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The failure to express a protein disulphide isomerase-like protein results in a floury endosperm and an endoplasmic reticulum stress response in rice

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Abstract

The rice somaclonal mutant *T3612* produces small grains with a floury endosperm, caused by the loose packing of starch granules. The positional cloning of the mutation revealed a deletion in a gene encoding a protein disulphide isomerase-like enzyme (PDIL1-1). In the wild type, *PDIL1-1* was expressed throughout the plant, but most intensely in the developing grain. In *T3612*, its expression was abolished, resulting in a decrease in the activity of plastidial phosphorylase and pullulanase, and an increase in that of soluble starch synthase I and ADP-glucose pyrophosphorylase. The amylopectin in the *T3612* endosperm showed an increase in chains with a degree of polymerization 8–13 compared with the wild type. The expression in the mutant's endosperm of certain endoplasmic reticulum stress-responsive genes was noticeably elevated. PDIL1-1 appears to play an important role in starch synthesis. Its absence is associated with endoplasmic reticulum stress in the endosperm, which is likely to underlie the formation of the floury endosperm in the *T3612* mutant.

Key words: ER stress, floury endosperm, protein disulphide isomerase-like 1-1, starch synthesis.

Introduction

Starch accounts for 60–80% of cereal grain endosperm. The analysis of starch synthesis mutants (commonly referred to as 'opaque' mutants) has shown that the products of *ISA-1* (Kubo *et al.*, 1999), *AGPS2b* (Kawagoe *et al.*, 2005), *Pho1* (Satoh *et al.*, 2008; Hwang *et al.*, 2010), *SSIIIa* (Fujita *et al.*, 2007), and *BEIIb* (Nishi *et al.*, 2001) are key enzymes in the process. A number of genes responsible for the floury endosperm phenotype have, however, proven to be not directly related to starch synthesis. One of these is *BiP*, which encodes a chaperone active in the endoplasmic reticulum (ER) where it interacts with nascent immature proteins to assist in their folding (Yasuda *et al.*, 2009; Wakasa *et al.*, 2011). In maize, mutated alleles of various

zein-encoding genes have also been shown to generate a floury endosperm, and a number of genes, including *BiP*, are known to be up-regulated in the endosperms of these mutants (Hunter *et al.*, 2002).

When an excess of unfolded or misfolded proteins aggregates in the ER, a process referred to as ER stress ensues, which is alleviated via the unfolded protein response (UPR) (Kaufman, 1999). The UPR of yeast and mammalian cells is rather well understood, but its equivalent in plant cells is still obscure. One branch of the mammalian UPR involves the protein PERK (protein kinase-like endoplasmic reticulum kinase) which acts to block protein synthesis via the phosphorylation of the eukaryotic

translation initiation factor 2α (Szegezdi *et al.*, 2006). However, no plant orthologue of PERK has yet been found. A second branch involves the transduction of the stress signal by a number of bZIP proteins from the ER to the nucleus, where a series of stress response genes are then induced (Liu *et al.*, 2007a, b; Iwata *et al.*, 2008), leading to the activity of various chaperones. The functions of this class of proteins have yet to be established in plants. Finally, the third branch involves programmed cell death (PCD), which is induced if the ER stress cannot be alleviated (Cacas, 2010). Protein disulphide isomerase (PDI) is a component of the UPR, and is involved via its active site WCGHC both in the catalysis of disulphide bond formation (reduction or isomerization) and in assisting polypeptide folding (Wilkinson and Gilbert, 2004; Gruber *et al.*, 2006). Cells lacking certain UPR components, including PDI and BiP, are prone to ER stress. In rice, for example, the absence of BiP1 induces an intense ER stress response (Wakasa *et al.*, 2011), while yeast *pdi1* loss-of-function mutants are lethal (Laboissiere *et al.*, 1995). In metazoans, persistent or severe ER stress induces PCD (Boyce and Yuan, 2006). The link between ER stress and PCD in plants has not yet been established.

Here, it is shown that abolishing the expression of a PDI protein (PDIL1-1) leads to the formation of a floury endosperm in rice, in which the frequency of short chain amylopectin molecules is markedly higher than in the wild type. The level of expression in the endosperm of a number of genes associated with starch synthesis has been compared between the mutant (*T3612*) and the wild type, and it has been possible to demonstrate a greater abundance of certain chaperones, co-chaperones, bZIP transcription factors, and ERAD-related proteins. Finally, it was possible to show the induction of the PCD marker gene *HSR203J* in the endosperm of the mutant. These results suggest that ER stress, induced by the absence of PDIL1-1, was probably the cause for the formation of a floury endosperm in *T3612*.

Materials and methods

Plant materials and phenotyping

The *T3612* mutant was regenerated from an *in vitro* culture of tissue from cv. Nipponbare. Plants were grown under natural conditions at Nanjing Agricultural University. The procedure used for scanning electron microscopy (SEM) was as described by Kang *et al.* (2005), and that for transmission electron microscopy (TEM) was as described by Wang *et al.* (2009). The starch content in the rice flour was measured with a starch assay kit (Megazyme, Wicklow, Ireland), according to the manufacturer's protocol. Amylose content was assessed following Liu *et al.* (2009), and that of lipids and proteins following Kang *et al.* (2005).

RNA extraction, RT-PCR, and qRT-PCR

Total RNA was extracted by means of an RNAprep pure Plant kit (TIANGEN Biotech, Beijing, China). A 1 μg aliquot of total RNA was reverse transcribed by priming with oligo(dT$_{18}$) in a 20 μl reaction based on the PrimeScript Reverse Transcriptase kit (Takara, Otsu, Japan). The rice *Actin1* sequence (GenBank accession no. NM_001057621) was employed as the RT-PCR

reference, and the rice *Ubiquitin* sequence (NM_001056014) as the qRT-PCR reference. Primer sequences used for both RT-PCR and qRT-PCR for the ER stress-related genes are given in Supplementary Table S1 available at *JXB* online. The qRT-PCR primers used to assay starch synthesis and grain protein storage synthesis-related genes have been documented elsewhere (Ohdan *et al.*, 2005; She *et al.*, 2010), as have those targeting *HSR203J*, *BiP-3*, and *Hsp70-2* (Wakasa *et al.*, 2011). qRT-PCRs were performed on an ABI PRISM 7900 device using SYBR Premix Ex Taq (Takara).

