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Protein Disulfide Isomerase Like 1-1 Participates In The Maturation Of Proglutelin Within Endoplasmic Reticulum In Rice Endosperm

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Abstract

The rice *esp2* mutation was previously characterized by the abnormal accumulation of elevated levels of proglutelin and the absence of an endosperm-specific protein disulfide isomerase like (PDIL1-1). Here we show that *Esp2* is the structural gene for PDIL1-1 and that this lumenal chaperone is asymmetrically distributed within the cortical endoplasmic reticulum (ER) and largely restricted to the cisternal-ER. Temporal studies indicate that PDIL1-1 is essential for the maturation of proglutelin only when its rate of synthesis significantly exceeds its export from the ER, a condition resulting in its buildup in the ER lumen and the induction of ER quality control processes which lower glutelin levels as well as for the other storage proteins. As proglutelin is initially synthesized on the cisternal ER, their deposition within prolamine protein bodies in *esp2* suggests that PDI1-1 helps retain proglutelin in the cisternal ER lumen until it is attains competence for ER export and, thereby, indirectly preventing heterotypic interactions with prolamine polypeptides.

Keywords: endoplasmic reticulum, endosperm, *Oryza sativa*, protein body, protein disulfide isomerase, storage protein

Introduction

Rice seed storage proteins consist mainly of two classes (Juliano 1972). One class consists of the acid-soluble glutelins, which are homologous to the 11S globulins of soybean and pea (Shotwell and Larkins 1989, Takaiwa et al. 1987, Zhao et al. 1983). The other class is the alcohol soluble prolamines, the storage protein class typically found in cereals (Ogawa et al. 1987, Shewry and Tatham 1999). Rice seed also accumulates a salt-soluble globulin which comprises of up to 5% of the total seed protein (Padhye and Salunkhe 1979).

Glutelins are initially synthesized as a 57 kD precursor on the endoplasmic reticulum (ER) (Yamagata et al. 1982). The precursor is then exported to the protein storage vacuole (PSV; also called protein body II, PB-II) where it is post-translationally processed into acidic and basic subunits interlinked by a disulfide chain (Krishnan and Okita 1986, Yamagata et al. 1982). The glutelin-containing PSV is characterized by its irregular shape with a diameter of about 3-4 μm and high uniform staining density (Beachtel and Juliano 1980, Tanaka et al. 1980).

The prolamines are also synthesized on the ER membrane but, unlike proglutelins, are retained in the ER lumen to form spherical intracisternal inclusions 1-2 μm in diameter called PB-I (Beachtel and Juliano 1980, Ogawa et al. 1987, Tanaka et al. 1980). Prolamines lack an ER retrieval signal and, hence, their retention within the ER lumen and

assembly into an intracisternal inclusion granule is due to other mechanisms. One process that facilitates prolamine ER retention is RNA sorting (Hamada et al. 2003, Li et al. 1993) whereby prolamine RNAs are localized specifically to the ER (PB-ER) membranes that delimit PB-I. The enrichment of prolamine RNAs on the PB-ER would effectively concentrate the newly synthesized polypeptides within a confined ER lumenal space, favoring protein-protein interactions and assembly to form an intracisternal inclusion granule (Okita and Rogers 1996). In contrast, glutelin RNAs are enriched on adjacent cisternal ER membranes which together with PB-ER constitute the cortical ER complex in developing rice endosperm cells (Hamada et al. 2003, Li et al. 1993).

A second process that facilitates the ER retention and assembly of prolamine polypeptides is the specific involvement of binding protein (BiP) (Muench et al. 1997). Although this lumenal chaperone is an excellent marker for ER, it is asymmetrically distributed within this membrane complex in rice endosperm cells (Li et al. 1993, Muench et al. 1997). BiP is highly enriched at the periphery of PB-I compared to the rest of the cortical ER (Li et al. 1993, Muench et al. 1997). Available evidence suggests that this lumenal chaperone facilitates the transport of the nascent prolamine polypeptide across the ER membrane and their folding and assemble into an intracisternal inclusion granule (Li et al. 1993, Muench and Okita 1997, Muench et al. 1997, Okita et al. 1998, Okita and Rogers 1996).

Other lumenal chaperones such as protein disulfide isomerase (PDI) are also likely to

be involved in storage protein folding and intracellular transport. PDI, a catalyst of disulfide-bond formation and rearrangement (Rowling and Freedman 1993), is also a molecular chaperone which facilitates polypeptide folding and has been suggested to have a role in storage protein biogenesis. (Bulleid and Freedman 1988). The rice *esp2* mutation was identified by the accumulation of abnormally large quantities of proglutelin with corresponding reductions in mature glutelin subunits (Kumamaru et al. 1987, Kumamaru et al. 1988). The *esp2* endosperm was also devoid of an endosperm-specific PDI (Accession no. AB039278, PDIL1-1), an observation suggesting a role for this lumenal chaperone in the folding and maturation of proglutelin to a conformation competent for ER export. In the absence of PDIL1-1, proglutelin and prolamines co-assembled via intermolecular disulfide bonds to form numerous small intracisternal aggregates within ER (Takemoto et al. 2002). Although the available evidence indicates that PDIL1-1 is involved in glutelin trafficking, the exact role of this molecular chaperone in this process is not known.

