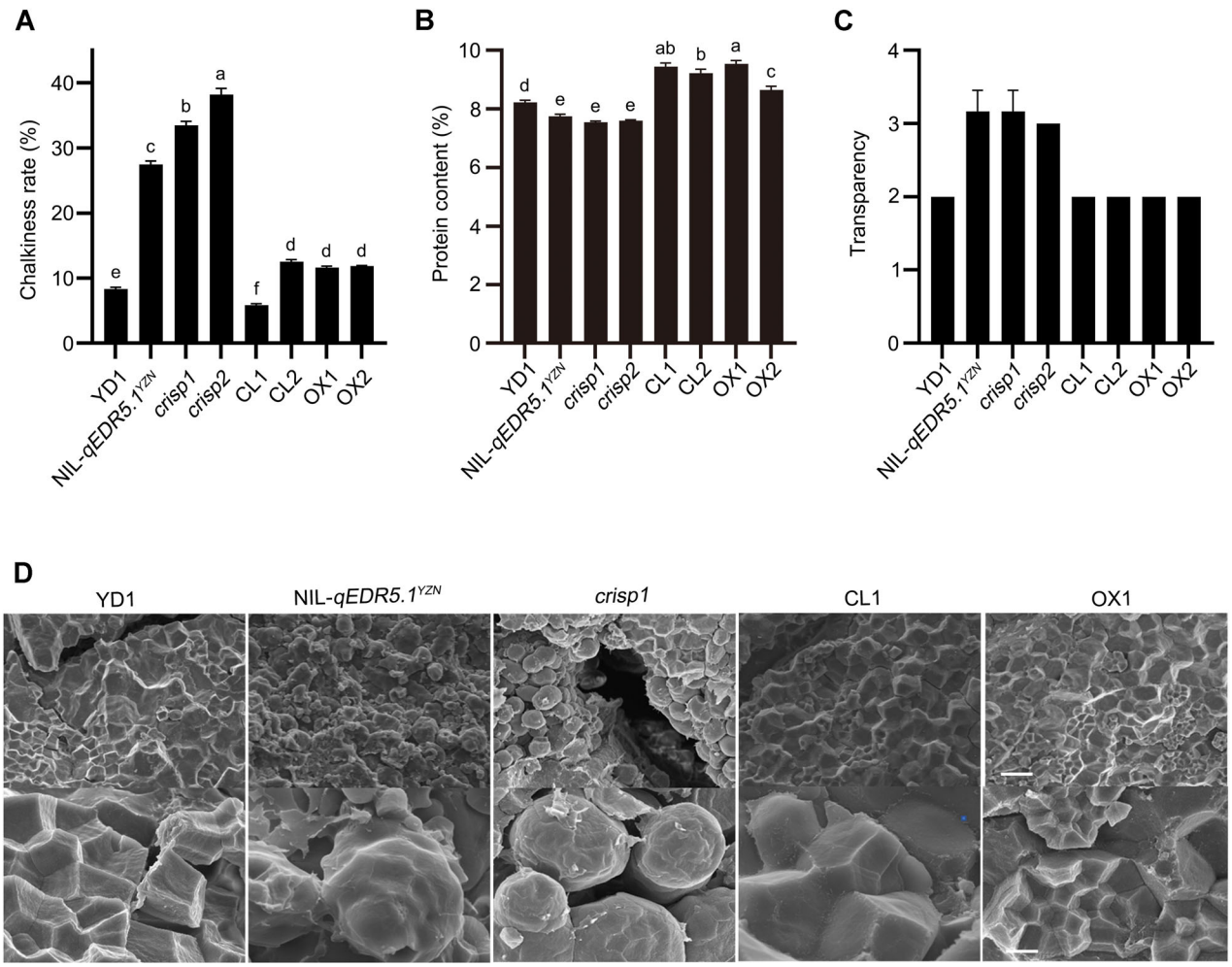


Figure 6. Effects of *EDR1* on the grain quality and morphology of starch granules

(A) Grain chalkiness rate, **(B)** protein content, and **(C)** transparency of mature seeds of Yundao1 (YD1), near-isogenic line (NIL)-*qEDR5.1*^{YZN}, *crisp1*, *crisp2*, CL1, CL2, OX1, and OX2. Different letters above the columns indicate significant differences (Tukey's test, $P \leq 0.05$). **(D)** Scanning electron microscopy (SEM) images of transverse sections from the endosperm bellies of mature seeds. Scale bars: 10 μm (above), 2 μm (below).