Enzyme assays and native PAGE/activity staining

A sample of 100 mg of developing endosperm [9 or 12 days after flowering (DAF)] was homogenized on ice in 1 ml of 50 mM HEPES NaOH (pH 7.4), 2 mM MgCl$_2$, and 12.5% (v/v) glycerol. The homogenate was centrifuged at 20 000 *g* for 10 min at 4 °C, and the resulting supernatant was used for all enzyme assays. ADP-glucose pyrophosphorylase (AGPase) and UGPase activity was determined following Nishi *et al.* (2001), and that of sucrose synthase following Doehlert *et al.* (1988). Debranching enzyme (DBE) and branching enzyme (BE) isozymes were separated by native PAGE and assayed according to Fujita *et al.* (2009). Starch synthase (SS) and phosphorylase (Pho) activities were generated in 7.5% polyacrylamide gels containing 0.8% (w/v) oyster glycogen (Sigma), according to Satoh *et al.* (2008). All electrophoretic separations were conducted at 4 °C at a constant voltage of 50 V.

DSA-FACE assessment of amylopectin chain length distribution

DSA-FACE (DNA sequencer-assisted flurophore-assisted carbohydrate electrophoresis) separation of mature grain starch was performed according to Nishi *et al.* (2001) and O'Shea and Morell (1996), with minor modifications. A 5 mg sample of rice flour was suspended in 200 μl of ultrapure water and boiled for 10 min. Then 800 μl of 50 mM sodium acetate was added, and the suspension was re-boiled for a further 10 min. After cooling, 0.1 U of *Pseudomonas amyloderamosa* isoamylase (ISA; Seikagaku Corporation, Tokyo) was added, and the mixture was incubated for 24 h at 37 °C. The resulting solution was then held at 100 °C for 2 min, and a 1 μl aliquot was combined with 1 μl of 0.1 M 8-aminopyrene-1,3,6-trisulphonic acid trisodium salt and 2 μl of 1 M sodium cyanoborohydride, and incubated for 24 h at 37 °C in the dark. The final solution was diluted with 21 μl of 6 M urea, 0.04 M boric acid, 0.04 M TRIS, pH 8.6. A 1 μl aliquot was loaded onto a 7.5% polyacrylamide gel and electrophoresed using an ABI PRISM 377 DNA sequencing device.

Genetic mapping and transformation

Ten F$_2$ progeny of the cross *T3612*×cv. NJ11 producing floury endosperms were genotyped with 242 genome-wide microsatellite markers. Fine mapping required the derivation of *de novo* molecular markers based on a comparison of the local genome sequences of cv. Nipponbare and cv. 93-11, and included both microsatellite and indel markers (Supplementary Table S1 at *JXB* online). For the complementation of the *T3612* mutation, the wild-type *PDIL1-1* cDNA sequence was cloned into the binary vector pPZP2Ha3 (+) (Fuse *et al.*, 2001) to generate the transformation cassette pPZP-35S-PDIL1-1. This plasmid was introduced into *Agrobacterium tumiefaciens* strain EHA105, which was then used to agroinfect rice calli (Hiei *et al.*, 1994). Hygromycin-resistant calli were regenerated and seedlings were grown in a greenhouse.

Heterologous expression of PDIL1-1

PDIL1-1 cDNA was cloned into the pGEX-4T-2 expression vector, which was introduced into *Escherichia coli* strain BL21 (DE3). Heterologous expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and shaking

the cultures at 28 °C for 12 h. The cells were pelleted by centrifugation (12 000 g, 5 min, 4 °C) and then resuspended in 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.5, lysed ultrasonically and recentrifuged (13 000 g, 10 min, 4 °C). The supernatant was used for the PDIL1-1 assay. Various concentrations of recombinant PDIL1-1 were added to 100 mM Na_2HPO_4, 2 mM EDTA, 1 mg ml^{-1} insulin, 2 mM dithiothreitol (DTT), kept at 30 °C, and the OD_{650} measured at 3 min intervals to assay for disulphide reductase activity. Its molecular chaperone activity was assayed in a 1 ml solution of 500 nM PDIL1-1, 40 mM HEPES NaOH, pH 7.5, 150 nM citrate synthetase, incubated at 42 °C. The OD_{360} of this solution was measured at 10 min intervals.

Detection of PDIL1-1 *product in rice*

To generate polyclonal antibodies recognizing PDIL1-1, recombinant PDIL1-1 protein was gel purified from lysed *E. coli* cells by SDS–PAGE. The gels were immersed in 250 mM KCl to allow excision of the band corresponding to the PDIL1-1 protein, and the excised gel fragment was then ground in liquid nitrogen. The powdered gel fragment was suspended in 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.5, and the suspension of 1 mg ml^{-1} PDIL1-1 was used as antigen for the production of rabbit polyclonal antibodies against PDIL1-1, using a standard protocol (Wang *et al.*, 2010). Western blot assays were based on frozen plant tissue (500 mg) ground in liquid nitrogen and homogenized in 1 ml of buffer with 50 mM TRIS-HCl, pH 8.0, 0.25 M sucrose, 2 mM DTT, 2 mM EDTA, and 1 mM phenylmethylsulphonyl fluoride (PMSF). The protein concentration in these preparations was determined using a commercial kit (Bio-Rad, Herclues, CA). A 10 μg aliquot of protein was resolved by electrophoresis (10% SDS–PAGE), and transferred electrophoretically to polyvinylidene difluoride membrane (0.45 μm; Millipore, www.millipore.com/) using a semi-wet transfer method (Wang *et al.*, 2010). The membranes were incubated in a 1:300 dilution of the polyclonal anti-PDIL1-1 antiserum in 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.5, and the hybridization signal detected by ECL via conjugation with an anti-rabbit IgG linked to horseradish peroxidase (BeyoECL Plus kit, obtained from Beyotime Institute of Biotechnology, Haimen, China).