In this study, we show conclusively that the *Esp2* locus is the structural gene for the PDIL1-1 and that the deficiency of this lumenal chaperone mediates the abnormal accumulation of proglutelin during rice endosperm development. The dependence on PDIL1-1 for the ER export of proglutelin is conditional and is influenced by temporal gene expression patterns of both glutelin and prolamine. As proglutelin and prolamine are normally restricted to distinct lumenal compartments due to the localization of their RNAs to specific ER subdomains, the abnormal interaction between these storage proteins

suggests that PDIL1-1 has an another role in addition to its disulfide isomerase and chaperone activities in facilitating the maturation of proglutelin to a state competent for ER export.

Results

Abnormal accumulation of proglutelin in the *esp2* mutant is caused by the deficiency of PDIL1-1

To confirm that *esp2* is a defective PDIL1-1 gene, we initiated several genetic studies. Gene dosage effect studies were carried out by generating F_1 seeds obtained from reciprocal crosses between an *esp2* mutant line "CM1787" and the wild type "Kinmaze" (Fig. 1). Densitometric measurement of PDIL1-1 protein obtained by immunoblot analysis showed that the amount of PDIL1-1 protein in the duplex (++*e*), simplex (+*ee*), nulliplex (*eee*) genotypes was 64%, 36% and 0%, respectively, of the wild type condition (Fig. 1B, C). Thus, the level of the PDIL1-1 protein increased linearly with the number of dominant *Esp2* alleles, indicating that the amount of PDIL1-1 protein corresponded to the gene dosage of *Esp2* allele. By contrast, the amount of proglutelin decreased according to the increase in the number of dominant *Esp2* alleles. Relative to the amount of proglutelin detected in nulliplex genotype, the amounts were 24% and 8% in the simplex and duplex genotypes, respectively, corresponding to an inverse relationship between proglutelin levels

and number of *Esp2* alleles. The level of mature glutelin subunits followed the increase in dominant *Esp2* alleles, *i.e.* 34% and 64% in the simplex and the duplex, respectively, in comparison to the triplex. In contrast to the increase in mature glutelin subunit levels, the extent of the decrease in proglutelin levels was not linear with the increase in number of the *Esp2* alleles.

To obtain further genetic evidence to show that elevated proglutelin levels were caused by deficiency in PDIL1-1, we analyzed 206 F_2 seeds derived from a cross between wild type Kinmaze and the *esp2* mutant EM44. The level of PDIL1-1 protein in all 154 F_2 seeds showing normal levels of proglutelin was the same as that detected in wild type (Table 1). On the other hand, PDIL1-1 was absent in the 52 F_2 seeds showing elevated levels of proglutelin. These results indicate that the abnormal accumulation of proglutelins in *esp2* mutants is due to the lack of PDIL1-1 protein.

To determine whether the *esp2* mutation was due to a lesion in the structural gene for PDIL1-1 itself or a gene regulating the transcription or modifying the expression of the gene coding the PDIL1-1, RFLP studies were conducted with progenies derived from a cross between *indica* rice cultivar "Kasalath" and the *esp2* mutant, CM1787, using the *PDIL1-1* clone (AB039278) as a probe. RFLP analysis of the F_2 population showed that all *esp2* homo genotypes co-segregated completely with a RFLP for the *PDIL1-1* of CM1787 (Table 2), suggesting that *esp2* is a mutation in the structural gene for PDIL1-1.

The *esp2* gene encodes PDIL1-1

A full length *PDIL1-1* cDNA clone was isolated from a cDNA library by using the partial cDNA clone (Accession No. AB039278) as a probe. The full-length cDNA clone (Accession No. AB373950) was 1,903 bp in length and contained a single open reading frame of 1,536 bp coding for 512 amino acids (Supplementary Fig. S1). PDIL1-1 contained a C-terminal ER retrieval tetra peptide KDEL, a potential glycosylation site and two thioredoxin active sites CXXC. The rice PDIL1-1 primary sequence showed 84.8%, 84.2% and 83.9% sequence identity to that of maize (Li and Larkins 1996), barley (Chen and Hayes 1994) and wheat (Shimoni et al. 1995) sequences, respectively.

The corresponding gene sequence was identified on the BAC clone OSJNBa0058P12 (AC139170) of chromosome 11 by "blast" searching the Rice Genome Automated Annotation system (RiceGAAS, http://ricegaas.dna.affrc.go.jp/) using the PDIL1-1 cDNA sequence as the query. The *PDIL1-1* gene, which spans 3,042 bp between the start to stop codons, consists of 10 exons and 9 introns (Fig. 2A). Comparison with the wild type gene sequences showed that each of the three *esp2* lines, CM1787, EM44, EM747, contained single nucleotide substitutions. In all lines, the mRNA of the *PDIL1-1* gene was not expressed (Takemoto et al. 2002). In CM1787, substitution of an A for a T occurred at nucleotide 194 resulting in the codon change from Lys^{65} to a termination stop codon in the third exon (Fig. 2B). The reduction of gene expression by nonsense-mediated mRNA decay (NMD) is well documented (Maquat 2004) and is the likely basis for the absence of

the *PDIL1-1* mRNA. Mutations in EM44 and EM747 were the substitution of G to A located at nucleotide positions 2327 and 2435, respectively. These mutations are located at the 3' end of intron 7 and the 5' end of intron 8, respectively (Fig. 2B). In both instances, the highly conserved border sequences of the splice sites were disrupted, which would result in incorrect splicing patterns leading to frame shifts or deletions in the mRNA (Brown 1996). These results demonstrate that *Esp2* is the structural gene for the PDIL1-1 protein and that its absence is responsible for the abnormal accumulation of proglutelin.

PDIL1-1 disrupts the accumulation and packaging of rice seed storage proteins

In addition to PDIL1-1 causing changes in proglutelin and mature glutelin subunits, *esp2* also affected the levels of the 26 kD α-globulin and prolamines. This effect is readily apparent for the prolamine polypeptide bands at 14 kD and 13 kD which are conspicuously reduced in the nulliplex genotype (Fig. 1A).

