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RESEARCH PAPER

Small GTPase Sar1 is crucial for proglutelin and α-globulin export from the endoplasmic reticulum in rice endosperm

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Abstract

Rice seed storage proteins glutelin and α-globulin are synthesized in the endoplasmic reticulum (ER) and deposited in protein storage vacuoles (PSVs). Sar1, a small GTPase, acts as a molecular switch to regulate the assembly of coat protein complex II, which exports secretory protein from the ER to the Golgi apparatus. To reveal the route by which glutelin and α-globulin exit the ER, four putative *Sar1* genes (*OsSar1a/b/c/d*) were cloned from rice, and transgenic rice were generated with *Sar1* overexpressed or suppressed by RNA interference (RNAi) specifically in the endosperm under the control of the rice glutelin promoter. Overexpression or suppression of any *OsSar1* did not alter the phenotype. However, simultaneous knockdown of *OsSar1a/b/c* resulted in floury and shrunken seeds, with an increased level of glutelin precursor and decreased level of the mature α- and β-subunit. *OsSar1abc* RNAi endosperm generated numerous, spherical, novel protein bodies with highly electron-dense matrixes containing both glutelin and α-globulin. Notably, the novel protein bodies were surrounded by ribosomes, showing that they were derived from the ER. Some of the ER-derived dense protein bodies were attached to a blebbing structure containing prolamin. These results indicated that OsSar1a/b/c play a crucial role in storage proteins exiting from the ER, with functional redundancy in rice endosperm, and glutelin and α-globulin transported together from the ER to the Golgi apparatus by a pathway mediated by coat protein complex II.

Key words: Endoplasmic reticulum, OsSar1, protein body, protein intracellular transport, protein storage vacuole, rice storage proteins.

Introduction

Rice seed storage proteins are mainly composed of three groups with distinct solubility profiles: alcohol-soluble prolamin, acid- and alkaline-soluble glutelin, and saline-soluble α-globulin. The prolamins are synthesized in the endoplasmic reticulum (ER) and retained in the ER lumen, forming a spherical protein body PB-I (Bechtel and Juliano, 1980; Tanaka *et al*., 1980; Yamagata and Tanaka, 1986). Glutelins are initially synthesized on the ER as 57 kDa precursors and are transported to the protein storage vacuole (PSV; also called PB-II) through or bypassing the Golgi apparatus. These precursors are then cleaved to 37–39 kDa acidic and 19–20 kDa basic subunits and stored in the PSV (Yamagata *et al*., 1982; Krishnan *et al*., 1986; Yamagata and Tanaka, 1986). The α-globulin is deposited together with glutelin in the PSV.

Many studies of glutelin intracellular transportation have focused on factors related to protein folding, movement from the Golgi apparatus to the PSV, and processing in the PSV. Protein disulphide isomerase (PDI) plays an essential role in proglutelin folding and the segregation of proglutelin and prolamin polypeptides within the ER lumen. In mutants with loss of function of PDI1-1 (*esp2*), glutelin heterotypically interacts with prolamin polypeptides within the ER lumen, which leads to the formation of new PBs containing

both glutelin and prolamin in the ER (Takemoto *et al*., 2002; Satoh-Cruz *et al*., 2010). OsRab5a, a small GTPase, is implicated in the intracellular transport of proglutelin from the Golgi to the PSV, and helps maintain the general structure of the endomembrane system in developing rice seeds. In *OsRab5a* mutants (*gpa1* and *glup4*), some of the glutelin and α-globulin is not delivered to the PSV but is secreted extracellularly to form unique paramural bodies (Wang *et al*., 2010; Fukuda *et al*., 2011). The vacuolar processing enzyme (VPE) is essential for proper PSV structure and compartmentalization of rice storage proteins in the PSV. In mutants of VPE (*W379* and *glup3*), glutelin is deposited in the PSV as a precursor and the PSV lacks the typical crystalline lattice structure (Wang *et al*., 2009; Kumamaru *et al*., 2010). In addition, independent mutants such as *glup1*, *2*, *5*, *6*, and *7* have been reported, which suggests that many genes participate in rice storage protein targeting, transport, and deposition (Ueda *et al*., 2010), although the genes have not been identified. However, little is known about how rice storage proteins are exported from the ER.

Golgi-dependent and -independent pathways are involved in storage protein transport from the ER to the PSV. The direct ER–PSV pathway involving precursor-accumulating (PAC) vesicles has been reported in developing pumpkin seeds (Hara-Nishimura *et al*., 1998) and in rice (Takahashi *et al*., 2005). However, most storage proteins travel through the Golgi apparatus to the PSV via dense vesicles (DVs) (Jolliffe *et al*., 2005; Vitale and Hinz, 2005). In rice, glutelin and α-globulin have been detected in Golgi-associated DVs in developing endosperm cells, which suggests that the Golgi apparatus plays an important role in rice storage protein transport to the PSV (Krishnan *et al*., 1986, 1992; Fukuda *et al*., 2011; Washida *et al*., 2012).

Protein is exported from the ER to the Golgi apparatus via the coat protein complex II (COPII) (Barlowe, 2002). The COPII consists of five proteins: secretion-associated, Ras-related protein 1 (Sar1), and Sec23, Sec24, Sec13, and Sec31. Sar1 is a small GTPase, and Sec23/24 and Sec13/31 are two structural heterodimers that form the inner and outer COPII coat (Barlowe *et al*., 1994). Sar1 plays a crucial role in initiating the COPII assembly on the ER. Sar1-GTP activated by Sec12 binds to the ER membrane and recruits Sec23/24 and then Sec13/31 heterodimers to form the COPII (Gurkan *et al*., 2006). Sar1 also participates in cargo selection, membrane curvature, and COPII vesicle fission, and controls membrane constriction to regulate ER export in mammalian cells (Giraudo and Maccioni, 2003; Lee *et al*., 2005; Sato and Nakano, 2005, 2007; Long *et al*., 2010). Sar1 is highly conserved in eukaryotes, and its homologues have been identified in *Arabidopsis* (d'Enfert *et al*., 1992; Bar-Peled and Raikhel, 1997; Vernoud *et al*., 2003), *Brassica campestris* (Kim *et al*., 1997), and tobacco (Takeuchi *et al*., 1998). The Sar1 dominant-negative mutant in the GTP-restricted form inhibits protein transport from the ER to the Golgi apparatus, which results in destroyed Golgi integrity in tobacco and *Arabidopsis* cells (Takeuchi *et al*., 2000; Phillipson *et al*., 2001; daSilva *et al*., 2004; Yang *et al*., 2005; Hanton *et al*., 2008; Osterrieder *et al*., 2009). *Arabidopsis* contains several Sar1 isoforms. Despite highly identical amino acid sequences, AtSARA1a and AtSARA1b show different localization and different levels of inhibition of soluble α-amylase secretion, which suggests functional heterogeneity among Sar1 isoforms in plants (Hanton *et al*., 2008). Functional studies of the Sec24 isoforms in *Arabidopsis* also suggested diverse functions of plant COPII components (Faso *et al*., 2009; Nakano *et al*., 2009; Conger *et al*., 2011). However, the components of the COPII vesicle have not been reported in rice.

In this study, the function of Sar1 in rice was identified by stable endosperm-specific knockdown transformation. Deficiency of OsSar1 in rice endosperm blocked the exit of glutelin and α-globulin from the ER, forming novel electron-dense PBs. The data indicated that the rice storage proteins glutelin and α-globulin were exported from the ER to the Golgi apparatus by a COPII-mediated pathway. The identification and characterization of OsSar1 can help in understanding the detailed mechanism of intracellular transportation of seed storage proteins.

Materials and methods

Isolation of OsSar1a/b/c/d and their promoters

Total RNA from rice was isolated by the TRIpure reagent method (Bioteke), and first-strand cDNA was generated as a template for amplification of *OsSar1a/b/c/d* coding sequences. Promoters of each *OsSar1* were amplified by PCR with rice genomic DNA used as a template. All primer sequences are listed in Supplementary Table S1 available at *JXB* online. The PCR products were cloned into a pMD18-T vector (Takara), then inserts were sequenced.

GFP and mCherry fusion constructs for transient expression in rice protoplasts

Green fluorescent protein (GFP) or mCherry was fused to the C-terminus of *Sar1a/b/c/d* (Sar1–GFP or Sar1–mCherry). The ER marker (mCherry–HDEL) was created by combining the signal peptide of AtWAK2 at the N-terminus of mCherry and the ER retention signal His–Asp–Glu–Leu at its C-terminus. The Golgi marker (GmMan1–mCherry) was created by combining the cytoplasmic tail and transmembrane domain (first 49 amino acids) of GmMan1 at the N-terminus of mCherry. The chimeric genes were subcloned into pBI221 under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter to obtain transient expression vectors, which were co-transformed into rice protoplasts as described (Chen *et al*., 2006). Transformed cells were examined under a confocal microscope (Leica TCS SP5) and digital images were recorded. The images were presented as stacks of neighbouring sections according to the cell diameter. The three-dimensional reconstruction functions were employed to compute projections of serial confocal sections.