Results

Phenotype of the T3612 *mutant*

The *T3612* mutant, recovered from cultured tissue of the *japonica* type rice cv. Nipponbare, was similar with respect to its general morphology and panicle size to the wild type (Fig. 1A, B), but produced grain with a floury endosperm (Fig. 1C) and achieved a markedly slower grain-filling rate (Fig. 2). SEM revealed that the starch granules in the mutant's endosperm were round and only loosely packed (Fig. 1E, F). Its mature grain weight was ~66% (Table 1) and its amylose content ~80% that of cv. Nipponbare. Starch contents per dry weight were similar for both the wild type and *T3612*. However, both the total protein and lipid content of the mutant's endosperm were ~15% above those of the wild type. Selfed progeny of plants heterozygous for the mutation segregated in the monogenic ratio three normal to one floury (Fig. 1D), showing that the mutation was based on a single recessive allele.

Fine mapping and complementation of the T3612 *mutation*

An F_2 population was created from the cross of *T3612* with the *indica* type cv. NJ11. The genotyping using 242 genome-wide markers of a sample of 10 progeny expressing the floury endosperm trait suggested the presence of linkage between the trait and the markers RM167 and B11-15, both of which are located on the short arm of chromosome 11 (Fig. 3). A subsequent fine-mapping exercise (details of all the markers used are listed in Supplementary Table S1 at *JXB* online) based on 1242 progeny located the mutation to a chromosomal segment bounded by T3612-31 and T3612-54, and identified T3612-55 as a co-segregating marker. This defined the position of the target gene as lying within a 61 kb region of bacterial artificial chromosome (BAC)
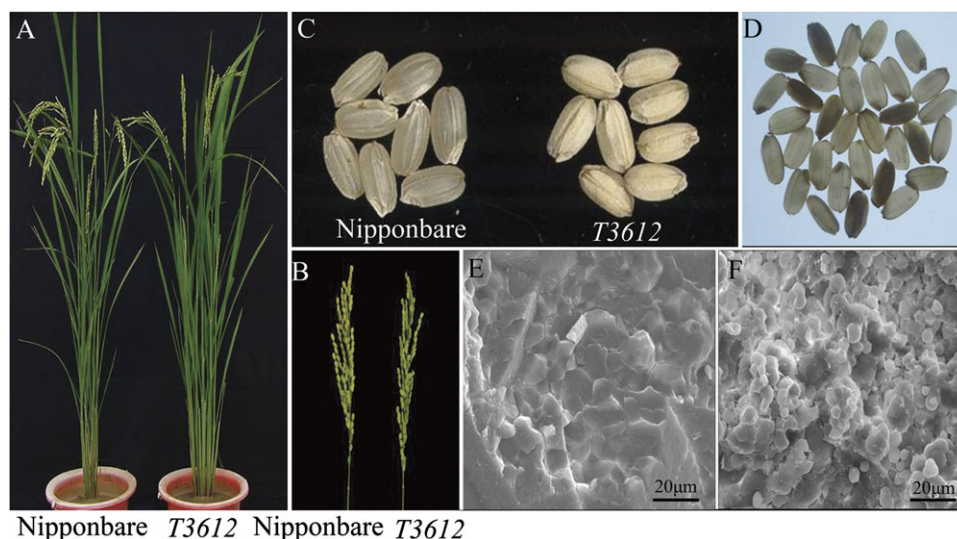

Fig. 1. The phenotype of the *T3612* mutant. (A) cv. Nipponbare and *T3612* plants. (B) Panicles of cv. Nipponbare and *T3612*. (C) The floury endosperm of *T3612* which (D) segregates in a monogenic fashion. Scanning electron micrographs of mature endosperms of (E) cv. Nipponbare and (F) *T3612*.
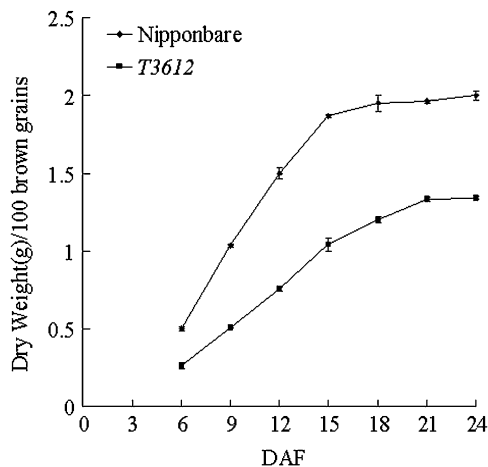
Fig. 2. The grain-filling process (weight in g per 100 grains) in *T3612* and cv. Nipponbare. DAF, days after flowering.

Table 1. Key characteristics of the cv. Nipponbare and T3612 grain

	Weight (g)	Amylose (%)	Starch (%)	Protein (%)	Lipid (%)
Nipponbare	18.69±0.18	14.68±0.26	75.00±0.42	10.88±0.02	2.56±0.06
T3612	12.39±0.25	12.24±0.39	74.55±0.64	12.5±0.02	2.95±0.07
Difference	−33.70%	−19.93%	−0.6%	+14.89%	+15.23%

OSJNBa0058P12. Gene prediction based on RiceGAAS software (ricegaas.dna.affrc.go.jp/) indicated the presence of 11 open reading frames (ORFs) in this segment (Supplementary Table S2). A set of RT-PCR analyses based on these 11 genes showed that the *PDIL1-1* transcript was absent in *T3612* (Fig. 3D). A genomic PCR (based on the primer pair DEL-1F/1R; the sequences of these, as well as of other primers referred to below, are given in Supplementary Table S1) showed that no amplicon was generated in *T3612*, while a PCR based on DEL-2F/-1R amplified the same size product in both *T3612* and cv. Nipponbare. PDI-2F/DEL-1R amplified an ~5 kb product from cv. Nipponbare, but an ~1 kb amplicon was produced from *T3612* (Fig 3D). The derived sequence of the ~1 kb *T3612* amplicon differed from the wild-type sequence by a 4145 bp deletion, starting 124 nucleotides downstream of the *PDIL1-1* ATG start codon. The section of *T3612* sequence around the deletion boundary is shown in Supplementary Fig. S1.