To obtain more insight on the relationship between storage protein accumulation and the PDIL1-1, the accumulation patterns of glutelin, prolamine and α-globulin were investigated and compared to the temporal accumulation patterns of PDIL1-1 as well as the lumenal chaperone BiP during seed development (Fig. 3). In developing wild type seeds, glutelin acidic and basic subunits are initially detected at 5 days after flowering (DAF) and their levels increase linearly between 10 to 18 DAF (Fig. 3A). Proglutelin and α-globulin levels were low at 5 to 10 DAF but began to increase at 10 DAF. Prolamine polypeptides,

especially the 14 kD and 16 kD cysteine-rich prolamines, were first detected at 10 DAF and increased significantly after 13 DAF, whereas 13 kD cysteine-poor prolamines started to accumulate somewhat later.

By contrast, proglutelin in *esp2* developing seeds exhibited a markedly different accumulation pattern. At 5 and 10 DAF, proglutelin levels are very low compared to those of its mature subunits. Hence in young developing seeds, proglutelin is efficiently exported from the ER and transported to PB-II where it is processed into acidic and basic subunits. At 13 DAF and later, however, proglutelin levels increased rapidly and exceeded those seen for individual glutelin acidic and basic subunits. The total amount of proglutelin and its mature subunits were significantly lower in *esp2* compared to wild type. The levels of prolamines and α-globulins in *esp2* were also significantly reduced, indicating that the deficiency of PDIL1-1 has a general suppressive effect on storage protein expression.

Fig. 3C shows the accumulation of PDIL1-1, BiP and the glutelin subunits during the development of wild type seeds. Glutelin acidic and basic subunits are first detected at 6 DAF while PDIL1-1 is detected much earlier at 2 DAF and attains a maximum level at 8 DAF (Fig. 3C). Although BiP was also detected at 2 DAF, its level remained low until 6 DAF where its relative levels increased, attaining a maximum level at 21 DAF. These results readily show that PDIL1-1 is expressed much earlier during seed development than the mature glutelin subunits.

PDIL1-1 is localized within the cisternal-ER

To establish a possible role for PDIL1-1, its intracellular localization in endosperm was investigated using both biochemical and microscopic approaches. Fig. 4 depicts a SDS polyacrylamide gel of fractions obtained from sucrose density gradient centrifugation of protein body-membrane fractions isolated from 15-20 DAF wild type developing seeds. PB-I containing prolamine was enriched in fractions 25 to 29 while PSV containing glutelin was detected in fractions 29 to 33 (Fig. 4A). It was previously shown that proglutelin localized in PB-ER fraction in the *esp2* mutant (Takemoto et al. 2002). Immunoblot analysis of the various fractions showed that BiP was distributed throughout the sucrose density gradient and especially prevalent in fractions 1 to 9 and fractions 23 to 29, the latter peak coinciding with prolamine PB-I, which is enriched for this lumenal chaperone (Muench et al. 1997). By contrast, PDIL1-1 was restricted mainly to fractions 1 to 13, which are enriched in light cisternal ER membranes. Very small amounts of PDIL1-1 were detected in PB-I fractions. These results indicate that PDIL1-1 is restricted mainly to the cisternal-ER membranes with very little, if any, associated with prolamine containing PB-I.

To verify the location of PDIL1-1 within the cisternal-ER and its exclusion from PB-I, both immunofluorescence and immunoelectron microscopy were performed. As predicted from the sucrose density gradient results, PDIL1-1 label was readily evident over the

cisternal ER when viewed by immunofluorescence microscopy (Fig. 5A, B and Supplementary Movie 1). Three-dimensional reconstructions from multiple z sections show that PDIL1-1 label is within the cortical ER network and is almost completely excluded from PB-I (Fig. 5A). Imaging of thin sections from 15 DAF seeds (Fig. 5B) clearly shows the presence of PDIL1-1 in discrete regions of the cisternal-ER immediately adjacent to the prolamine-containing PB-I. Fig. 5C more clearly depicts the relationship between PDIL1-1 and rhodamine signals within the cortical ER, the merged image suggesting that protein bodies are depressed within the surface of the cisternal ER since the central portion of the PB remains magenta in color. Fig. 5D also clearly shows the relationship between PDIL1-1 and BiP, with BiP being localized to the PB-I surface (Muench et al. 1997) whilst PDIL1-1 is present within the cis-ER. Further analysis of endosperm tissue by immunoelectron microscopy once again confirmed that, PDIL1-1 was found within the cisternal ER (Fig. 5E, F, arrows). Although PDIL1-1 was largely excluded from PB-I, there was one instance where it was detected in PB-I. Its distribution was not random but decidedly concentrated in a localized region on the surface of an intracisternal inclusion granule (Fig. 5F, arrowhead). Since PDIL1-1 is largely distributed to the cisternal ER, the association of PDIL1-1 with PB-I may be at junctions connecting these two membrane subdomains.

We had previously shown that *esp2* endosperm lacks the normal prolamine-containing PB-I but, instead, contains numerous small PBs exhibiting low electron density surrounded

by rough ER (Takemoto et al. 2002). Detailed analysis at the electron microscopy level showed that these small variant PBs, which contained aggregate mixtures of proglutelin and prolamine, are clusters of intracisternal inclusions granules contained within the ER membrane complex (Takemoto et al. 2002). Immunoelectron microscopic studies showed that these variant PBs in *esp2* also contained α-globulin (Fig. 5G), which is normally deposited in the PSV (Krishnan et al. 1992, Tanaka et al. 1980). Hence, the global effect on storage protein gene expression seen in *esp2* endosperm is likely due to ER quality control processes as well as the disruption of normal protein targeting and deposition within the endomembrane system.