The soluble and membrane fractions of transformed protoplasts were prepared as described (Hanton *et al*., 2008).

Construction of the binary vectors and rice transformation

To construct the *OsSar1a/b/c/dpro-GUS* vector, promoters were introduced upstream of the *gusA* gene, which encodes β-glucuronidase (GUS) in the binary vector of pCAMBIA1300. To construct the gene overexpression vector, the *OsSar1a/b/c/d* fragments were inserted in the binary vector pGPTV-GluC-GUS-35S-HPT (Qu *et al*., 2008) containing the endosperm-specific *GluC* promoter by replacing the *GUS* gene. To construct the RNA interference (RNAi) vector, the specific sequence was amplified and ligated to the GluA2-pTCK303 vector, with the endosperm-specific GluA2 promoter replacing the

ubiquitin promoter (Wang *et al*., 2004). All primer sequences used above are listed in Supplementary Table S1 at *JXB* online.

The binary vectors were introduced into rice (*Oryza sativa* cv. Kitaake) by *Agrobacterium tumefaciens*-mediated transformation as described (Qu *et al*., 2005). Successful transformants were verified by PCR and grown in a greenhouse as described (Qu *et al*., 2008).

Analysis of GUS expression

Histochemical analysis of GUS expression was conducted as described (Jefferson *et al*., 1987). Different tissues of *OsSar1a/b/c/dpro-GUS* transgenic lines were vacuum-infiltrated for 15 min in GUS staining buffer and then incubated at 37 °C for 12 h. The organs were destained in 70% ethanol until chlorophyll was removed.

Real-time quantitative PCR

Total RNA extraction and first-strand cDNA synthesis was as described above. Real-time quantitative PCR (qRT-PCR) involved the LightCycler480 real-time PCR system (Roche). The rice *Actin-1* gene was used as the endogenous control, and all experiments involved at least three biological replicates. All primer sequences used for qRT-PCR are given in Supplementary Table S1 at *JXB* online.

Protein extraction, SDS–PAGE, and western blot analysis

Total protein was extracted from seeds with use of 0.125 M TRIS-HCl, pH 6.8, 4% SDS, 4 M urea, and 2% β-mercaptoethanol. Proteins were resolved and separated by 13.6% SDS–PAGE, then transferred onto a polyvinylidene difluoride (PVDF) membrane, which was blocked with non-fat milk and incubated with antibodies, then alkaline phosphatase-conjugated secondary antibodies for immunodetection.

Transmission electron microscopy and immunogold localization

Transverse sections of developing seeds were fixed in PIPES buffer (pH 7.2) as described (Kumamaru *et al*., 2010). Samples were embedded in Spurr's low-viscosity resin and sectioned into semi-thin and ultra-thin sections. Semi-thin sections were prepared for immunofluorescence analysis. Ultra-thin sections were stained with uranyl acetate and lead citrate solution, then observed by transmission electron microscopy (TEM; JEM-1230, Hitachi).

For immunogold localization, ultra-thin sections were blocked with 1% bovine serum albumin (BSA) in TRIS-buffered saline (TBS) buffer, then incubated with diluted antibodies. With non-specifically bound antibodies rinsed off, sections were incubated with gold-conjugated secondary antibodies (15 nm or 5 nm gold particles) and stained with uranyl acetate, then observed by TEM.

Immunofluorescence microscopy

Semi-thin sections were treated with blocking buffer containing 0.9% BSA in phosphate-buffered saline (PBS), then incubated with diluted antibody in blocking buffer. After non-specifically bound antibodies were washed off, sections were incubated with secondary antibody with fluorescein isothiocyanate (FITC) or rhodamine red (Invitrogen), then examined by confocal microscopy (Zeiss 510).

Antibodies

For OsSar1 antibody production, *OsSar1c* cDNA was cloned into the pGEX-2T vector, and the glutathione *S*-transferase (GST)-tagged OsSar1 fusion protein was purified. Polyclonal antibody against OsSar1 was then raised in mice and diluted 1:1000. Antibody against glutelin basic subunits was raised in mice and diluted 1:5000. Antibodies against α-globulin, 14 kDa prolamin, and OsBip1 were raised in rabbits and diluted 1:1000. Antibodies against α-tubulin (Sigma) and OsCBL (Beijing protein innovation, China) were diluted 1:1500.

Accession numbers

OsSar1a (Os01g23620), OsSar1b (Os12g37360), OsSar1c (Os01g15010), OsSar1d (Os06g12090), AtSar1a (At1g09180), AtSar1b (At1g56330), NtSar1a (BAA13463), NtSar1b (AAF17254), BcSar1a (AAC49716), BcSar1b (AAC49717), LeSar1 (AAA34168), ScSar1 (CAA35978), CeSar1 (CCD73074), MmSar1a (AAH05549), MmSar1b (AAH82550), HsSar1a (AAH03658), and HsSar1b (AAH02847).

Results

Isolation of OsSar1 genes from rice

A survey of the rice (*O. sativa*) genome revealed four *Sar1* homologues, named *OsSar1a*, *OsSar1b*, *OsSar1c*, and *OsSar1d*. The first three homologues encode a polypeptide with 193 amino acids and the latter encodes a polypeptide with 194 amino acids. The amino acid sequences of OsSar1a, b, and c are nearly 90% identical to that of AtSar1 and NtSar1. The amino acid sequence of OsSar1a shares 96, 93, and 78% identity with those of OsSar1b/c/d, respectively (Fig. 1A). A Neighbor–Joining phylogenetic tree was constructed for the 17 Sar1 proteins from plants, animals, and yeast (Fig. 1B). Although Sar1 is highly conserved in eukaryotes, 17 proteins could be classified into two clades, with all of the plant Sar1 proteins in the same clade. OsSar1a, b, and c belong to a group with AtSar1a and AtSar1b, while OsSar1d does not. The phylogenetic analysis suggests that Sar1 proteins are evolutionarily conserved in plants, and OsSar1a, b, and c may function similarly to AtSar1.

OsSar1 isoforms were distributed on the ER and ER export sites (ERES) in rice protoplasts

To investigate the subcellular localization of OsSar1, GFP was fused to the C-terminus of the four Sar1 isoforms and the constructs were transiently expressed in rice protoplasts. Co-expression of each Sar1 isoform with the ER marker mCherry–HDEL showed that the green fluorescence of OsSar1–GFP was partly merged with the red fluorescence of mCherry–HDEL, which indicated that the OsSar1 proteins distributed on the ER. In addition to the reticular structures of OsSar1 proteins, multiple punctate structures were observed (Supplementary Fig. S1A at *JXB* online).

To determine whether the OsSar1 punctate structures were similar to those of NtSar1 and AtSar1 (daSilva *et al*., 2004; Hanton *et al*., 2008), each OsSar1–GFP was co-expressed with the Golgi marker Man1–mCherry. In cells transformed with both OsSar1–GFP and Man1–mCherry, most of the punctate structures of OsSar1–GFP co-localized with the Golgi marker (Fig. 2A). To examine whether the OsSar1 was distributed at ERES, OsSar1 was fused with mCherry (OsSar1–mCherry) and co-expressed with yellow fluorescent protein (YFP)–AtSec24, which labels ERES (Hanton *et al*.,

2009), in rice protoplast cells. The punctate structures labelled with OsSar1–mCherry coincided with YFP–AtSec24 fluorescence (Fig. 2B), and the distribution pattern of the four Sar1 proteins was similar. These data indicated that the OsSar1 isoforms were distributed at ERES, at the ER, and in the cytosol.

In *Arabidopsis*, AtSARA1b is more membrane associated than is AtSARA1a (Hanton *et al*., 2008). To determine the intracellular partitioning of OsSar1, soluble and membrane proteins were extracted from OsSar1–GFP-expressing protoplasts for western blot analysis with antibody against GFP. Each Sar1 isoform was detected in soluble and membrane fractions, but the expression of all isoforms was higher in the soluble than in the membrane fractions (Supplementary Fig. S1B at *JXB* online). This subcellular distribution of OsSar1 isoforms was similar to that of AtSar1a but differed from that of AtSar1b (Hanton *et al*., 2008).

OsSar1a, b, and c are expressed universally in rice

qRT-PCR of each *OsSar1* revealed that *OsSar1a*, *b*, and *c* were expressed in all examined tissues; however, the expression of *OsSar1d* was barely detectable (Supplementary Fig. S2 at *JXB* online). *OsSar1a* was highly expressed in vegetative tissues such as seedlings, leaf sheaths, and leaves. Expression was lower for *OsSar1b* than for *OsSar1a* in most tissues, but higher in leaves and leaf sheaths than in other tissues. *OsSar1c* expression was abundant in leaves, leaf sheaths, developing embryo, and endosperm.