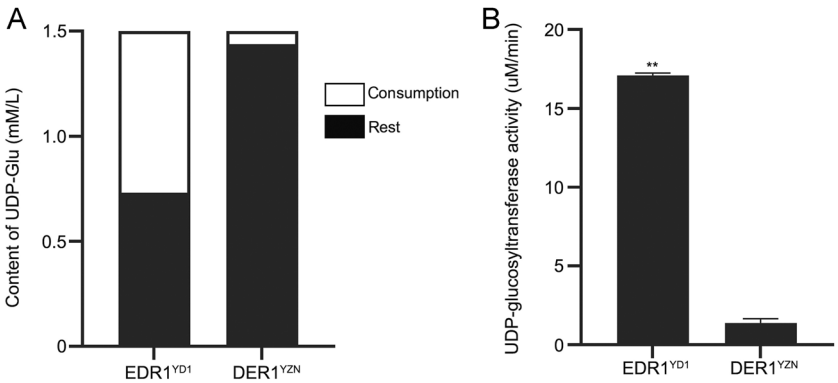


Figure 7. Determination of uridine 5′-diphospho (UDP)-glucosyltransferase activity
(A) The content of UDP-glucose before and after the UDP-glucosyltransferase Endosperm Development in Rice (EDR1)-mediated enzymatic reaction. **(B)** The UDP-glucosyltransferase activity of EDR1^{YD1} and EDR1^{YZN}. **Highly significantly different values (Student's *t*-test, $P \leq 0.01$).

EDR1 influences grain quality

Defects in endosperm development always cause grain quality changes (Wang et al., 2008; Zhang et al., 2016; Wu et al., 2019; Yu et al., 2021). The above results indicated that *EDR1* influences endosperm development in rice, so grain quality was analyzed. The seeds of NIL-*qEDR5.1*^{YZN} and two knockout transgenic lines, *crisp1* and *crisp2*, all showed a significant decrease in protein content and transparency but an increase in grain chalkiness (Figure 6A–C). On the other hand, two complementation transgenic lines and two overexpression transgenic lines showed similar YD1 phenotypes (Figure 6A–C). Scanning electron microscopy (SEM) images of grains with high chalkiness from NIL-*qEDR5.1*^{YZN} and *crisp1* indicated that the endosperm was filled with loosely packed spherical starch granules with large air spaces, whereas the grains with low chalkiness from YD1, CL1 and OX1 consisted of densely and regularly packed polyhedral starch granules (Figure 6D). These results demonstrated that *EDR1* influences grain quality, including grain chalkiness, protein content, grain transparency, and the morphology of starch granules.

Natural variation in *EDR1* reduces glucosyltransferase activity of EDR1^{YZN}

It has been reported that glucosyltransferases are critical enzymes in glycosylation, and mediate the glycosylation of proteins, lipids, and secondary metabolites, such as monolignols and flavonoids, thus regulating their physical and chemical properties, such as activity, solubility, and stability (Ross et al., 2001; Le Roy et al., 2016; Dong et al., 2020). Here, *EDR1* is predicted to encode a UDP-glucosyltransferase that mainly uses UDP-glucose as a glucosyl donor. To investigate whether the natural variation in *EDR1* influences the glucosyltransferase activity of EDR1 between YD1 and YZN, we heterologously expressed EDR1 in *Escherichia coli* and performed liquid chromatograph–mass spectrometry (LC-MS) analysis of the UDP-glucose content in *in vitro* EDR1-mediated reactions, where UDP-glucose was a glycosyl donor and flavonoids and monolignol were used as receptors. We found that the UDP-glucose content consumed in the EDR1^{YD1}-mediated reaction was much greater than that in the EDR1^{YZN}-mediated reaction (Figure 7A). By calculating the enzyme activity, we found that the glucosyltransferase activity of EDR1^{YD1} was more than 12 times higher than that of EDR1^{YZN} (Figure 7B). The above results suggested that natural variation in the amino acid sequence of EDR1 greatly reduced the glucosyltransferase activity of EDRI^{YZN}.