PDIL1-1 interacts with proglutelin

To provide additional evidence for a relationship between PDIL1-1 and proglutelin, co-immunoprecipitation experiments were conducted. Extracts from developing seeds were incubated with Protein A agarose beads bound with either PDIL1-1 or glutelin antibody and the resulting captured material was then analyzed by western blotting using the same antibodies. The degree of background binding was assessed by using anti-GFP-Protein A in addition to no antibody negative control reactions.

Fig. 6 shows that both glutelin and PDIL1-1 antibodies are able to co-immunoprecipitate protein complexes containing PDIL1-1 (lane 4) and glutelin, (lane 6) indicating a interaction between these proteins. Furthermore, neither protein was detected

in the GFP control nor in the extract controls. This result is significant since a similar interaction between PDI and the soybean ortholog of the rice proglutelin has previously only been observed using chemical cross-linking to stabilize protein-protein interactions (Wadahama et al. 2007, Wadahama et al. 2008).

Attempts were also made to isolate PDIL1-1-prolamine co-immunoprecipitate complexes by, however any interaction, if present, was below our limit of detection (results not shown). This is perhaps not surprising given the abundance of PDIL1-1 within the glutelin-containing cisternal ER and its almost complete absence from prolamine-containing PB-I as well as the lower solubility of prolamines in aqueous solutions. These results suggest that PDIL1-1 interacts with proglutelin, thereby potentially preventing the deleterious interactions between proglutelin and prolamine within the ER lumen.

Discussion

The *esp2* mutant is characterized by its abnormally high levels of proglutelin which are deposited together with prolamine in small ER-derived inclusions (Takemoto et al. 2002). The inability to export proglutelin from the ER and to transport it to the PSV where it is processed into acidic and basic subunits results in abnormal increases in proglutelin levels and a simultaneous reduction in the levels of acidic and basic subunits. Despite the increases in the amounts of proglutelin, total glutelin levels as well as for

prolamines and globulins are lower in *esp2* compared to wild-type. BiP levels are elevated in *esp2* endosperm (Takemoto et al 2002), suggesting a condition reflecting an unfolded protein response (Kaufman et al. 2002). The reduction in glutelin accumulation is likely mediated by ER quality control processes induced by the unfolded protein response, which include the retrotranslocation of unfolded proglutelins and their subsequent turnover in the cytoplasm and the attenuation of protein synthesis. The latter is likely responsible for the reduction in the accumulation of prolamine and globulin.

Three independent *esp2* lines lacked PDIL1-1, indicating that the mutation is due to the loss of this lumenal chaperone and not due to an indirect modifying activity. This relationship is also supported by co-segregation analysis of an F_2 population between *esp2* (EM44) and wild type Kinmaze (Table 1), by RFLP analysis between *esp2* (CM1787) and the indica cultivar, Kasalath (Table 2), and by gene dosage studies (Fig. 1). This initial assignment of the *Esp2* locus as being the PDIL1-1 gene was substantiated by molecular analysis of three available independent *esp2* lines. Each of the three *esp2* lines contained a point mutation in the PDIL1-1 structural gene, which would affect its expression. Overall, this molecular analysis unequivocally demonstrates that *Esp2* is the structural gene for PDIL1-1.

Results from sucrose density gradient analysis of ER membranes (Fig. 4) and microscopic analysis (Fig. 5) indicate that PDIL1-1 is predominantly located in the cisternal ER. It is largely excluded from mature prolamine PBs with the exception of

small localized patches of PDIL1-1 on the surface of the prolamine PBs (Fig. 5B-F). The absence or very low levels of PDIL1-1 in the prolamine containing PB-I in wild-type suggests that PDIL1-1 is not involved in the folding and maturation of cysteine-rich prolamine polypeptides (Fig. 4 and 5). At a much lower frequency, PDIL1-1 was observed to be distributed uniformly around prolamine inclusion bodies (Fig. 5A). These PBs were smaller and stained less intensely with rhodamine, suggesting that they were at an earlier stage of development. It is not clear whether the presence of PDIL1-1 in younger prolamine PBs denotes a role in prolamine PB initiation or whether it simply indicates that it has not yet been excluded from this ER subdomain as it is differentiating from the cisternal ER.

As the cisternal ER membranes are the site of glutelin synthesis, an initial assumption is that PDIL1-1, which is mainly restricted to these membranes, is required for maturation of proglutelin to a conformation that is competent for export from the ER to the Golgi (Krishnan et al. 1986) and eventually to the PSV where it is proteolytically processed to acidic and basic subunits by the vacuolar processing enzyme (Kumamaru et al. 2010, Wang et al. 2009). In addition to its isomerase activity PDIL1-1 can serve as a chaperone in facilitating polypeptide folding, a role supported by the co-immunoprecipitation experiments presented in Fig. 6. The corresponding increase in proglutelin and the decrease in acidic and basic subunits in *esp2* suggest that in the absence of PDIL1-1, the proglutelin cannot efficiently attain the requisite protein conformation required for export