The expression pattern of *OsSar1* homologues was also verified by a stable transgenic strategy using GUS as a reporter gene driven by the native promoter of each *OsSar1*. GUS signals were detected in callus, young leaves, rootlet, sheath, mature leaves, stems, nodes, glume, stamen, pistil, and seeds of constructs containing the promoter for *OsSar1a/b/c* (Supplementary Fig. S3 at *JXB* online). *OsSar1d* GUS signal was not detected in any tissues because of its low expression. These results were consistent with the qRT-PCR results and indicate that *OsSar1a*, *b*, and *c* are ubiquitously expressed in plants, with *OsSar1a* mainly expressed in vegetative tissues and *OsSar1c* abundant in seeds.

No phenotype alteration with endosperm-specific overexpression or knockdown of a single OsSar1

To reveal the function of each OsSar1 in ER export of rice storage proteins *in vivo*, transgenic rice were generated with *OsSar1* specifically overexpressed or suppressed in endosperm under the control of glutelin *GluC* and *GluA-2* promoters, respectively. Successful transformants were screened by RT-PCR. At least five independent transgenic lines were obtained for each construct. The transcription of each *OsSar1* was stronger in *OsSar1a/b/c/d*-overexpressing transformants than in the wild type (Supplementary Fig. S4 at *JXB* online). The phenotypes of *OsSar1*-overexpressing seeds were normal. The protein profiles of *OsSar1a/b/c/d*-overexpressing transformants did not differ from that of the wild type, especially

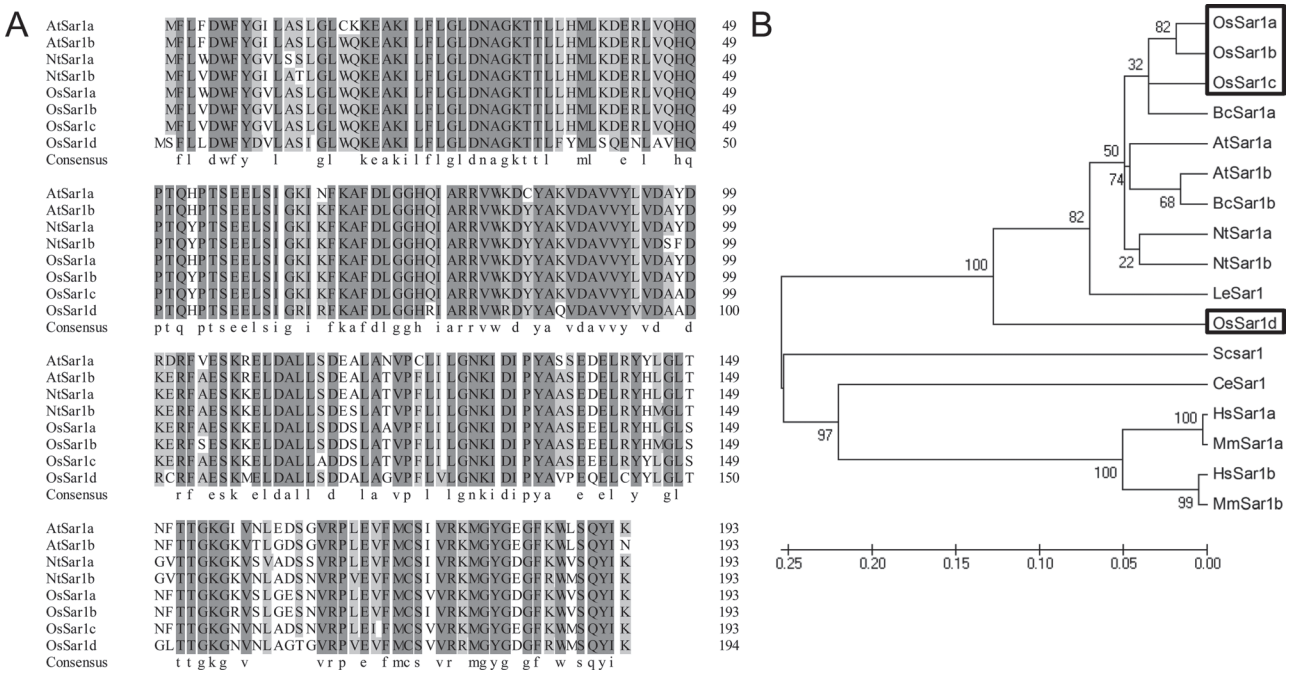

Fig. 1. Multiple sequence alignment of Sar1. (A) Amino acid sequence alignment of OsSar1a/b/c/d by the DNAMAN program. (B) Phylogenetic tree of Sar1. Species designations: animals, Ce, *Caenorhabditis elegans*; Mm, *Mus musculus*; Hs, *Homo sapiens*; yeast, Sc, *Saccharomyces cerevisiae*; plants, At, *Arabidopsis thaliana*; Bc, *Brassica campestris*; Le, *Lycopersicon esculentum* (*Solanum lycopersicum*); Nt, *Nicotiana tabacum*; Os, *Oryza sativa*. The phylogenetic tree was constructed by use of the MEGA program (Tamura *et al*., 2011) and used full-length protein sequences by the Neighbor–Joining method with the following parameters: bootstrap (1000 replicates; random seed), pair-wise deletion, and Poisson correction.

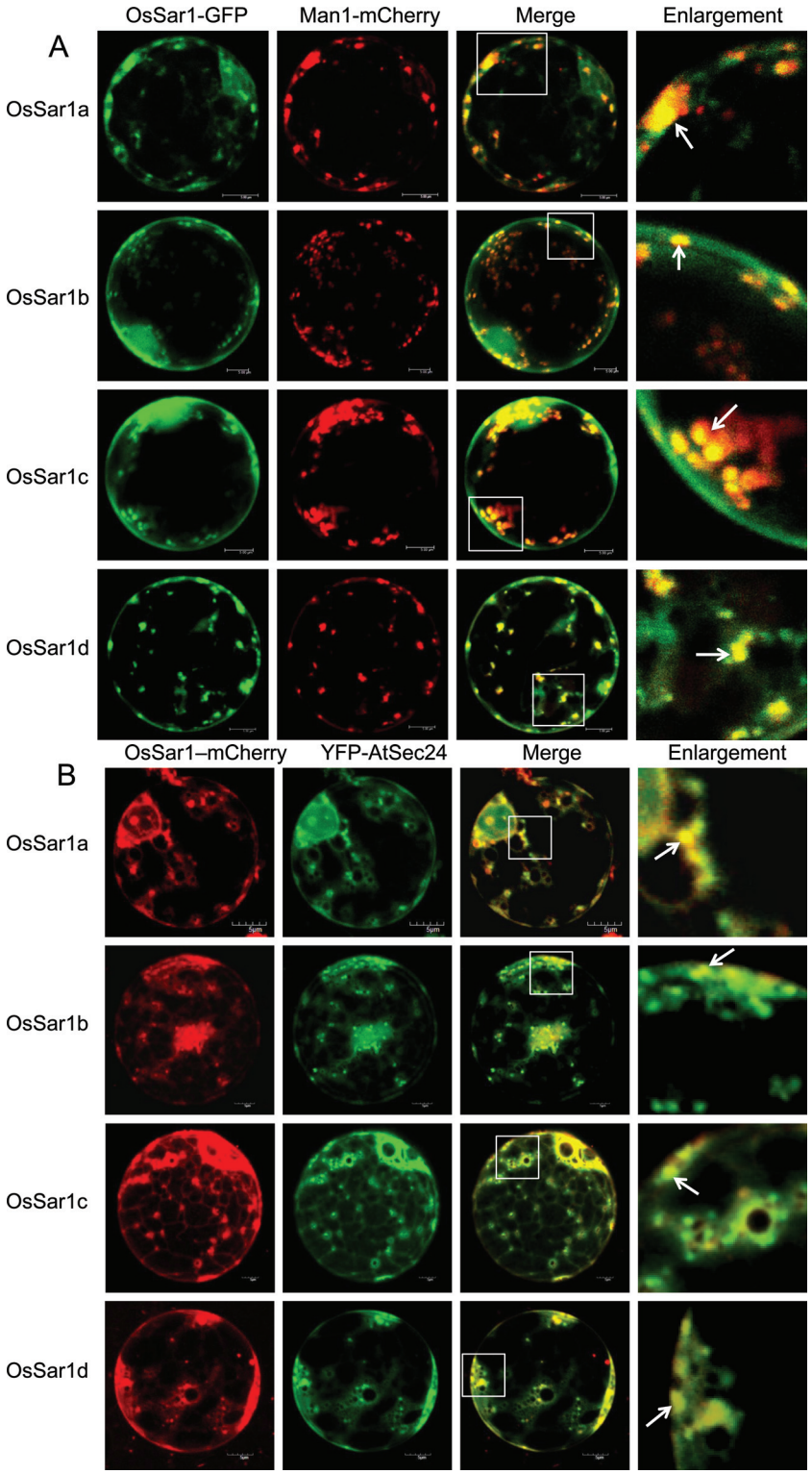

Fig. 2. Subcellular localization of Sar1 in rice protoplasts. Rice protoplasts were co-transformed by OsSar1a/b/c/d–GFP with GmMan1–mCherry (A) or OsSar1a/b/c/d–mCherry with YFP–AtSec24 (B). Confocal images of GFP/YFP signal (green), mCherry signal (red), and merged signal. The white arrow indicates the merged punctate structures. Bars=5 μm.

the 57 kDa glutelin precursor (Supplementary Fig. S5A). This implied that wild-type seeds contained enough OsSar1 for COPII assembly to transport the storage proteins.