Natural variation of EDR1 in different rice varieties

To investigate the natural variations in EDR1 in different rice varieties, we sequenced the coding region sequence in 51 accessions, including the different subpopulations and two ecotypes of paddy rice and upland rice (Table S1; Petit et al., 2009). Based on amino acid polymorphisms, we identified seven haplotypes (Figure 8A). Among these haplotypes, Hap1, having the same amino acid sequence as EDR1^{YZN}, and Hap2, having the same amino acid sequence as EDR1^{YD1}, were the two major haplotypes (Figure 8A). Rice varieties with Hap1 amino acid sequences showed a significantly higher rate of a high chalkiness grain phenotype than those with Hap2 amino acid sequences (Figure 8B), indicating that EDR1 indeed plays an important role in regulating endosperm development. Among the investigated upland rice varieties, Hap1 was 88.9%, but it was 39.4% among the investigated paddy rice varieties (Figure 8C), suggesting that EDR1 is conserved in upland rice but segregates in paddy rice. Now that there are 25 amino acid polymorphism sites (Figure 8A), we investigated which sites play a decisive role in determining the grain chalkiness phenotype. The correlation coefficient between each amino acid polymorphism site and phenotype was analyzed, and the correlation ($0.3 < R < 0.8$) suggests that nine sites might be responsible for endosperm development (Figure 8D).