from the ER to the PSV. However, this dependence on PDIL1-1 for the folding of proglutelin polypeptide is temporally conditional and is only conspicuously evident at the mid and later stages of seed development, when the rates of proglutelin synthesis and accumulation are at their highest. In *esp2* developing seeds less than 10 days old, proglutelin levels are very low compared to those of mature subunits (Fig. 3B), indicating that the precursor has obtained a structural conformation competent for ER export to the PSV where it is efficiently proteolytically processed. This lack of PDIL1-1 dependence in younger *esp2* developing seeds indicates that proglutelin has an inherent native capacity to form disulfide bonds and fold to a structural conformation competent for ER export and that PDIL1-1 is not required for these processes. Alternatively, a different PDI activity may compensate for the loss of PDIL1-1 during early endosperm development. The rice genome has 19 *PDI* sequences (Houston et al. 2005) and at least 10 of them including PDIL1-1 are expressed in developing seeds (Supplementary Fig. S2). Of these ten, five possess a C-terminal KDEL, and one more has a C-terminal NDEL (Houston et al (2005), Supplementary Fig. S2). In soybean cotyledons, two PDI isoforms have been suggested to facilitate the polypeptide folding and maturation of proglycinin (Wadahama et al. 2007, Wadahama et al. 2008). Hence, one or more of these other PDI activities may serve in a capacity similar to that of PDIL1-1 in the cisternal ER in young developing rice seeds. The dependence on OsEro1, which donates disulfide bonds to PDI, for efficient maturation of proglutelin supports the involvement for one or more lumenal PDIs other than PDIL1-1

(Onda et al. 2009).

The presence and capacity of prolamines to interact with proglutelins readily account for the conditional accumulation of proglutelin in *esp2*. Moreover, it supports the view that PDIL1-1 is not absolutely essential for proglutelin maturation prior to its ER export. If PDIL1-1 is not essential for maturation of the proglutelin via promotion of intramolecular disulfide bond formation and polypeptide folding, then what role does it play? Under normal conditions, glutelins and prolamines are spatially separated from each other in the ER lumen as they are synthesized on the PB-ER and cisternal-ER, respectively. Although these membrane subdomains are continuous, these storage proteins never interact to any significant degree, presumably because the rates of ER export for glutelin and self-assembly for prolamines are much greater than the rates of their diffusion within the ER lumen. However, the efficient export of proglutelins only in the absence of prolamines but not in their presence in *esp2* indicates that diffusion rates can be similar, if not greater, than the rate of ER export and that some other mechanism is responsible for maintaining segregation of these storage proteins within their respective ER subdomains.

The microscopy results presented in Fig. 5 together with the supplementary movie provide significant insights. Except in young expanding prolamine PBs, PDIL1-1 is largely excluded from the prolamine-containing PB-I and is distributed to the cisternal ER. This localization pattern differs substantially from that observed for BiP (Fig. 5D), which is concentrated within PB-I. Three-dimensional co-localization studies of PDIL1-1 and

prolamine protein bodies allow us to speculate on the exact spatial relationship between PB-ER and cisternal ER subdomains (Fig. 5A and Supplementary movie). Although there are likely to be a large number of possible arrangements, our observations suggest that most protein bodies are positioned in accordance with one of the two models shown in Fig. 7A where the prolamine PB is associated with lamellar ER (model 1) or tubular ER (model 2). It is important to note that in both models the lower surface of the intracisternal inclusion granule lies beneath the upper surface of the cisternal ER membrane to which it is connected. This spatial arrangement depicted in model 1 explains that fact that when single optical sections are viewed, prolamine protein bodies frequently appear to be present within holes in the cisternal ER (Fig. 5A, C). This phenomenon is most obvious when looking at an image which lies in the same plane as cortical ER directly beneath the plasma membrane**.** In other cases, 3D reconstructions clearly show protein bodies on the surface of ER tubules in agreement with model 2 (see supplementary movie).

Fig. 7B shows that in both models, the region of potential interaction between prolamine and proglutelin is not limited to just a single point in space, but actually encompasses a significant amount of surface area. This interaction surface represents an important region in which protein-protein interactions are likely to be carefully modulated. The asymmetric localization patterns of PDIL1-1 and BiP within PB-ER and cisternal-ER subdomains observed in mid-stage developing endosperm is depicted in Fig. 7B and explains why the lack of PDIL1-1 in the *esp2* mutant allows deleterious heterotypic

interactions between prolamine and proglutelin polypeptides.

Material and Methods

Plant Material

The rice parent cultivar Kinmaze and the three *esp2* mutant lines, CM1787, EM44 and EM747, produced by N-methyl-N-nitrosourea (MNU) mutagenesis, were described previously (Kumamaru et al. 1987, Kumamaru et al. 1988, Takemoto et al. 2002). Rice plants were grown at the Kyushu University Farm field plots under natural conditions. Rice vegetative tissues and developing seeds harvested at 2, 3, 4, 6, 8, 10, 13, 15, 18, 21, 25, 30 and 35 days after flowering (DAF) were immediately frozen in liquid nitrogen and stored at -80 ºC until use.

For the gene dosage effect analysis of *Esp2* allele, endosperm tissue containing 3 (+++), 2 (++*e*), 1 (+*ee*) and 0 (*eee*) copies of *Esp2* allele was obtained from the self-pollination of progeny from an initial cross between Kinmaze (♀) and CM1787 (♂) or CM1787 (♀) and Kinmaze (♂), and the self-pollination of CM1787, respectively.

SDS-PAGE and Western analyses

SDS-PAGE of the crude protein extract and western blotting were performed as described previously (Takemoto et al. 2002).

Antibody

An antibody against BiP was raised against a 14 amino acid synthetic peptide of rice BiP. Antibodies against the rice PDIL1-1 and against GFP were raised in rabbits with purified bacterial-expressed recombinant protein. Antibodies against acidic and basic glutelin subunits, and prolamine were raised in rabbit and mouse, respectively.