To show that the absence of the *PDIL1-1* transcript was responsible for the floury endosperm trait, its ORF was fused to the *Cauliflower mosaic virus* (CaMV) 35S promoter and then introduced into the *T3612* mutant. Grain harvested from T_1 plants segregated for endosperm type (Fig. 3F). Using a screen based on the hygromycin resistance selection marker, it was possible to show that the vitreous grains carried the transgene, while the floury ones did not. Two genomic PCRs—the first based on the *PDIL1-1* coding region targeted primer pair PDI-2F/-2R, and the second, on PDI-2F/DEL-1R, targeted the *T3612* deletion—-

produced the expected amplicons in the primary transformants (Supplementary Fig. S2 at *JXB* online). A western blot analysis showed that the PDIL1-1 protein was present in vitreous grain harvested from T_1 plants (Fig. 3G). Thus the transgenic constitutive expression of *PDIL1-1* was able to complement the floury endosperm trait.

In order to determine whether PDIL1-1 protein possessed any disulphide reductase and/or molecular chaperone activity it was provided with either insulin or citrate synthetase as a substrate. In the presence of DTT, PDI reduces the intermolecular disulphide bonds between the insulin A and B chains. Precipitation of the insoluble B chain can be measured photometrically by an increase in the absorbance at 650 nm. The assay was performed with recombinant glutathione *S*-transferase (GST)-tagged PDIL1-1. As shown in Fig. 4A, GST–PDIL1-1 was able to accelerate the reduction of insulin, and the rate of this reduction was intensified by increasing the concentration of GST–PDIL1-1, clearly demonstrating that PDIL1-1 possesses disulphide reductase activity *in vitro*. The GST–PDIL1-1 was also able to protect citrate synthetase effectively from thermal denaturation at 42 °C. Precipitation of citrate synthetase decreased by 64.9% in contrast to GST control (Fig. 4B).

Expression of PDIL1-1 in plants

RT-PCR analysis showed that *PDIL1-1* was expressed in the root, culm, leaf, sheath, and young panicle of cv. Nipponbare. Among these organs, the level of expression was lowest in the leaf and highest in the young panicle. T3612 was free of PDIL1-1 transcript (Fig. 5A). In the developing endosperm, peak expression occurred at 12 DAF (Fig. 5C). Immunoblotting showed that PDIL1-1 expression in the wild type occurred in the culm, young panicle, and developing endosperm at 12 DAF (Fig. 5B, D), but was absent from any part of the *T3612* plant.