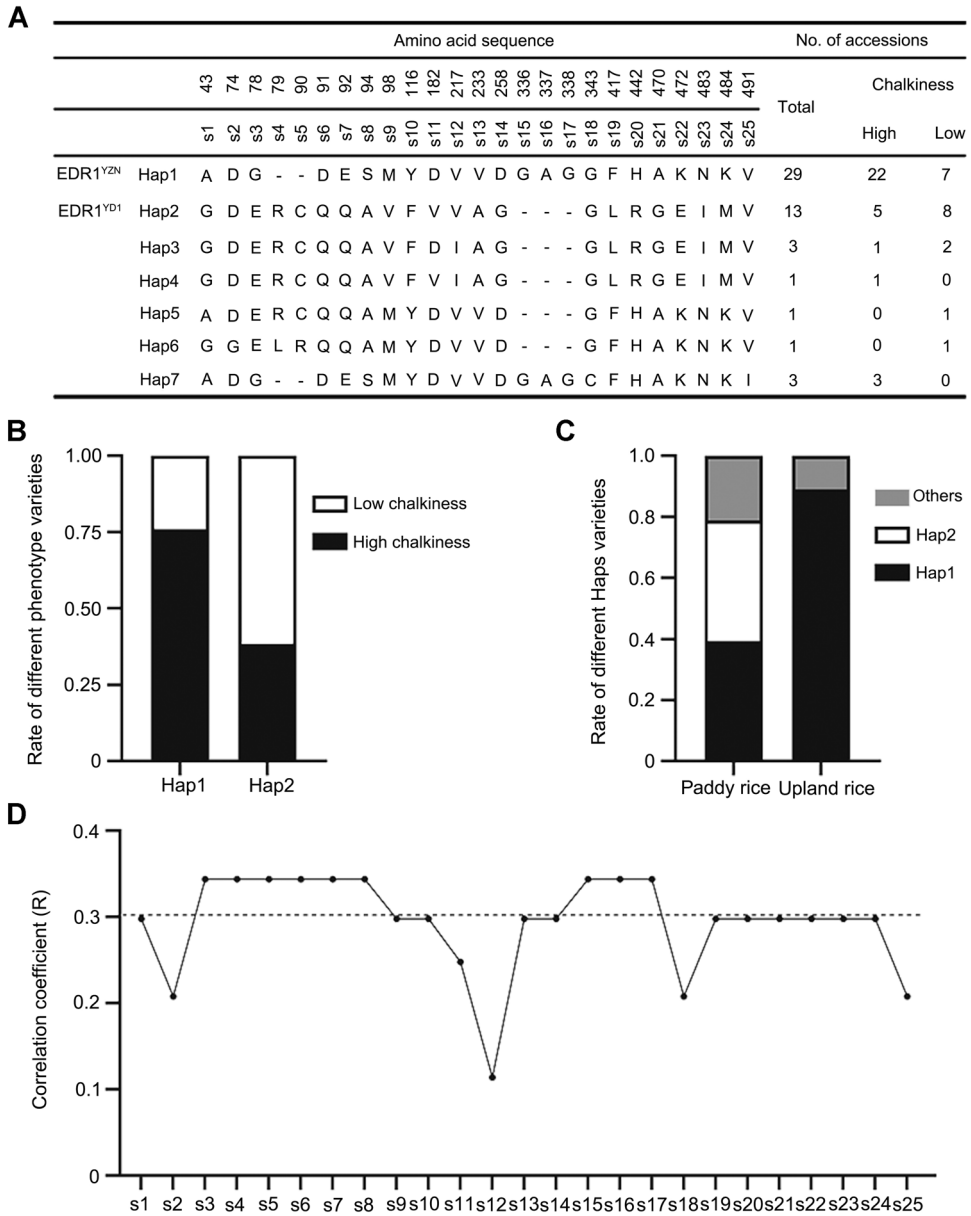


Figure 8. Natural variations of Endosperm Development in Rice (EDR1)

(A) Haplotype analysis of EDR1 in 51 rice varieties based on amino acid polymorphisms. In total, 25 sites and seven haplotypes were identified. **(B)** The distribution of high chalkiness grain and low chalkiness grain phenotypes in Hap1 and Hap2 varieties. **(C)** The distribution of different EDR1 haplotypes between upland and paddy rice varieties. **(D)** Correlation analysis between each amino acid polymorphism site and phenotype. $0.3 < |R| \leq 0.8$ means weak correlation, $0.8 < |R| \leq 1$ means strong correlation, and $0 \leq |R| \leq 0.3$ means no correlation.

DISCUSSION

The issue of food security has always been an unresolved problem globally (Yin et al., 2020). Although since the green revolution, the output of grain has been greatly improved, it still cannot meet the growing population's demand for food (Hedden, 2003; Liu et al., 2020). Rice is the staple food for nearly half of the world's population, so it is particularly important to improve the rice yield (Kusano et al., 2012, 2021). There are two main ways to increase the rice yield. One is to breed new varieties to increase the yield per unit area; the other is to expand the cultivation area of rice. By semidwarf breeding, hybrid breeding, molecular breeding, and other methods, breeders have greatly increased the yield of rice per unit area, so it will be more difficult to further increase the yield per unit area of rice (Peng et al., 1999; Sasaki et al., 2002; Hedden, 2003). Moreover, the increase in rice yield per unit area is accompanied by the extensive use of chemical fertilizers and pesticides, which has caused substantial environmental pollution. On the other hand, arable land suitable for traditional paddy rice cultivation is

limited, so it is almost impossible to increase the rice cultivation area. Compared with traditional paddy rice, the yield per unit area of upland rice is less than 20%, so the cultivation area of upland rice is still small (Garrity et al., 1992; Phapumma et al., 2020). Therefore, there is great potential to improve the yield of upland rice. Upland rice usually shows insufficient grains resulting from abnormal endosperm development, which is one of the reasons for its low yield (Fageria et al., 1982; Cavite et al., 2020). Many genes influencing endosperm development have been reported, but few studies have been performed on upland rice. In this study, we identified and cloned the *EDR1* gene; additionally, the natural allele of *EDR1* from YZN caused abnormal endosperm development in NIL-*qEDR5.1*^{YZN}, which is characterized by insufficient grains and changes in grain quality. Our results provide new insight into rice breeding for improving grain quality and yield.

The endosperm is an important part of rice seeds and the main part of nutrient storage. The endosperm development state can often affect the size of the seed, which in turn affects the yield. Flo10 is a PPR protein, and Flo18 is a TPR protein; they both play an important role in the post-transcriptional splicing of mitochondrial genes (Wu et al., 2019; Yu et al., 2021). The mutation of Flo10 and Flo18 resulted in abnormal endosperm development, with a significant decrease in 1,000-grain weight and a significant increase in chalkiness (Wu et al., 2019; Yu et al., 2021). Flo7 regulates the development of peripheral endosperm by regulating starch synthesis and starch granule formation. The phenotype of the flo7 mutant was consistent with that of the flo10 mutant and flo18 mutant (Zhang et al., 2016). Here, by phenotype analysis, NIL-*qEDR5.1*^{YZN} showed a significant decrease in 1,000-grain weight, grain size, and grain filling rate (Figures 4C, S2) but an increase in grain chalkiness compared with YD1 (Figure 4D), indicating that *EDR1* is involved in regulating rice endosperm development. Complementation of *EDR1*^{YD1} in NIL-*qEDR5.1*^{YZN} could rescue the phenotype of NIL-*qEDR5.1*^{YZN}, and knockout of *EDR1* in YD1 showed the same phenotype as NIL-*qEDR5.1*^{YZN}, which further proved that *EDR1* plays an important role in regulating rice endosperm development (Figure 4C, D). However, when *EDR1*^{YD1} was overexpressed in the YD1 background, the 1 000-grain weight and grain chalkiness did not vary depending on the expression of *EDR1* (Figure 4C, D), indicating that EDR1 as an enzyme has no dosage effect on the regulation of endosperm development when it is enough for the substrates, but when it is insufficient, little change in its content may have notable effects.