Fractionation of PBs by Sucrose Density Gradient Centrifugation

0.5 g of dehulled developing rice seed was homogenized on ice using a mortar and pestle in 2 mL of 10 mM Tris-HCl, pH 7.5, containing 0.4 M sucrose. The homogenate was filtered through cheesecloth and centrifuged at 100 *g* for 10 min. One mL of the supernatant was layered onto a 40%, 45%, 50%, 52%, 54%, 56%, 58%, 60%, 62%, 64% (w/w) stepwise sucrose density gradient and centrifuged at 32,000 rpm for 1.5 hours in a BECKMAN SW40-Ti rotor and L-70 ultracentrifuge. Fractions (150 μL) were collected from the top of the gradient and analyzed by SDS-PAGE and immunoblotting.

DNA sequence analysis

Total genomic DNA from the leaves of *esp2* lines and two wild types, Kinmaze and Taichung65 was obtained using a CTAB (cetyltrimethylammonium bromide) method (Murray and Thompson 1980). DNA sequence was determined using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Ltd.). DNA sequence analysis was

performed using EditView1.0.1 and AutoAssembler 2.1. Comparisons between wild type and mutants sequences were performed using CLUSTALW of DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/top-e.html).

Transmission Immunoelectron Microscopy

Transmission immunoelectron microscopy was performed as described by Takemoto et al., (2002).

Indirect Fluorescence Labeling of Developing Rice Seeds for Confocal Microscopy

Freshly-harvested mid-developing rice seeds were submerged in MTSB (50 mM Pipes-KOH, pH 6.9, 10 mM EGTA, 10 mM $MgSO_4$, 1% DMSO, 0.1% Triton X-100) and sectioned to 60 µM using a vibratome prior to fixation for 30 min in MTSB containing 4% paraformaldehyde. Following three brief washes with MTSB, cell wall digestion was performed by incubating sections with 100 µL of pre-warmed 1% w/v cellulase (Onosuka R-10, SERVA) solution (0.4 M mannitol, 0.1% Triton X-100, 0.3 mM PMSF and 5 mM EGTA, pH 5.5) at 37°C for 5 min. The cellulase solution was then replaced with PBS containing 0.5% Triton X-100 and samples were incubated for 15 min at room temperature. Prior to incubation with antibodies, seed sections were treated with blocking buffer (2% BSA in PBS, 0.1% Triton X-100) for 1 hour to prevent non-specific binding. Rabbit anti-PDIL1-1 and mouse anti-BiP antibodies were added at a dilution of 1:200 in blocking

buffer and sections were incubated for 3 hours before washing three times (for 5 min each time) using PBS containing 0.01% Triton-X100. Secondary antibody (fluorescein goat anti-rabbit and mouse IgG (H+L), Invitrogen) was added at a dilution of 1:200 in blocking buffer and sections were incubated for 1 hour with Rhodamine B hexyl ester being added to the sample volume after 40 min at a final concentration of 1 μM. Following three 5 min washes with PBS containing 0.1% Triton X-100 and a final wash with PBS alone, a single drop of Vectashield (Vector Laboratories, Inc. Burlingame, CA) anti-fade mounting medium was added to each well and a number one coverslip was added and sealed with nail polish.

Confocal microscopy was performed on a Zeiss 510META laser scanning confocal microscope (Jena, Germany) or a Bio-Rad View Scan DVC-250 laser scanning confocal microscope using fluorescein and rhodamine filter sets. Image processing was performed using Adobe Photoshop (Mountain View, CA), ImageJ, or Microsoft PowerPoint (Redmond, WA).

Immunoprecipitation

Freshly harvested mid-developing rice seed was dehulled and 1 g was extracted in 6 mL IP buffer (20 mM Tris-HCl pH 7.5, 75 mM NaCl, 1 mM EDTA, 0.1% NP-40). The extract was then centrifuged for 10 min at 20,000 *g* and the supernatant removed to a new tube and subjected to additional centrifugation using the same conditions. Protein A resin

which had previously been incubated overnight with either 1xPBS (no antibody control), anti-GFP, anti-glutelin or anti-PDIL1-1 antibody was washed three times with 1 mL IP buffer prior to the addition of 1.5 mL of the cleared supernatant described above. Extracts and antibody were incubated for 2 hours prior to washing five times with 1 mL IP buffer. The Protein A was then boiled in SDS-containing sample buffer prior to western blotting with both anti-glutelin and anti-PDIL1-1 antibodies at a dilution of 1:1000.

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Short legends for supplementary material

The following materials are available in the online version of this article.

Supplementary Movie S1. 3D Movie of Localization of PDIL1-1 in developing rice endosperm.

3D reconstruction using maximum intensity projections of PDIL1-1 (green) localization in

developing rice endosperm obtained by indirect immunofluorescence confocal microscopy. PB-ER is stained with Rhodamine (magenta). This movie shows the spatial relationship between PB-ER and C-ER sub-domains based on the contrasting localizations of PDI and Rhodamine staining, respectively.

Supplementary Fig. S1 cDNA and deduced amino acid sequence of PDIL1-1 in Kinmaze. The deduced amino acid sequence is shown below the nucleotide sequence using the single-letter code. The stop codon is indicated with an asterisk. The putative signal peptide is highlighted. Thioredoxin active sites (-CXXC-), N-linked glycosylation site (-NXF/T-) and the C-terminal ER-retention signal (KDEL) are indicated.

Supplementary Fig. S2 Spatial expression of the various rice PDI homologs as assessed by RT-PCR.