The absence of PDIL1-1 affects major starch synthesis enzyme activity

A comparison between levels of enzyme activity in the mutant and the wild type showed that there were no significant differences with respect to ISA, Pho2, SSIIIa, or BE (Fig. 6). However, Pho1 activity appeared to be lower in the mutant than in the wild type at 9 DAF. Pullulanase (PUL) activity was greatly reduced in the mutant, and the proteins appeared to have a lower electrophoretic mobility. The activity of SSI was substantially increased. However, qRT-PCR-based evaluations of gene expression suggested that PUL and SSI levels were similar in the mutant and wild type, while that of *Pho1* was increased by only 1-fold (Supplementary Fig. S3 at *JXB* online). Thus it appears possible that the effect of PDIL1-1 is felt post-transcriptionally. UGPase activity was somewhat lower in the mutant than in the wild-type endosperm, while those of SS and AGPase were, respectively, ~30% and 146% higher than in the wild type (Table 2). The expression of *DPE-1* was only ~50% of the level in the wild type. Thus, overall, it was clear that the deletion of *PDIL1-1*
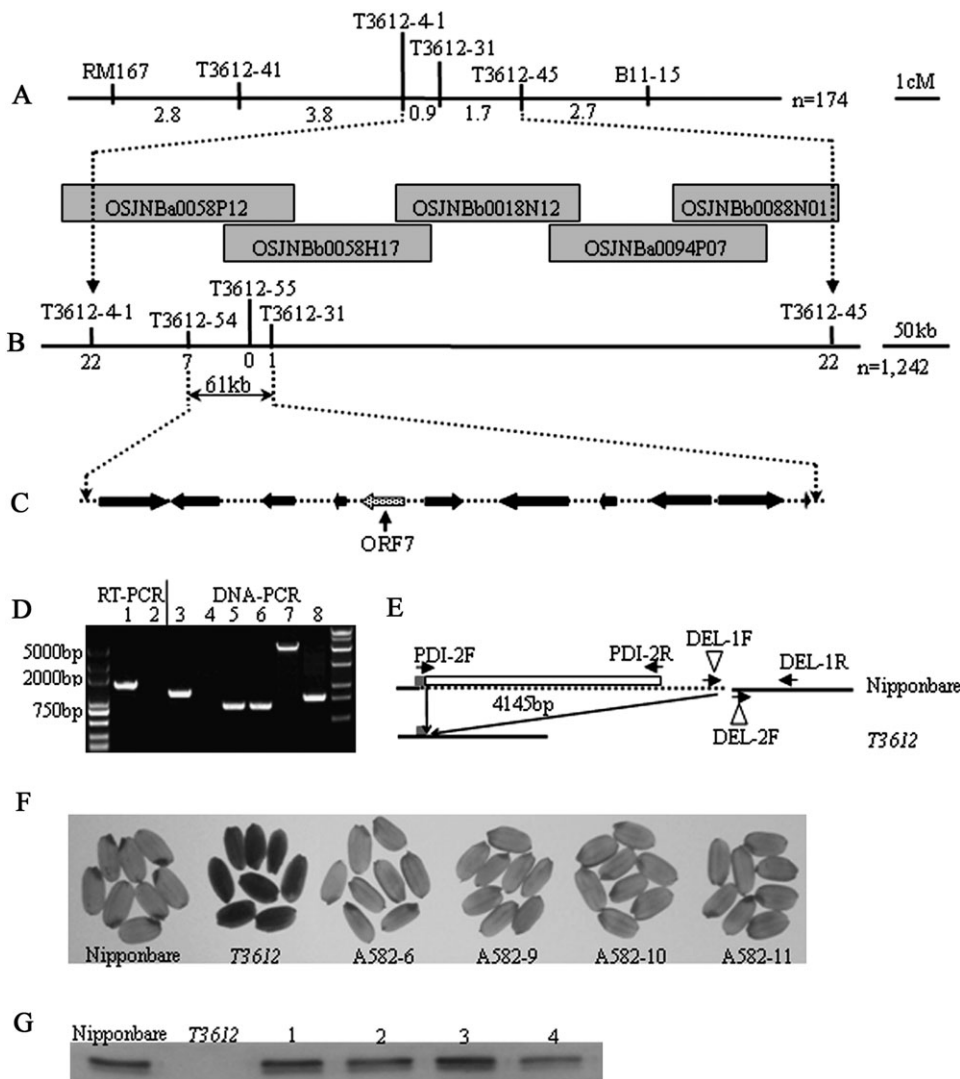
Fig. 3. *OsPDIL1-1* is the gene responsible for the *T3612* mutant phenotype. (A) The mutated gene was mapped to the short arm of chromosome 11 between molecular markers T3612-4-1 and T3612-45. Genetic distances and names of the molecular markers are indicated (*n* =174). OSJNBa0058P12, OSJNBb0058H17, OSJNBb0018N12, OSJNBa0094P07, and OSJNBb0088N01 are BAC clones covering this locus. (B) The location of the mutation was narrowed to a 61 kb genomic region between T3612-54 and T3612-31. The number of recombinants is indicated below the molecular marker (*n* = 1242). (C) Gene prediction indicates 11 open reading frames in the critical region. The candidate gene (ORF7) is shown by an arrow. (D) PCRs based on cDNA (lanes 1,2) and genomic DNA (lanes 3–8) reveal the deletion in the *T3612 PDIL1-1* allele. Lanes 1 and 2, PDI-2F/PDI-2R; lanes 3 and 4, DEL-1F/DEL-1R; lanes 5 and 6, DEL-2F/DEL-1R; lanes 7 and 8, PDI-2F/DEL-1R; lanes 1, 3, 5, and 7, cv. Nipponbare; lanes 2, 4, 6, and 8, *T3612*. (E) The deletion sequence (broken line) and primer annealing sites (indicated by arrows). The white box shows the deleted part of *PDIL1-1*. (F) Phenotypes of T_1 kernels in four independent complemented lines. (G) PDIL1-1 protein was present in vitreous T_1 grains. Lanes 1–4 represent four complementation lines shown in (F).

affected the activities of certain starch synthesis enzymes, and that the loss of Pho1 activity may be a major determinant of the formation of the floury endosperm in the *T3612* mutant.

The effect of the PDIL1-1 deletion on amylopectin structure

DSA-FACE (O'Shea and Morell, 1996; Nishi *et al.*, 2001) was applied to contrast the chain length distribution in the amylopectin synthesized in the *T3612* and wild-type endosperm. The analysis suggested that the mutant amylopectin consisted of a higher proportion of short chains in the range degree of polymerization (DP) 8–13 and a lower proportion of DP 6–7 chains (Fig. 7B). This shift in amylopectin chain lengths agrees with the enhanced SSI and depressed PUL activities in the mutant (Fig. 6A,C).

Analysis of the ER stress-responsive genes

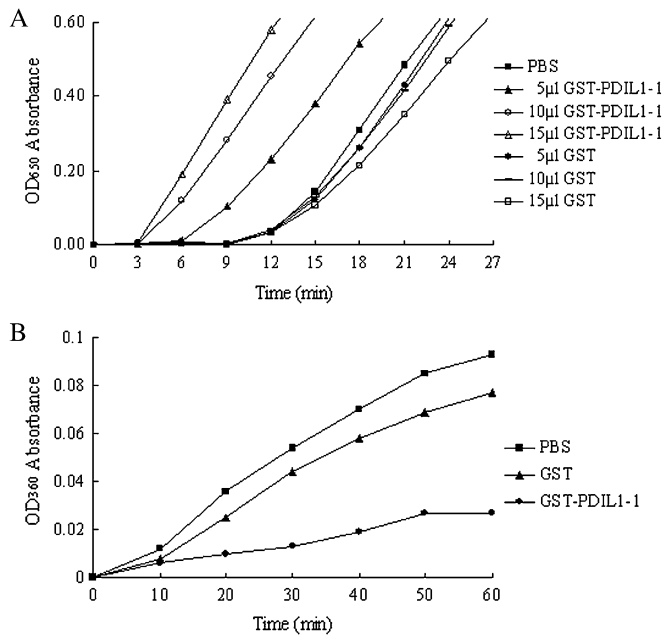As PDIL1-1 is a chaperone protein involved in the formation of inter- and intramolecular disulphide bridges