An endosperm-specific RNAi knockdown vector was constructed using the 3'-untranslated regions (UTRs) of *OsSar1a*, *b*, or *c* because of the high similarity of *OsSar1* coding sequences. The expression of *OsSar1a*, *b*, or *c* was significantly knocked down in seeds of RNAi transformants (Supplementary Fig. S6 at *JXB* online). The phenotype of *OsSar1a*, *b*, or *c* RNAi transformant seeds was identical to

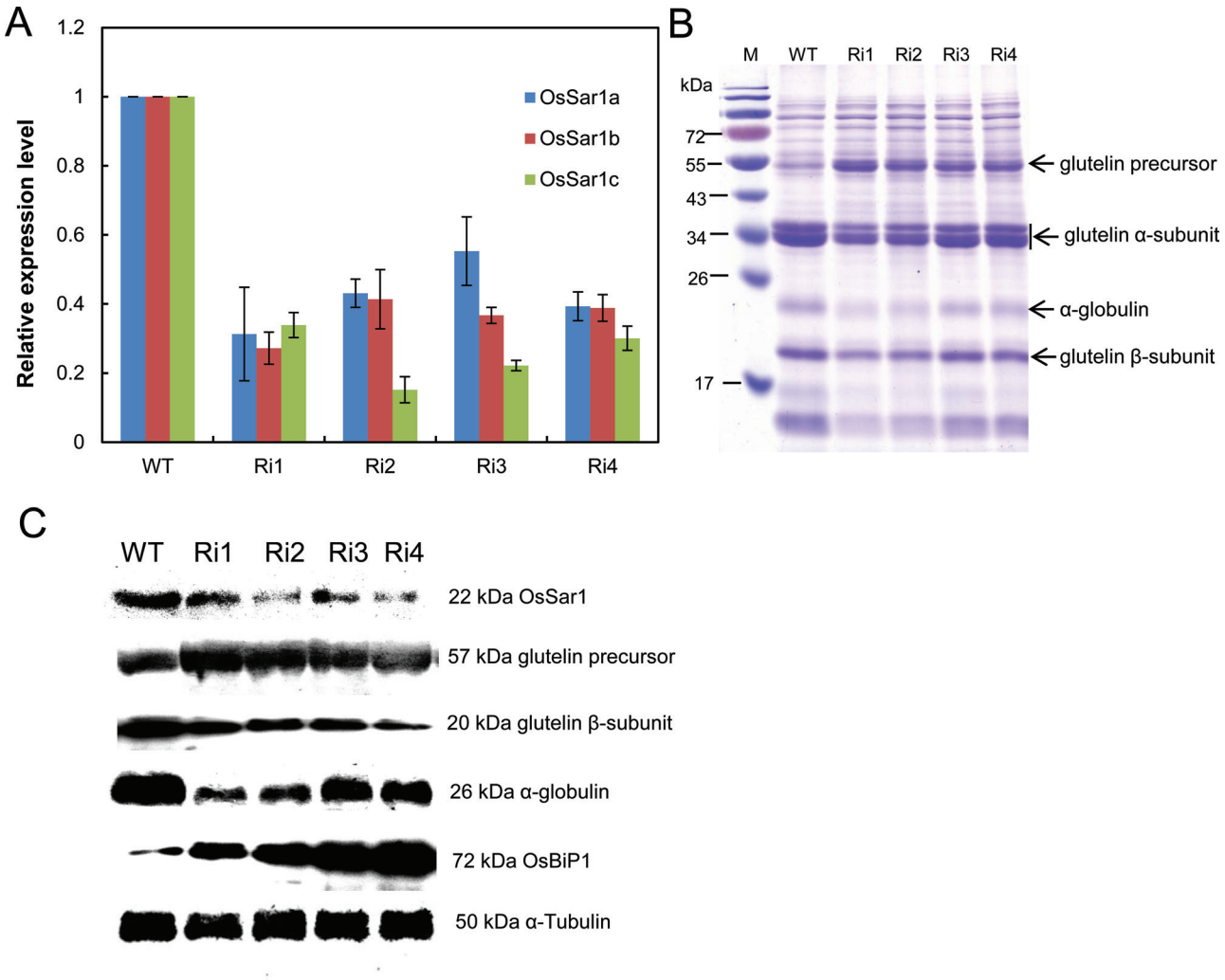

Fig. 3. Expression of OsSar1 in *OsSar1abc* RNAi seed. (A) Quantitative RT-PCR (qRT-PCR) analysis of mRNA levels of *OsSar1a*, *b*, and *c* at 15 DAF in seeds of transformants. (B) SDS–PAGE analysis of seed storage protein in *OsSar1a/b/c* RNAi transformant seeds. (C) Western blot analysis of protein levels of OsSar1, glutelin precursor and basic subunit, α-globulin, and OsBiP1 in *OsSar1abc* RNAi transformant seeds. The α-tubulin was used as a loading control. (This figure is available in colour at *JXB* online.)

that of the wild type, with no difference between the wild type and transformants in seed storage protein profiles (Suppelementay Fig. S5B). Therefore, deficiency of any OsSar1 isoform did not affect storage protein transport, and functional redundancy may exist among OsSar1a/b/c in rice endosperm. Because the transcription of *OsSar1d* was low and GUS signal was not detectable in seeds, *OsSar1d* RNAi knockdown was not performed.

OsSar1abc RNAi produces abnormal seeds and accumulates proglutelin

To elucidate the function of OsSar1 in endosperm, transgenic rice were produced with *OsSar1a*, *b*, and *c* knocked down simultaneously by RNAi in endosperm. An *OsSar1abc* RNAi vector was constructed by using a conserved part of the *OsSar1c* coding sequence fragment (427 bp) under the control of the endosperm-specific promoter *GluA-2*. Eighteen independent transformants were produced and the expression of *OsSar1a*, *b*, and *c* in the endosperm was measured by qRT-PCR. The mRNA levels of *OsSar1a*, *b*, and *c* were lower in RNAi transgenic lines than in the wild type (Fig. 3A). The western blot analysis revealed that the protein level of OsSar1 was greatly decreased in transgenic endosperm (Fig. 3C).

The growth and development of *OsSar1abc* RNAi lines were almost identical to those of the wild type, because *OsSar1a*, *b*, and *c* were simultaneously knocked down in seeds but not other tissues. However, most mature seeds of the *OsSar1abc* RNAi lines were opaque, with a floury and shrunken appearance, and failed to germinate. Furthermore, some seeds were even pre-harvest sprouting (Fig. 4A, B). Because the features of seeds were significantly altered in the *OsSar1abc* RNAi transformants, starch granules were observed in the seeds of transformants by scanning electron microscopy (SEM). In the wild type, the endosperm appeared to be tightly packed with irregularly polyhedral starch granules (Fig. 4C). In contrast, endosperm in the *OsSar1abc* RNAi transformants appeared to be a loosely packed and