Supplementary Table 1. The primers used for expression analysis via RT-PCR.

Figure Legends

Fig. 1 Gene dosage effects of *Esp2* gene on PDIL1-1 and seed storage proteins. + and *e* indicate wild type and mutation genes for *Esp2*, respectively. +++, ++*e*, +*ee* and *eee* indicate endosperm genotype possessing three, two, one or null wild type *Esp2* allele(s),

respectively. A: Coomassie brilliant blue staining of SDS-polyacrylamide gel containing seed extracts. The seed extracts in panel A were prepared by first extracting crushed seeds with 0.5 M NaCl to remove globulins which interfered with the resolution of glutelin and prolamines on polyacrylamide gels. The high salt-washed residue was then prepared in SDS sample buffer and analyzed. Numbers on the left refer to the molecular weights in kD. B: Immunoblot with PDIL1-1 and glutelin acidic subunits antibodies. C: Relative protein levels obtained by densitometric scanning of the immunoblot signal. The protein level of PDIL1-1 and glutelin acidic subunit in +++ genotype and *eee* genotype were defined as 100% and 0%, respectively. The level of proglutelin (glutelin precursor) in the *eee* genotype was defined as 100%.

Fig. 2 Schematic representation of the PDIL1-1 gene and base substitutions detected in three *esp2* mutant lines. ATG and TGA indicate the initiation and termination codons, respectively. Black boxes indicate exons. CM1787 contains a premature stop codon while EM44 (Kinmaze origin) and EM747 (Taichung65 origin) have nucleotide substitutions at exon splice sites.

Fig. 3 Temporal accumulation patterns of seed storage proteins during the development of rice endosperm. A and B indicate the wild type c.v. Kinmaze, and *esp2* mutant, CM1787, respectively. Total protein extracted from developing seeds was separated by SDS-PAGE

(upper panels) and protein quantities were measured by densitometry (lower panels). The staining intensity of the 80 kD polypeptide at 35 DAF indicated by an arrowhead was defined as 100%. C: Immunoblot analysis using BiP, PDIL1-1 and glutelin acidic/basic subunit antibodies.

Fig. 4 Fractionation of protein bodies and ER membranes by sucrose density gradient centrifugation. A: CBB stained SDS-PAGE gel containing odd-numbered fractions from a sucrose density gradient centrifugation of 15 DAF developing rice seeds. The positions of cisternal ER membranes, PB-I and protein storage vacuoles (PSV) are indicated. M = molecular weight markers, K = Kinmaze total extract. B: Immunoblot analysis using antibodies against PDIL1-1 and BiP.

Fig. 5 Localization of PDIL1-1 in developing endosperm of wild type rice.

A: PDIL1-1 (green) and prolamine protein bodies (magenta) were visualized by fluorescent labeling using a FITC conjugated secondary antibody and Rhodamine B hexyl ester respectively. The larger panel (x-y) shows a single 1 µm optical section from a stack of 18 consecutive images. The upper and left panels show x-z and y-z orthogonal views through all 18 sections along the dashed lines shown in the main image. Arrows indicate protein bodies which are sectioned in the orthogonal views. The arrows in the upper left corner show immature protein bodies which contain significant amounts of PDIL1-1 protein. Scale bar = 5 µm.

B: Localization of PDIL1-1 and prolamine within 15 DAF rice endosperm 200 nm sections. Double immunostaining was performed using rabbit PDIL1-1 and mouse anti-prolamine antibodies labeled with FITC (green) and rhodamine (magenta) respectively. The inset panel shows a higher magnification view in which PDIL1-1 within cisternal tubular ER membranes is seen in direct contact with prolamine protein bodies. Scale bar = 5 µm.

C: Single channel images and a merged image showing the spatial relationship between rhodamine-stained prolamine protein bodies (magenta) and PDIL1-1 labeled with FITC-conjugated secondary antibody (green). Four consecutive 1 µm optical sections were stacked. Scale bars = 5 µm.

D: The images are projections from 4 consecutive 1 micron images single channel images

and a merged image showing the spatial relationship between BiP labeled with Alexa 488-conjugated secondary antibody (magenta) and PDIL1-1 labeled with FITC-conjugated secondary antibody (green). Four consecutive 1 µm optical sections were stacked. Scale bars = 5 µm.

E and F: Immunoelectron microscopy of developing wild type rice seed at 15 DAF. Anti-PDIL1-1 antibody labeled with gold particles was used to localize PDIL1-1. Panels E and F show that PDIL1-1 is found both within cisternal-ER membrane (C-ER, arrows) and is present at high concentrations in discrete regions of protein bodies (PB-I, arrowhead). Scale bars = 500 nm.

G: Immunoelectron microscopy of the developing *esp2* rice seed at 15 DAF. Anti-globulin and anti-glutelin labeled with gold particles were used to localize globulin (10 nm, closed arrowheads) and glutelin (20 nm, open arrowheads). Note that both globulin and glutelin are contained within the ER in *esp2* and the protein granule is smaller than PB-I in wild type. Scale bar = 500 nm.

Fig. 6 Co-immunoprecipitation of PDIL1-1 and glutelin. A total rice extract from developing seed was immunoprecipitated using either GFP (lane 2), glutelin (lane 4) or PDIL1-1 (lane 6) antibodies (as indicated above the lanes) prior to analysis of the captured material by SDS-PAGE and western blotting using both PDIL1-1 and glutelin antibodies at same time. Lane 1 is a positive control showing the presence of both PDIL1-1 and

glutelin in the extract. Notice that complexes containing PDIL1-1 and glutelin are immunoprecipitated with both glutelin and PDIL1-1 antibodies (lane 4, 6). The arrowhead and open arrowhead depict PDIL1-1 and glutelin captured by anti-glutelin and anti-PDIL1-1, respectively. The GFP and no antibody control lanes (lanes 2,3 and 5) show that there is no background binding of either PDIL1-1 or glutelin to the Protein A resin. In western analysis, PDIL1-1 and glutelin antibodies were used.