Fig. 4. Assay for recombinant GST-tagged PDIL1-1 activity. (A) Assay for disulphide reductase activity of GST–PDIL1-1 using the turbidimetric assay of insulin reduction. Phosphate-buffered saline (PBS) and GST are the controls. (B) Assay for molecular chaperone activity of GST–PDIL1-1. PDI can protect citrate synthetase from thermal denaturation in 42 °C. PBS and GST are the controls.
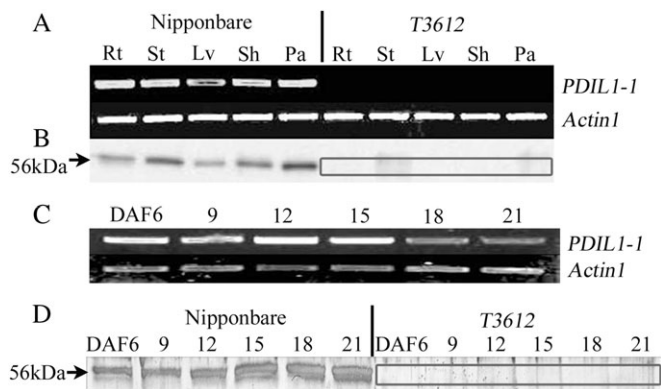

Fig. 5. Expression of *PDIL1-1*. (A) Expression as assayed by RT-PCR. *Actin1* was used as the reference. Rt, root; St, stem; Lv, leaf; Sh, sheath; Pa, panicle. (B) Western blot analysis based on anti-PDIL1-1 polyclonal antibodies. The absence of PDIL1-1 protein in the mutant is highlighted. (C) Expression of *PDIL1-1* in the developing [6–21 days after flowering (DAF)] endosperm of cv. Nipponbare assayed by RT-PCR. (D) Western blot analysis of the presence of PDIL1-1 in the developing endosperm. The absence of PDIL1-1 protein in the mutant is highlighted.

(Wilkinson and Gilbert, 2004; Satoh-Cruz *et al.*, 2010), qRT-PCR and semi-quantitative RT-PCR were used to investigate the expression of a set of ER stress- and PCD-associated genes. Genes encoding 17 chaperone-related (eight PDILs, three BiPs, two Hsp70s, one Hsp90, one CNX, and two CRTs) and four co-chaperone-related (NEF,
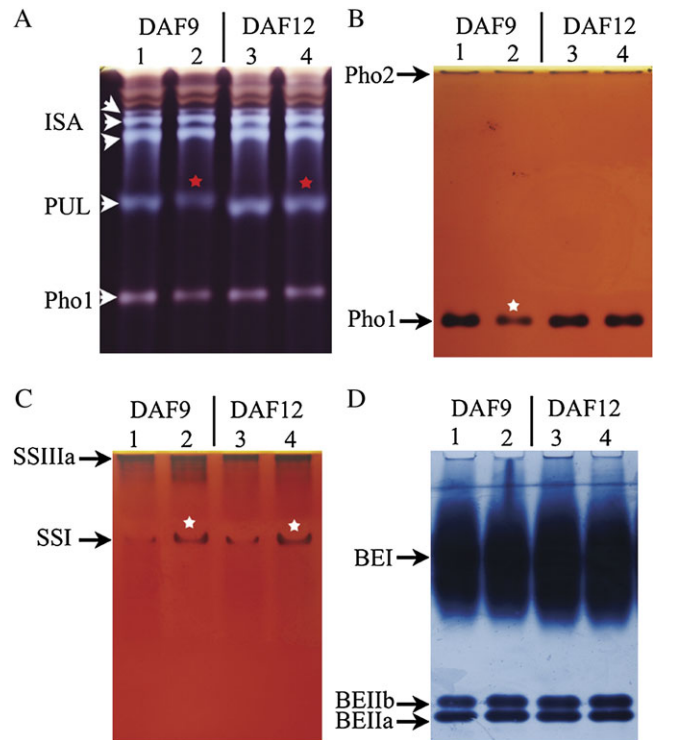

Fig. 6. Enzyme activity in the developing endosperm. (A) Native PAGE/debranching enzyme activity. ISA, PUL, and Pho activity are indicated by arrowheads. (B) Native PAGE/starch phosphorylase activity staining. Pho1 and Pho2 are indicated by arrowheads. (C) Native PAGE/starch synthase activity. SSIIIa and SSI activity are indicated by arrowheads. (D) Native PAGE/branching enzyme activity. BEI, BEIIa, and BEIIb activity are indicated by arrowheads. Lanes 1 and 3, cv. Nipponbare; lanes 2 and 4, *T3612*.

Table 2. The effects of the *T3612* mutation on the activities of enzymes involved in starch and sucrose metabolism in the developing endosperm (12 d after flowering)

Values represent the means ±SD from two replicates.

	Enzyme activity (nmol min^{-1} mg^{-1} FW)		
	AGPase	**UGPase**	**Sucrose synthase**
Nipponbare	5.15±0.40	25.07±1.35	2.88±0.05
T3612	12.7±1.86	23.28±0.25	3.73±0.01

ERDJ3-like, Stt3a, and UDP glucose transporter) proteins, three membrane-associated bZIP transcription factors (bZIP17, bZIP28, and especially bZIP60), the ERAD-related protein (Derlin), and the PCD marker HSR203J were more highly expressed in *T3612* than in wild-type endosperms (Fig. 8). The level of expression of *BiP-2*, both *Hsp70* genes, *CRT-2*, and *NEF* was >5-fold higher in the mutant than in the wild-type endosperm. This suggested that the absence of PDIL1-1 induced a level of ER stress. Of the remaining PDIL genes, only *PDIL2-3* was noticeably up-regulated (4-fold). Nevertheless, large amounts of a 57 kDa proglutelin accumulated in the *T3612* endosperm

(Supplementary Fig. S4 at *JXB* online), which suggested that PDIL2-3 and PDIL1-1 each have their own distinct role in the folding of storage proteins (Onda *et al.*, 2011).

Fig. 7. Amylopectin chain length distribution in the *T3612* and cv. Nipponbare endosperm. (A) Estimated molar chain length distribution for DP 6–56 starch chains. (B) Difference in the amylopectin chain length distribution between *T3612* and cv. Nipponbare.