UDP-glycosyltransferases are a superfamily of enzymes that mediate the transfer of glycosyl residues from activated nucleotide sugars to acceptor molecules, such as proteins, lipids, and secondary metabolites, thus regulating their physical and chemical properties, such as activity, solubility, and stability (Huang et al., 2008; Dong et al., 2020). UDP-glucosyltransferases belong to the superfamily of UDP-glycosyltransferases found in animals, plants, bacteria, and viruses (Burchell and Coughtrie, 1989; Mackenzie et al., 1997; Meech and Mackenzie, 1997). In plants, UDP-glucose is the most common glucosyl donor in UDP-glucosyltransferase-mediated reactions. We identified 25 hypothesized UDP-glucosyltransferase genes, including *EDR1* and *GSA1*, in rice from the National Center for Biotechnology Information and found that both of them belong to different lineages based on evolutionary analysis (Figure S3A). They contained the Plant Secondary Product Glycosyltransferase (PSPG) box, which was conserved in UDP-glucosyltransferases, although the amino acid sequences were only 29% similar (Figure S3B). In this study, UDP-glucose, total lignin, and flavonoids extracted from plants were used as glucosyl donor and receptors respectively, and confirmed that EDR1^{YD1} possessed UDP-glucosyltransferase activity, suggesting that EDR1 and GSA1 might have similar functions. The activity of EDR1^{YD1} was 12 times higher than that of EDR1^{YZN} (Figure 7B), suggesting that natural variations changed the activity of EDR1, thereby influencing rice endosperm development. Since natural variations reduce the activity of EDR1^{YZN}, 11 sites were discovered that might be responsible for the change in UDP-glucosyltransferase activity by analyzing the correlation between 25 acid polymorphism sites and phenotypes in 34 rice varieties (Figure 8A, D). However, if more rice varieties are analyzed, one or more accurate sites responsible for the change in UDP-glucosyltransferase activity might be found. Although the glycosyl donor in the EDR1-mediated reaction was identified, the details of putative receptors are still elusive. More work remains to be done to identify the detailed glucosyl receptors.

Breeding varieties with high yield and high biotic/abiotic resistance has always been the long-term goal of breeders (Kissoudis et al., 2016; Deng et al., 2017). However, high yields and high biotic/abiotic resistance are usually incompatible due to internal trade-off mechanisms in plants (Deng et al., 2017). Under suitable environmental conditions, most of the energy would be used for growth and development in rice; however, when rice suffered from biotic or abiotic stresses, more energy would be allocated to resist those stresses at the expense of retardant growth and development. If grown in upland fields, rice might inevitably suffer from abiotic stresses, such as water shortages and high salt across the whole life cycle. Hence, it is reasonable that rice growth in upland fields would reduce the energy used for grain filling and endosperm development, thereby reducing grain size. Many genes are involved in the response to trade-off mechanisms. *MiR-156* directly targets *IPA1* and *OsSPL7* and negatively regulates their expression. Down-regulation of miR-156 or overexpression of *IPA1* and *OsSPL7* could improve disease resistance against bacterial blight but reduce rice yield (Liu et al., 2019). Appropriate indoleacetic acid (IAA) plays an important role in promoting the growth of rice. *GH3-8*, which encodes an IAA–amino synthetase, has been confirmed to increase conjugated IAA accumulation. Overexpression of *GH3-8* results in enhanced disease