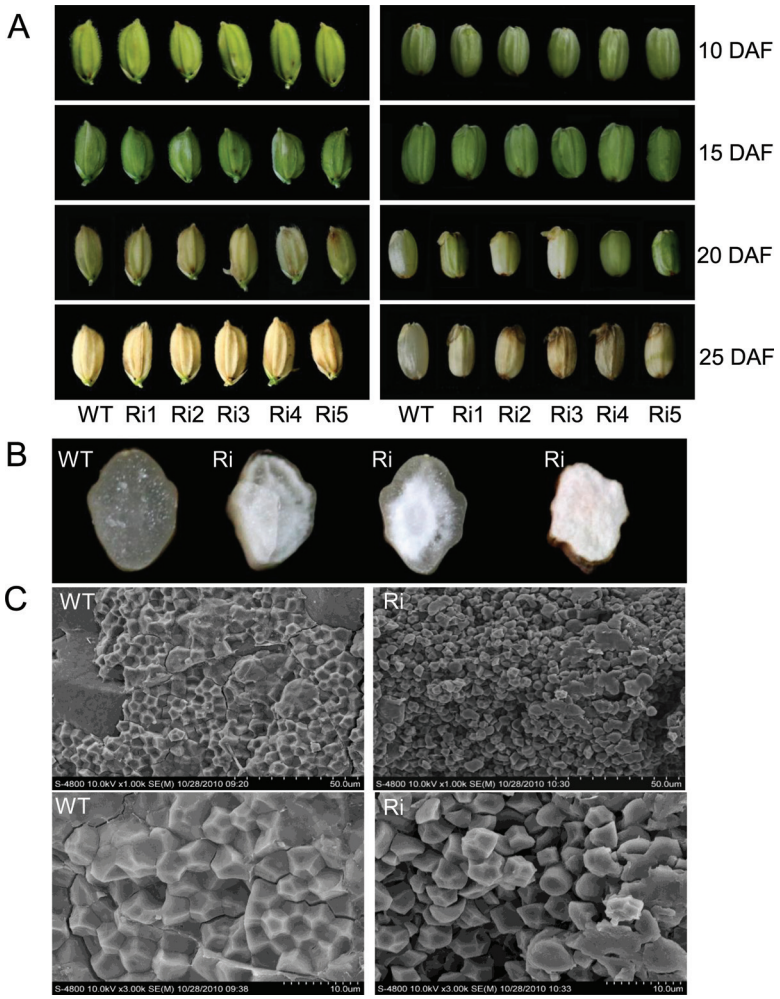

Fig. 4. Phenotype analysis of *OsSar1abc* RNAi transgenic seeds. (A) Morphology of *OsSar1abc* RNAi seeds at 10, 15, 20, and 25 days after flowering (DAF). The right panel shows the seeds on the left without glumes. (B) Transverse sections of grains. (C) Scanning electron microscopy of endosperm. WT, wild type, Ri, *OsSar1abc* RNAi lines. (This figure is available in colour at *JXB* online.)

fragile structure with spherical starch granules. These morphological changes in the starch granules may be one of the reasons for the floury and shrunken features of the *OsSar1abc* RNAi transformants.

SDS–PAGE and western blot analyses of developing seeds at 15 days after flowering (DAF) were used to examine whether suppression of *OsSar1a/b/c* affected accumulation of proteins. The level of the 57 kDa glutelin precursor was greatly increased in *OsSar1abc* RNAi transgenic plants, while the levels of the acidic and basic subunits were greatly reduced (Fig. 3B). In addition, the levels of 26 kDa α-globulin and prolamin were reduced. Western blot analysis revealed significant accumulation of the 57 kDa glutelin precursor, with the level of the basic subunit lower than that of the wild type. Furthermore, accumulation of the 26 kDa α-globulin was suppressed in transformants. However, the level of OsBiP was markedly increased in *OsSar1abc* RNAi seeds (Fig. 3C). These results implied that the intracellular transport of storage proteins was inhibited and the 57 kDa glutelin precursor was blocked in the ER in *OsSar1abc* RNAi seeds, which enhanced the level of the glutelin precursor in the ER and decreased that of mature glutelin acidic and basic subunits.

Novel ER-derived electron-dense protein bodies formed in OsSar1abc RNAi endosperm

Because the profile of seed storage protein was greatly altered in *OsSar1abc* RNAi transformants, the intracellular structures of developing endosperm cells in transformants was observed by immunofluorescence microscopy and TEM. Developing rice endosperm has two types of PBs: spherical, electron-lucent PB-I containing prolamin; and irregularly shaped, electron-dense PB-II containing glutelin and α-globulin. Therefore, PB-I was detected by anti-prolamin antibody and PB-II by anti-glutelin antibody. Prolamin and glutelin in the wild type were packaged into separate PBs, with prolamin in a red, spherical, smaller, dot-like PB-I (Supplementary Fig. S7B at *JXB* online) and glutelin in a green, irregularly shaped, larger PB-II (Supplementary Fig. S7A). In *OsSar1abc* RNAi endosperm, PB-I was identical to that in the wild type; however, in addition to the normal PB-II, many novel, spherical,

smaller glutelin PBs were observed, which were separated with PB-I (Supplementary Fig. S7H).

TEM of the endosperm of 15 DAF wild-type seeds revealed that the ER-derived prolamin-containing PB-I was spherical, electron-lucent, and surrounded by rough ER membrane with ribosomes, whereas the glutelin- and α-globulin-containing PB-II was irregularly shaped with high electron density (Fig. 5A). In endosperm cells of *OsSar1abc* RNAi transformants, in addition to the normal PB-I and PB-II (Fig. 5B), many smaller and spherical novel structures with high electron density and surrounded by ribosomes were observed (Fig. 5C, D). The attachment of the ribosomes on the surface of the novel structures indicated that they were derived from the ER, so these novel structures were designated as ER-derived electron-dense PBs (dPBs). Furthermore, electron-lucent blebbing structures were attached to some of the dPBs (Fig. 5D).

Export of proglutelin and α-globulin from the ER was affected in OsSar1abc RNAi endosperm cells

To determine whether the formation of dPBs was due to the accumulation of proglutelin in the ER, the intracellular localization of glutelin was examined. In wild-type endosperm cells, glutelin was detected in PB-II (Fig. 6A). In *OsSar1abc* RNAi endosperm cells, glutelin was detected in normal PB-II (Fig. 6B) and in dPBs (Fig. 6C), but not in the electron-lucent blebbing structures (Fig. 6D). These results confirmed that when the expression of *OsSar1* was suppressed, a large part of glutelin precursors were not able to transport to the PB-II but were blocked in the ER and formed dPBs, which suggested that the glutelin transport from the ER to PSV depended on COPII.

Rice 26 kDa α-globulin is also synthesized in the ER and deposited in PB-II. To determine whether the export of α-globulin from the ER was affected in *OsSar1abc* RNAi endosperm cells, the subcellular co-localization of α-globulin and glutelin was examined by immunofluorescence and immunoelectron microscopy. In the wild type, α-globulin and glutelin were packaged together in irregularly shaped, larger PB-II, with the red fluorescence of α-globulin mainly distributed in the peripheral, amorphous matrix area, and the green fluorescence of glutelin located in the bulky crystalloid regions. The red and green fluorescence were almost not overlapping in the wild type (Fig. 7A–D). However, in *OsSar1abc* RNAi endosperm cells, in addition to distribution in the normal PB-II, the red fluorescence of α-globulin and the green fluorescence of glutelin were uniformly distributed in dPBs, with the expression of both proteins merged completely. The appearance of yellow fluorescence in dPBs implied no spatial partitioning of α-globulin and glutelin within the dPBs (Fig. 7E–H).

Immunocytochemistry revealed that in the wild type, α-globulin accumulated mainly in the peripheral regions of PB-II, whereas glutelin was mainly distributed in the inner region (Fig. 8A, B). The α-globulin and glutelin were not detected in the PB-I of the wild type (Fig. 8C). Their distribution in normal PB-II was similar in *OsSar1abc* RNAi and wild-type cells (Fig. 8D, E). However, α-globulin was not distributed in the peripheral regions of dPBs but rather randomly in the inner areas with glutelin (Fig. 8F–H). The α-globulin and glutelin were not detected in the blebbing structures, with low electron density of dPBs (Fig. 8I). Therefore, a large proportion of proglutelin and α-globulin was not transported to PSVs but accumulated in the ER and was sequestered in the smaller, spherical dPBs.

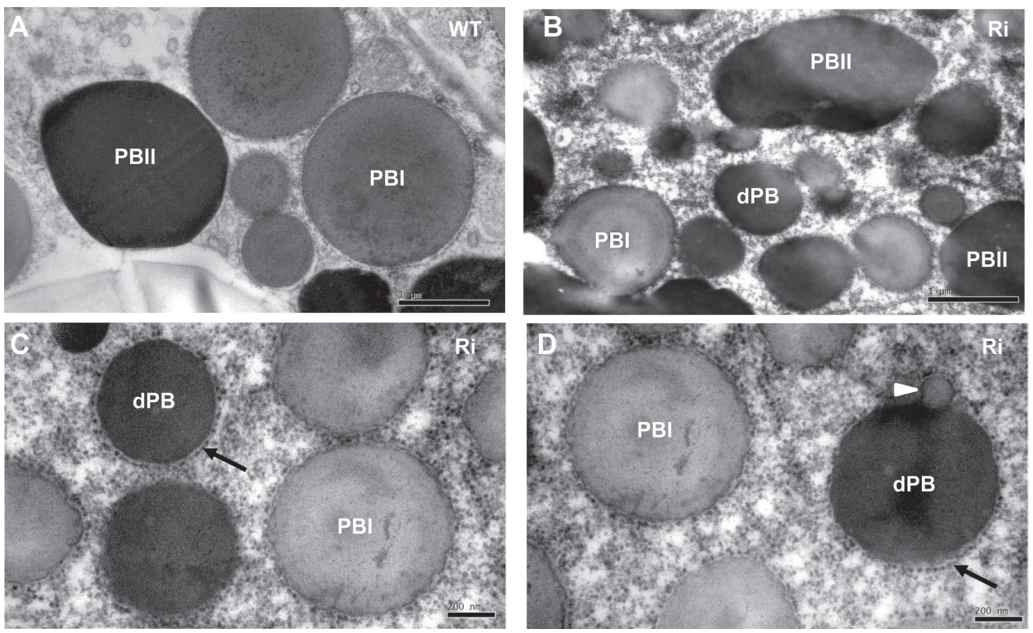

Fig. 5. Transmission electron microsopy (TEM) of protein bodies in developing endosperm. (A) Wild type; (B–D) *OsSar1abc* RNAi transformants. The black arrow indicates the ER-derived dense protein body (dPB) surrounded by rough ER membrane with ribosomes. The white arrowhead indicates some dPBs with blebbing structures. Scale bars=1 μm in (A, B) and 200 nm in (C, D).