Discussion

The deletion of PDIL1-1 results in the formation of a floury endosperm

The current picture of cereal starch synthesis has been established from the study of opaque mutants, largely in maize and rice (Myers *et al.*, 2000; Toyota *et al.*, 2006; Hennen-Bierwagen *et al.*, 2008, 2009). However, as further opaque endosperm mutants continue to be uncovered and analysed, a number of genes have been identified which cannot readily be incorporated into the established scheme, thus requiring a re-assessment of the model. The *T3612* mutant is distinct from previous floury endosperm mutants, and has implicated a role for PDIL1-1 in the starch synthesis network operating in the rice endosperm. The only documented association of PDI with starch synthesis in plants relates to the product of *AtPDIL1-3* which is involved in the synthesis of transitory starch in the leaves of *Arabidopsis thaliana* (Lu and Christopher, 2006).

The level of activity of most of the known starch synthesizing enzymes in the *T3612* endosperm was hardly different from the wild-type levels—with the exception of that of Pho1, AGPase, SSI, and PUL. The activity of Pho1 was markedly diminished in *T3612*. Although the specific molecular mechanism of Pho1 in the synthesis of starch is still controversial, circumstantial evidence suggests the involvement of Pho1 in starch synthesis in storage tissues. Pho1 enzyme activity and protein levels are coordinated
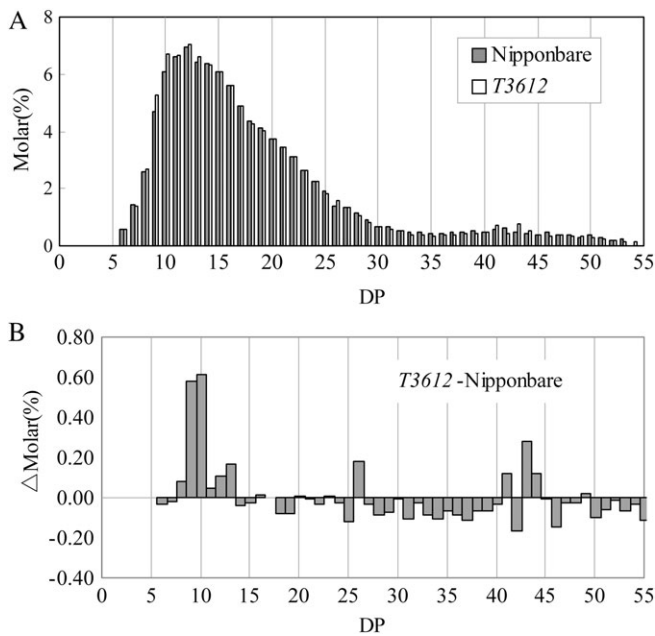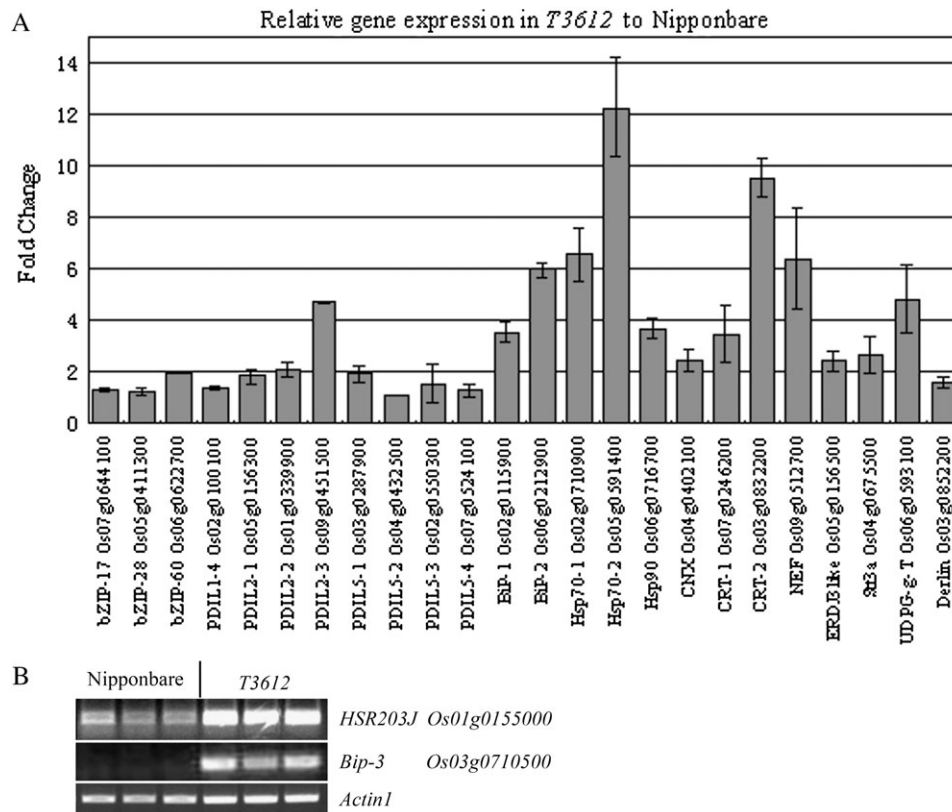

Fig. 8. Expression levels of selected ER stress-associated genes (A) and RT-PCR analysis of expression of the chaperone *BiP-3* and the PCD marker *HSR203J* in *T3612* and cv. Nipponbare (B). Each value reported is the mean ±SD of three biological replicates.

with starch production in potato tuber (Kossmann *et al.*, 1991; St-Pierre and Brisson, 1995), maize (*Zea mays*) endosperm (Ozbun *et al.*, 1973), and wheat endosperm (Schupp and Ziegler, 2004).

Another gene which was substantially down-regulated in *T3612* was *DPE-1*; the primary expression site of this gene is the amyloplast in developing endosperm cells (Ohdan *et al.*, 2005; Bresolin *et al.*, 2006). In *Chlamydomonas reinhardtii*, lack of D-enzyme activity (the *sta11* mutant) results in significantly reduced starch deposition (Colleoni *et al.*, 1999*a*). Although in higher plants the role is unclear, the partially purified active D-enzyme from the endosperm of developing wheat grain was able to elongate glycogen or amylopectin (Bresolin *et al.*, 2006), which share the same function as D-enzyme in *C. reinhardtii* (Colleoni *et al.*, 1999*b*). Future studies on the mutants that lack D-enzyme in rice grains will help elucidate its role in starch synthesis *in vivo*.