resistance to the rice pathogen *Xanthomonas oryzae* pv *oryzae* but retards rice growth and development (Ding et al., 2008). Previous studies revealed that upland rice generally exhibited abnormal endosperm development; however, upland rice exhibited higher tolerance to abiotic and biotic stresses (Yang, 2002; Deng et al., 2017). In our study, an upland rice variety, YZN, was used as the donor parent, which exhibited abnormal endosperm development and poor grain quality. *EDR1* was highly expressed not only in developing seeds but also in radicles and coleoptiles during germination, indicating that *EDR1* might play an important role in seed germination (Figure 5A). Due to its adoption in upland fields, upland rice is susceptible to abiotic stresses, such as water shortages and high salt during germination. These results implied that $EDR1^{YZN}$ might also be responsible for the abiotic stress tolerance of upland rice during germination.

MATERIALS AND METHODS

Plant materials and natural field experiment conditions

The paddy rice variety Yundao1 was used as the recurrent parent, and the upland rice variety Yanzhinuo was used as the donor parent. All rice (*Oraza sativa* L.) seeds used in this study were cultivated in Xishuangbanna (Yunnan Province, China) under natural field conditions twice a year in the spring and fall. For better measurement of the rice phenotypes and grain quality, all rice seedlings were planted in triplicate, and seeds were harvested in the middle of each plot when they were ripe.

Greenhouse experimental conditions

To facilitate water management, the phenotypic experiments were arranged in a rain shelter greenhouse, which was sheltered at the top and open around. Experiments were divided into two groups and cultivated in paddy field and upland field conditions. For the paddy field treatment, sowing and transplanting single seedlings were performed, and water holding was maintained. The water covered 2–5 cm above the soil before rice ripened. For upland field treatment, direct sowing with three to four seeds per hole was performed and thinned to one seedling at the three-leaf stage. Subsequently, to maintain an aerobic environment, the study plots were watered regularly to maintain normal seeding growth instead of water covering above the soil. The soils used for greenhouse experiments were from a natural field. The temperature and light duration of the greenhouse were the same as those of the natural environment in Xishuangbanna (Yunnan Province, China) from May to October. Both paddy fields and upland fields received the same dose of compound NPK fertilizer (2.0 kg/100 m^2). The planting density of all rice plants was 15 cm × 25 cm. Paddy field and upland field treatments were performed with the same experimental design, and each experiment was conducted with three replications.

Primers

All primers for polymerase chain react ion (PCR) and quantitative real-time reverse-transcription PCR (qRT-PCR) used in this study were designed using primer5 (V 5. 5. 0) and CE Design (V 1. 03); these primers are listed in Table S2.

Vector construction and transformation

To obtain the complementation construct, the 3.5-kb genomic fragment covering the entire CDS and 2 kb promoter of $EDR1^{YD1}$ was inserted into the plant binary vector pCAMBIA1300 at the *Eco*RI and *Bam*HI sites. To obtain the overexpression construct (OX), the entire CDS of $EDR1^{YD1}$ was transformed into the plant binary vector pCAMBIA1301 at the *Bam*HI and *Sac*I sites. The mutation construct was obtained using the CRISPR/Cas9 system. PCR was carried out in a 50-μL reaction volume using Phanta Max Super-Fidelity DNA Polymerase (Vazyme). Amplification procedures consisted of 33 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for *x* min (1 kb/min). All the generated vector transformations into rice plants were performed by Biorun (Wuhan, China).

DNA and RNA extraction

Rice genomic DNA was extracted from young leaves with the sodium dodecyl sulfate method and RNA was extracted from different plant tissues with the water-saturated phenol method. We obtained first-strand complementary DNA (cDNA) by reverse-transcription using the Prime ScriptTM RT reagent Kit (TaKaRa) according to the manufacturers' instructions.

Gene expression analysis

Quantitative real-time RT-PCR analysis was performed with three technical replicates per template. Each sample was analyzed with three independent biological replicates, and the average value represented relative expression levels. Quantitative real-time RT-PCR was carried out in a 10-μL reaction volume using SYBR GreenI (TaKaRa). Osactin was used as the internal control, and the relative expression level was calculated by $2^{-\Delta\Delta C_t}$.

Phenotypes and grain quality analysis

All rice seeds for phenotype and grain quality analyses were air-dried. Grain size and weight were measured with a YieldTraits Scorer (YTS-5DS), and grain quality, such as grain chalkiness, protein content, and grain transparency, was determined by CHINA SEED, Hubei Branch (Wuhan, China).