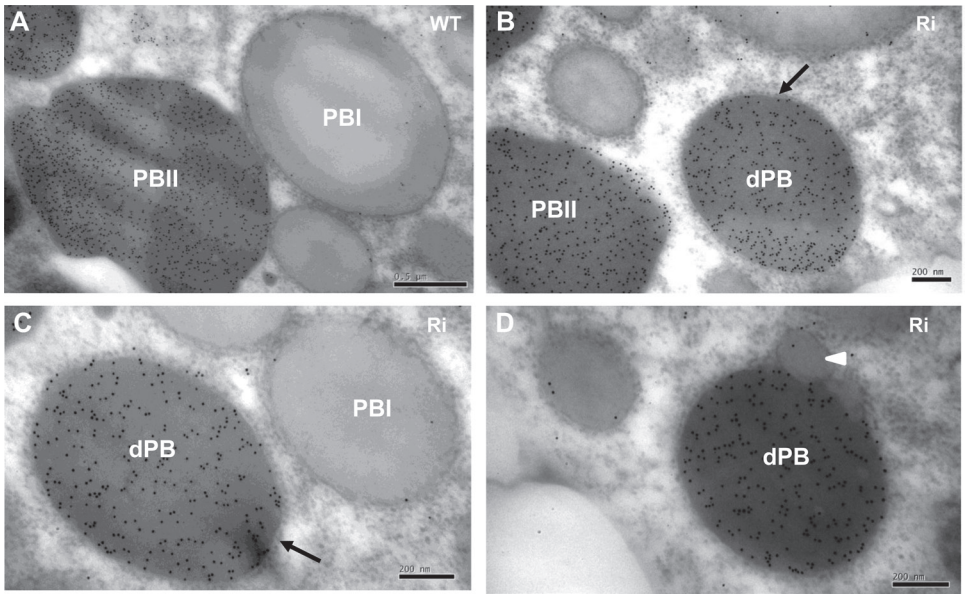

Fig. 6. Immunocytochemistry of glutelin in protein bodies. (A) Glutelin in PB-II in the wild type. (B, C) Glutelin localized on the normal PB-II and dPB in *OsSar1abc* RNAi lines. The black arrow indicates the dPB surrounded by rough ER membrane with ribosomes. (D) The white arrowhead indicates that glutelin did not accumulate in the blebbing structures of dPBs. Gold particles (10 nm) indicate the reaction of glutelin antibody. Scale bars=0.5 μm in (A) and 200 nm in (B–D).

To make sure that the abnormal accumulation of glutelin and α-globulin in ER is due to the specificity of COPII assembly malfunction, and not a result of the secondary effect from ER stress response, OsCBL, a homologue of AtCBL6 whose transport to the vacuole was COPII independent (Batistic *et al.*, 2010; Bottanelli *et al.*, 2011), was selected as a negative control. The results of western blot analysis showed that the expression levels of OsCBL in *OsSar1abc* RNAi seeds did not differ from that of the wild type (Supplementary Fig. S8 at *JXB* online), indicating that the abnormal accumulation of glutelin and α-globulin in the ER was caused by COPII deficiency.

Blebbing structures of dPBs were PB-I derivatives

OsSar1abc RNAi endosperm cells showed no distribution of α-globulin and glutelin in electron-lucent blebbing structures of dPBs. Therefore, if prolamin was distributed in the blebbing structures, it may have caused the low electron density. To support this assumption, the co-localization of prolamin

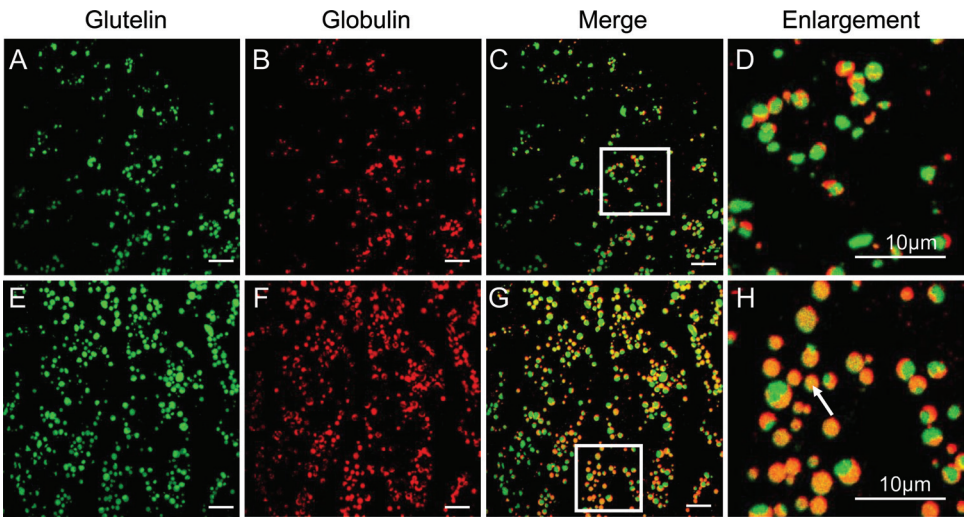

Fig. 7. Immunofluorescence analysis of glutelin and α-globulin in rice endosperm. Secondary antibodies labelled with FITC (green, A and E) and rhodamine (red, B and F) were used to visualize glutelin and α-globulin, respectively, in wild-type (A–D) and *OsSar1abc* RNAi endosperm (E–H). (C and G) Merged images. (D and H) Enlarged images of areas inside the white boxes in (C, G). A white arrow indicates α-globulin and glutelin merged in dPBs. Bars=10 μm.

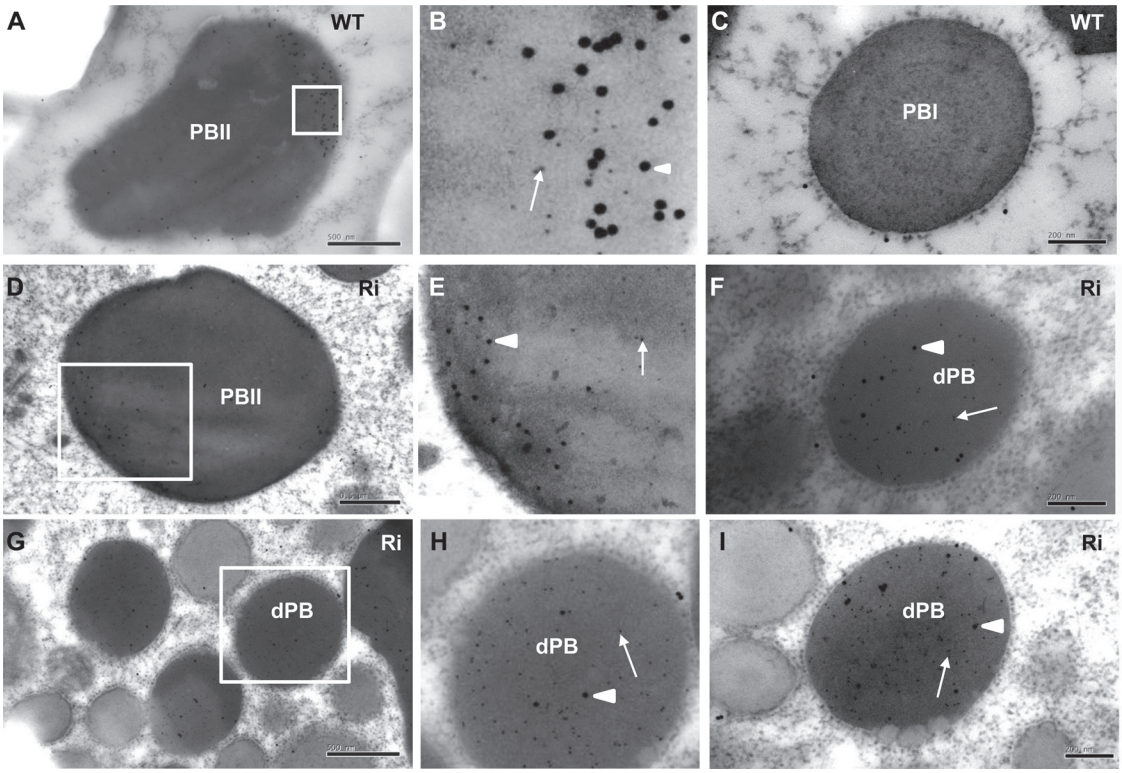

Fig. 8. Immunocytochemistry of glutelin and α-globulin in protein bodies. (A–C) Wild type; (D–I) *OsSar1abc* RNAi transformants. (B, E, H) Enlarged images of areas inside the boxes in (A, D, G), respectively. Glutelin and α-globulin antibodies are labelled with 5 nm (white arrow) and 15 nm (white arrowhead) immunogold particles, respectively. Scale bars=500 nm in (A, D, G) and 200 nm in (C, F, I).