Both AGPase and SSI activities in *T3612* were higher than those of the wild type, and similar observation was made in the rice *SSIIIa* mutant (Fujita *et al.*, 2007). High levels of sugars enhance the expression of the maize genes *sh2* and *bt2* that encode the large and small subunits of AGPase (Giroux and Hannah, 1994). Therefore, the high level of sugars in *T3612* (data not shown) may enhance the expression of the genes *OsAGPL2*, *OsAGPL3*, *OsAGPS2b*, and *OsAGPS2a* which led to the higher AGPase activity.

Not only was PUL activity lowered in the mutant, but its electrophoretic mobility was also changed. A similar change to PUL has been noted in an *ISA1* mutant (Kubo *et al.*, 2005). The *PUL*-null mutation had no effects on the seed morphology and crystallinity of starch granules, except that the short chains (DP ≤13) of amylopectin were increased. The *T3612* mutant overproduced amylopectin chains in the range DP 8–13 and underproduced the shorter DP 6–7 chains. The change in the mature endosperm amylopectin structure was consistent with the observed increase in SSI activity and decrease in PUL activity (Figs 6, 7).

Therefore, further work will be needed to elucidate whether the reduced activity of Pho1 and the down-regulation of *DPE-1* had a major effect on starch synthesis in the *T3612* endosperm.

The absence of PDIL1-1 had a deleterious effect on storage protein synthesis

The fine mapping and complementation analysis provided ample evidence that the floury endosperm phenotype of the *T3612* mutant was due to the absence of PDIL1-1. The rice *esp2* mutant also apparently lacks a functional allele of *PDIL1-1* (Takemoto *et al.*, 2002), while its mature endosperm accumulates large quantities of a 57 kDa proglutelin, which is mislocalized to an unusual, small ER-derived protein body I (PBI). This observation has been taken to suggest that PDIL1-1 has a role in grain storage protein trafficking in the developing endosperm. No mention has been made as to whether the *esp2* mutant produces floury endosperm. The *T3612* endosperm accumulated large

quantities of a 57 kDa proglutelin (Supplementary Fig. S4 at *JXB* online) and produced an unusually small PBI (Supplementary Fig. S5). In addition, the expression of a number of grain storage protein genes was markedly suppressed in the mutant's endosperm at 9 DAF, a time when grain storage proteins began to appear in abundance in cv. Nipponbare (Supplementary Fig. S6). PDIL1-1 was thus clearly involved in the synthesis of both grain storage protein and starch.

In the ER lumen, PDIL proteins are thought to assist in the folding of immature secretory proteins, in co-operation with other chaperones such as the BiPs, CNX, and CRT. PDI and BiP are the two main classes of chaperone in the ER lumen involved in the folding of grain storage proteins (Satoh-Cruz *et al.*, 2010; Wakasa *et al.*, 2011). Glutelin is abundant enough in the endosperm to allow defects, such as in *T3612* and *esp2*, to be readily detectable by SDS–PAGE. Of course, many other proteins—perhaps including those involved in starch synthesis—may be similarly misfolded, but this can be hard to identify. In *A. thaliana*, a proteomics approach has shown that >8% of the chloroplast proteins carry signal peptides directing translocation to the ER (Kleffmann *et al.*, 2004). One suggestion, therefore, is that some at least of such proteins are incorrectly folded in the *T3612* mutant as a result of the absence of PDIL1-1, with consequences for starch metabolism in the plastids.

ER stress interacts with grain starch synthesis

The severe suppression of *BiP1* is known to result in the formation of a floury endosperm; along with a reduction in starch accumulation, these endosperms suffer from acute ER stress. Microarray and RT-PCR-based analyses have shown that many of the genes involved in ER stress are strongly up-regulated (Wakasa *et al.*, 2011), and many of these same genes were also up-regulated in the *T3612* endosperm. It is assumed, therefore, that the deletion of *PDIL1-1* resulted in severe ER stress. In the maize opaque mutants *fl2*, *Mc*, and *De-B30*, ER stress is induced by the accumulation of several abnormal zeins (Lending and Larkins, 1992; Kim *et al.*, 2004). The expression of chaperone genes such as *BiP* and *PDI* is also increased in these mutants (Hunter *et al.*, 2002). The overexpression of *BiP1* or human *amyloid β* also generates ER stress and produces floury endosperms, which achieve a reduced level of starch accumulation (Oono *et al.*, 2010; Wakasa *et al.*, 2011). In all of these examples, opaque mutant endosperm cells are prone to ER stress, which suggests that it may be the ER stress itself which contributes to the formation of the opaque phenotype. The overall conclusion is that the mutant endosperm suffers from persistent and/or acute ER stress, and this both serves as a trigger for PCD and compromises the normal starch synthesis process. The forward focus is on the identification of the amyloplast proteins which interact with PDIL1-1 and the demonstration that PCD is occurring in the *T3612* endosperm.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Part of the sequence of the deletion boundary in *T3612*.

Figure S2. Genomic PCR from four independent T_1 complemented lines (A582-6, -9, -10, and -11). Lane 1, cv. Nipponbare; lane 2, *T3612*.

Figure S3. Expression levels of various genes involved in starch synthesis. Each value reported is the mean ±SD of three biological replicates.

Figure S4. SDS–PAGE analysis of total mature grain protein.

Figure S5. TEM images of the developing endosperm of (A) cv. Nipponbare and (B) *T3612*.

Figure S6. Expression levels of various genes involved in grain storage protein synthesis. Each value reported is the mean ±SD of three biological replicates.

Table S1. Primer sequences used.

Table S2. The 11 predicted genes present in the candidate region of chromosome 11 containing the floury endosperm mutation of *T3612*.

Acknowledgements

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