Subcellular localization

For transient expression analysis in *N. benthamiana* leaf epidermal cells, full-length $EDR1^{YD1}$ without a stop codon was amplified and cloned into pCAMBIA30-GFP and driven by the strong CaMV 35S promoter to produce *35S::*$EDR1^{YD1}$*-GFP* fusion constructs. Moreover, blank pCAMBIA30-GFP vectors were used as controls. Subsequently, these constructs were introduced into *Agrobacterium. tumefaciens* strain EHA105 and then infiltrated into *N. benthamiana* leaves as described

previously (Waadt and Kudla, 2008). After 48 h, epidermal cell nuclei were stained with 4′,6-diamidino-2-phenylindole and imaged using an LSM700 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

Scanning electron microscopy

For SEM, rice samples were processed as described previously (Imai et al., 2006) and then imaged using a ZEISS EVO LS10 scanning electron microscope (Carl Zeiss). All procedures were performed according to the manufacturer's protocol.

Enzyme activity assay

The full-length cDNA sequences of $EDR1^{YD1}$ and $EDR1^{YZN}$ were cloned into a pGEX-4T vector. The construct was then transferred into *Escherichia coli* strain BL21, and the transformants propagated in 50-mL Luria-Bertani liquid medium with 100 mg/mL ampicillin at 37°C until the optical density at 600 nm reached 0.6. Twenty-five microliters of isopropylthio-β-galactoside (0.5 mol/L) was added and the expression of EDR1 protein was induced by incubating at 16°C for 20 h. The cells were harvested by centrifugation (5,000 *g*, 10 min) at 4°C and resuspended in 5 mL of phosphate-buffered saline. Then, 100 μL of protease inhibitor (PI, 50×) was added to the suspension. The cells were lysed by using a sonication homogenizer (80 W, five cycles) and centrifuged (11,000 *g*, 20 min) at 4°C to obtain the supernatant. Finally, we purified the supernatant using a glutathione S-transferase adsorption column and obtained a 200-mL EDR1 protein solution. The enzyme activity assay was performed in a 200-μL aliquot of reaction mixture containing 10 mmol/L $MgCl_2$, 1.5 mmol/L UDP-glucose as the glycosyl donor, 250 μmol/L monolignol and flavonoid as the glycosyl acceptor, 50 μL EDR1 protein solution and 200 mmol/L glycine-NaOH buffer (pH 8.5) as the reaction buffer. The reaction mixtures were incubated at 37°C for 45 min, and 200 μL of methanol was added to quench the reaction. The enzymatic reaction mixtures were centrifuged (11,000 *g*, 20 min) at 4°C. The supernatant was then subjected to LC-MS analysis.

Statistical analysis

QTL IciMapping software was used to construct a genetic linkage map based on the genotypes and phenotypes, and Excel and SPSS (version 22) were used for data analysis. All samples were subjected to three replicate measurements. The data are presented as the mean ± *SD*. Student's *t*-test was performed to determine the significant differences compared to the control, where * and ** indicate statistical significance at $P \leq 0.05$ and ≤ 0.01, respectively.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

D.Y. and P.X. conceived the project and designed the experiments. Z.W. wrote the manuscript and performed the experiments with assistance from X.Z., G.C., J.Y., J.W., F.W., D.T., J.Z and L.S. All authors interpreted and discussed the data, and approved the manuscript.

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SUPPORTING INFORMATION

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Figure S1. Comparison of the coding DNA sequence (CDS) of LOC_Os05g47950 from Yundao1 (YD1) and near-isogenic line (NIL)-qEDR5.1YZN Natural variations in the CDS are shown in red

Figure S2. One thousand-grain weight of developing seeds at different stages after flowering

Figure S3. Alignment of hypothesized uridine 5′-diphospho (UDP)-glucosyltransferase amino acid sequences in rice

(A) Neighbor-joining of hypothesized UDP-glucosyltransferase in rice. **(B)** Amino acid sequence alignment of the Plant Secondary Product Glycosyltransferase (PSPG) box from EDR1 and GSA1. The red line indicates the PSPG box domain.

Table S1. Genotype and phenotype of 51 rice varieties

H represents high chalkiness, L represents low chalkiness

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