and glutelin was examined, and glutelin was found to be distributed in irregularly shaped PB-II of the wild type and normal PBs of *OsSar1abc* RNAi, with high electron density (Fig. 9A–D), whereas prolamin was detected only in the spherical PB-I with low electron density (Fig. 9A, E). Most dPBs showed no prolamin, and only glutelin was detected in the spherical dPBs (Fig. 9F). For the blebbing dPBs, glutelin accumulated in the inner areas of dPBs, with high electron density, and some prolamin was in the blebbing structures, with low electron density (Fig. 9G). Therefore, the blebbing structure was an ER-derived PB-I derivative.

Discussion

Rice glutelin is synthesized in the ER and then transported to PSVs through or bypassing the Golgi apparatus for final

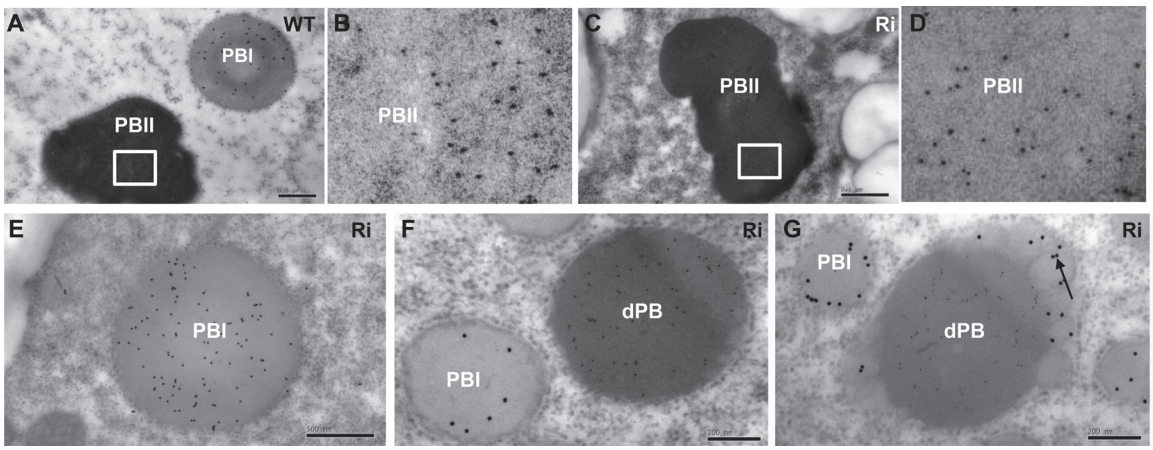

Fig. 9. Immunocytochemistry of glutelin and prolamin in protein bodies. (A, B) Wild type; (C–G) *OsSar1abc* RNAi transformants. (B, D) Enlarged images of areas inside the boxes in (A, C). Glutelin and prolamin antibodies were labelled with 5 nm and 15 nm immunogold particles, respectively. The black arrow indicates prolamin accumulated in the blebbing structures of dPBs. Scale bars=500 nm in (A, C, E) and 200 nm in (F, G).

processing to mature acid and basic subunits. Research into glutelin intracellular transportation has focused on factors such as chaperones that affect protein folding, factors affecting glutelin transport from the Golgi to PSVs, and glutelin processing in PSVs (Takemoto *et al*., 2002; Wang *et al*., 2009, 2010; Satoh-Cruz *et al*., 2010; Kumamaru *et al*., 2010; Fukuda *et al*., 2011). Little is known about the factors regulating glutelin export from the ER to the Golgi apparatus.

Subcellular distribution of OsSar1 and functional redundancy in rice endosperm

We focused on the function of Sar1 GTPases in storage protein transport in rice because Sar1 plays an essential role in the formation of COPII vesicles in ER to Golgi traffic in yeast, mammalian, and plant cells (Takeuchi *et al*., 2000; Hanton *et al*., 2006; Marti *et al*., 2010). Different subcellular localization of Sar1 isoforms in plant cells has been reported in tobacco and *Arabidopsis*, which suggests that Sar1 proteins might be functionally distinct in plant cells. In tobacco leaf epidermal cells, NtSar1 is distributed predominantly in the cytosol but is visibly recruited to ERES on co-expression of membrane cargo proteins destined for the Golgi apparatus (daSilva *et al*., 2004). In *Arabidopsis*, AtSARA1a and AtSARA1b are distributed at multiple punctate structures and localize at ERES, independently of co-expression of Golgi-destined membrane cargo (Hanton *et al*., 2008). In this study, we describe four Sar1 isoforms with high amino acid sequence identity in rice. Transient expression of all OsSar1 isoforms revealed a punctate pattern in rice protoplast cells. On co-expression of OsSar1–GFP with the *cis*-Golgi marker GmMan1–mCherry, the punctate structures labelled with OsSar1 were associated with the Golgi area, while co-expression of OsSar1–mCherry with YFP–AtSec24 confirmed that OsSar1 localized at ERES (Fig. 2). The localization of OsSar1 at ERES is similar to that of AtSARA1, because punctate structures were also observed in the absence of Golgi cargo. Furthermore, the two AtSar1 isoforms show different subcellular distribution and activity despite near-identical protein sequences. However, the subcellular distribution of the four Sar1 isoforms in rice was consistent with that of AtSARA1a but differed from that of AtSARA1b, since they mainly existed in the soluble fraction (Supplementary Fig. S1 at *JXB* online). This may be because the amino acid residue at the C-terminus of all OsSar1 isoforms is lysine (K), which is conserved in AtSARA1a (Hanton *et al*., 2008). Although the C-termini of Sar1 may contribute to the membrane association of the protein, the exchange of GDP for GTP on Sar1 triggers the exposure of its N-terminal amphipathic α-helix, which constitutes a membrane anchor to the ER, and then initiates the COPII coat assembly (Bielli *et al*., 2005; Lee and Miller, 2007). Therefore, GFP/mCherry should be fused to the C-termini of Sar1 for subcellular distribution similar to AtSARA1 (Hanton *et al*., 2008).

The similar subcellular localization and distribution of the four OsSar1 isoforms raised the question of tissue specificity and redundancy for OsSar1 isoforms in rice. qRT-PCR and stable transgenic analysis with the GUS reporter gene revealed *OsSar1a*, *b*, and *c* expressed universally in rice without tissue specificity (Supplementary Figs S2, S3 at *JXB* online). To avoid a detrimental effect on growth and development, transgenic plants were produced by endosperm-specific overexpression or suppression of the *OsSar1* genes. Endosperm-specific suppression of any *OsSar1* meant that rice endosperm could not accumulate proglutelin in the ER, but suppression of the three *OsSar1* genes simultaneously caused abnormal seeds and proglutelin accumulation. These results suggest functional redundancy of OsSar1a, b, and c in rice endosperm, which is different from AtSec24, another COPII component. AtSec24 isoforms have incompletely overlapping functions, because a missense point mutation of AtSec24A induced the formation of clusters of the ER and Golgi apparatus, and total loss of AtSec24A function was lethal; the phenotypes could not be complemented by AtSec24B and AtSec24C (Faso *et al*., 2009; Nakano *et al*., 2009; Conger *et al*., 2011). The difference in functional redundancy of OsSar1 and AtSec24 may be related to their different functions. Sar1 acts as a molecular switch to control the assembly of COPII (Memon, 2004), whereas Sec24 selects and captures cargo via specific binding sites of ER export motifs (Sato and Nakano, 2007). The different AtSec24 isoforms may interact with specific cargo that participates in different pathways in cells. The present data suggest that the OsSar1 isoforms are functionally redundant in rice endosperm, but they might have different roles in other tissues.

Co-suppression of OsSar1abc blocked the storage proteins in the ER, forming novel ER-derived electron-dense PBs

Rice glutelins are synthesized on the ER as 57 kDa precursors, and finally transported to the PSV, where they are processed into mature acidic and basic subunits. If any step in glutelin transport and processing is defective, the glutelin will remain as 57 kDa precursors (Takemoto *et al*., 2002; Wang *et al*., 2009, 2010; Fukuda *et al*., 2011). The accumulation of most mature glutelin, α-globulin, and some prolamin was severely inhibited in *OsSar1abc* RNAi transformants. Much glutelin was deposited as precursors, and post-translational processing into mature subunits was suppressed (Fig. 3B, C). The intracellular structures of endosperm cells were significantly disrupted by the formation of spherical electron-dense PBs, many of which were surrounded by ribosomes that were never observed in the wild type (Fig. 5). Similar ER-derived PBs were observed in transgenic soybean seeds in which the expression of α and α' subunits of β-conglycinin was co-suppressed (Kinney *et al*., 2001). Of note, the dPBs were smaller than the PB-II, and some dPBs were connected to electron-lucent blebbing structures (Figs 5, 6, 8, 9). The highly electron-dense matrix of dPBs contained glutelin and α-globulin, and the low electron-dense blebbing structures contained prolamin (Figs 6, 8, 9). Therefore, dPBs were derived from the ER, and dPBs with blebbing structures may be formed by the adjacent area of the PB ER and cisternal ER, with the blebbing structures as PB-I derivatives.

The dPBs formed in the *OsSar1abc* RNAi lines contained glutelin and α-globulin but not prolamin. The dPBs differ

from those in the rice PDI1-knockout mutant *esp2* and BiP-overexpressing lines, which contain both glutelin and prolamin (Takemoto *et al*., 2002; Yasuda *et al*., 2009). In *esp2*, because of loss function of PDI1-1, the glutelin precursor could not fold correctly but, instead aggregated with prolamin via interachain disulphide bonds (Takemoto *et al*., 2002; Satoh-Cruz *et al*., 2010). In BiP-overexpressing lines, the high concentrations of BiP might perturb entry into the ER and the folding and sorting of secreted proteins (Yasuda *et al*., 2009). In *OsSar1abc* RNAi lines, proglutelin could fold correctly and separate with prolamin but could not be transported from the ER perhaps because of COPII deficiency. ER-derived PBs (MAG bodies) were also found in *Arabidopsis mag2* and *mag4* mutants, with reduced efficiency of protein transport from the ER to the Golgi complex (Li *et al*., 2006; Takahashi *et al*., 2010). However, the distribution of storage proteins in dPBs differed from that in MAG bodies. Within the MAG bodies, two major storage proteins were separately localized with 2S albumin in the core and 12S globulin in the matrix region, whereas in dPBs, α-globulin and glutelin were distributed uniformly without spatial partitioning (Figs 7, 8).

The glutelins and α-globulins are transported to PSVs by a Golgi-dependent pathway in rice

Two pathways were reported for storage protein transport from the ER to PSVs, the Golgi-dependent pathway in which the storage proteins travel through the Golgi apparatus into the PSV via DVs, and the Golgi-independent machinery with the storage proteins aggregated within the ER and transported directly to PSVs by PAC. PAC has been reported in pumpkin (Hara-Nishimura *et al*., 1998), but the report in rice remains unique (Takahashi *et al*., 2005) since these vesicles have not been verified in other ultrastructural studies of rice PB biogenesis (Fukuda *et al*., 2011). Moreover the detection of glutelin and α-globulin in DVs suggested that the Golgi apparatus may mediate the rice storage protein transport (Krishnan *et al*., 1986, 1992; Fukuda *et al*., 2011; Washida *et al*., 2012). Using endosperm-specific RNAi technology, it was confirmed that rice storage protein transport from the ER to PSV depended on COPII. The deficiency of *OsSar1* may perturb the formation of COPII, which results in blocking glutelin precursors in the ER and in the formation of dPBs in the ER lumen (Figs 6, 8, 9). Some of the dPBs were connected to but not surrounded by prolamin-containing electron-lucent blebbing structures (Fig. 9), which differed from PAC-like structures reported in rice (Takahashi *et al*., 2005). It is not surprising that some glutelins and α-globulin were exported from the ER and transported to the PSV in *OsSar1abc* RNAi lines because RNAi is a knockdown but not knockout technology. The results, together with glutelin and α-globulin being detected in Golgi-associated DVs in developing endosperm cells, indicated that the rice storage protein transport to the PSV is a Golgi-dependent pathway. Further investigation on how the rice storage proteins selectively packaged into the vesicles will be required. The ER export signal and vacuolar sorting determinants in rice glutelin and α-globulin are being identified.

Rice α-globulin RNAs, like those of prolamin, are targeted to the PB ER, whereas glutelin RNAs are enriched on the adjacent cisternal ER (Choi *et al*., 2000; Crofts *et al*., 2004, 2005; Washida *et al*., 2012). However, α-globulin is deposited together with glutelin in the PSV, whereas prolamin is deposited in the ER-derived PB-I. The α-globulin and glutelin are mainly sequestered in the matrix and the crystalloid regions of PSVs, respectively (Kumamaru *et al*., 2010). Previous studies suggested that α-globulin might be exported directly from the PB ER to the Golgi apparatus because α-globulin was detected only on the PB ER and not on the cisternal ER (Washida *et al*., 2012). In the *OsSar1abc* RNAi transformants, α-globulin could not exit from the ER but was distributed uniformly with glutelin in the smaller, spherical ER-derived dPBs without spatial partitioning (Fig. 8). The newly synthesized α-globulin might move from the PB ER lumen to export sites on the cisternal ER and then be transported to PSVs together with glutelin via COPII. How α-globulin moves from the PB ER lumen to export sites on the cisternal ER remains to be investigated.

Deficiency of OsSar1 results in ER stress response

Secretory proteins must move from the ER to reach a final destination for their proper function. When the exit of proteins is disturbed, the proteins will accumulate in the ER, where the high concentrations will cause ER stress. Severe ER stress induces cell death in plant cells (Iwata and Koizumi, 2005). BiP is involved in a signalling pathway as a sensor of the ER stress response and an indicator of ER stress. Overexpression of BiP in transgenic rice seed resulted in an opaque phenotype and produced ER-derived PB-like compartments filled with both prolamin and glutelin (Yasuda *et al*., 2009; Wakasa *et al*., 2011). The increased expression of BiP and the formation of dPBs in the *OsSar1abc* RNAi lines (Figs 3, 5) suggested that the cell might sense a severe stressful condition in the ER because the secretory proteins were blocked in the ER. *OsSar1abc* RNAi showed reduced levels of prolamin compared with the wild type, similar to that in the proglutelin-accumulating mutants *esp2*, *glup4*, *glup5*, and *glup6* (Ueda *et al*., 2010), which might be a general phenomenon of ER stress response. Deficiency of OsSar1 in rice endosperm induced physiological damage leading to an opaque phenotype, even pre-harvest sprouting. *OsSar1abc* RNAi lines showed inhibited sorting of glutelin and α-globulin from the ER to PB-II and inhibited normal transport of other secretory proteins. Accumulation of secretory proteins in the ER resulted in ER stress, thus leading to changes to an opaque seed phenotype, with floury and shrunken features (Fig. 4). The seed morphological features of *OsSar1abc* RNAi lines was similar to that of ER stress lines caused by BiP overexpression (Yasuda *et al*., 2009), whereas that of the dPBs differed from that of PB-like structures in BiP-overexpressing transformants. The appearance of pre-harvest sprouting suggested that the transport of some proteins involved in seed germination and dormancy was defective in *OsSar1abc* RNAi seeds, but the identity of the specific proteins is not clear.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Distribution of OsSar1 in rice protoplasts.

Figure S2. The qRT-PCR analysis of relative mRNA expression of *OsSar1a*, *b*, *c*, and *d* in rice tissue.

Figure S3. Expression pattern of *OsSar1* in rice.

Figure S4. RT-PCR analysis of mRNA expression of *OsSar1* in wild-type (WT) and overexpressng transgenic seeds.

Figure S5. SDS–PAGE of total seed protein level in *OsSar1a* overexpression lines (A) and *OsSar1c* RNAi lines (B).

Figure S6. RT-PCR analysis of mRNA expression of *OsSar1a* (A), *b* (B), and *c* (C) in seeds of relative RNAi transgenic lines.

Figure S7. Immunofluorescence analysis of glutelin and prolamin in rice endosperm.

Figure S8. Western blot analysis of protein levels of OsCBL in *OsSar1abc* RNAi transformant seeds.

Table S1. Primer sequences used in this study.

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