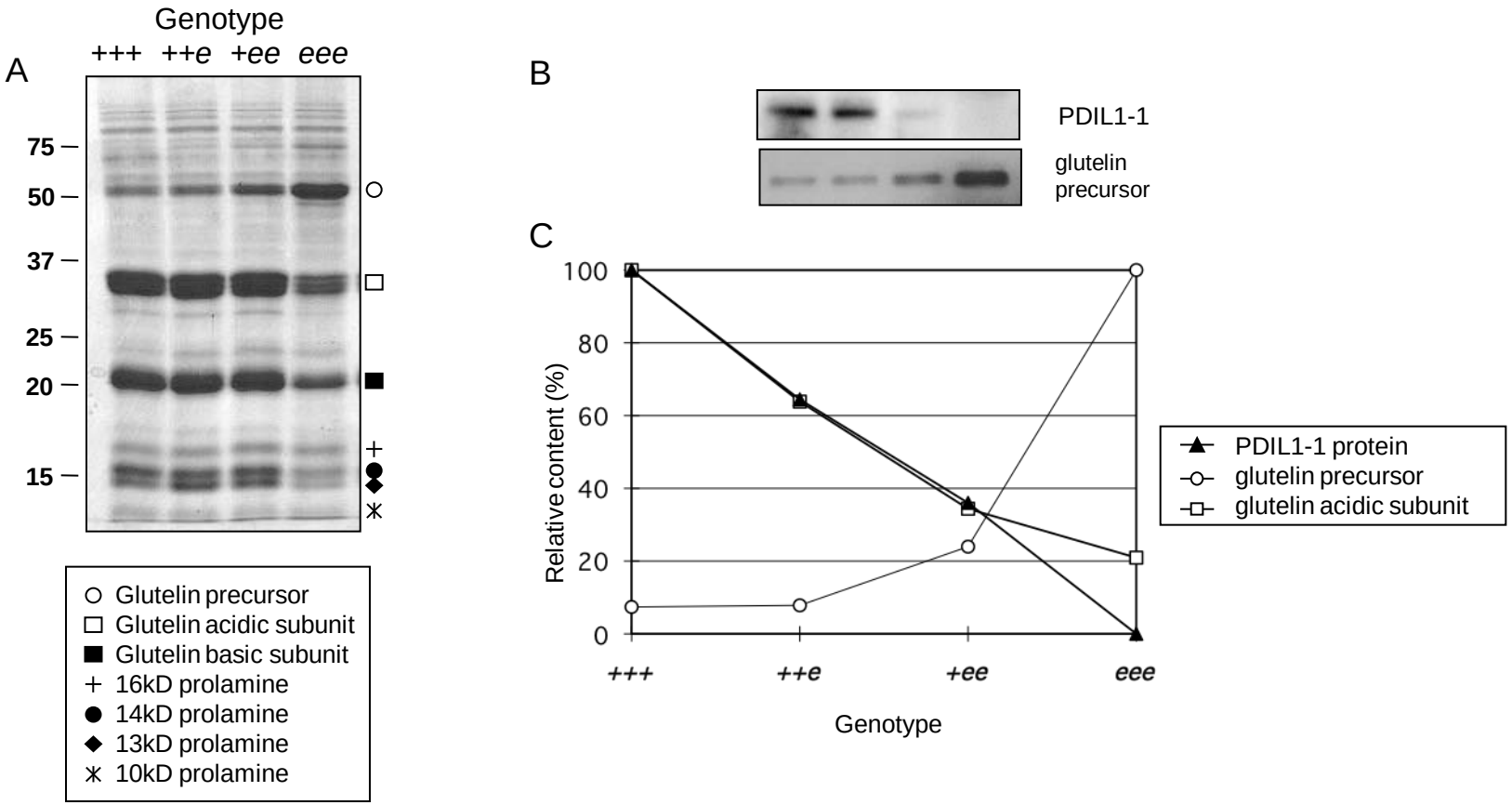
Fig. 7 The spatial relationship between PB-ER and cisternal ER subdomains.

A: Two schematic models are shown which depict spatial relationships between cisternal ER (C-ER) and protein body ER (PB-ER) subdomains as suggested by confocal microscopy. Model 1 shows a protein body, the surface of which is sunken below the surface of a lamellar sheet of ER. Such a model explains the fact that protein bodies appear to lie within holes in the cisternal ER (see Fig. 5A and C). Model 2 shows a protein body lying on an ER tubule, again with the lower surface of the spherical protein body beneath the upper surface of the tubule on which it sits. Further evidence in support of both of these models can be found in the supplementary movie.

B: Schematic representation of a cross section through a mid-stage prolamine protein body and adjacent cisternal ER. Ribosomes translating mRNA encoding prolamine and the resulting prolamine polypeptides are shown in black. Ribosomes translating glutelin messages and the resulting proglutelin polypeptides are shown in white. Note the

asymmetric distribution of both prolamine and glutelin messages and of the lumenal chaperones BiP (see Fig. 5D) and PDIL1-1. This distribution would allow PDIL1-1 to interact with proglutelin and prevent it from interacting with prolamine.

Figure 1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50
37
25
20
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Glutelin precursor
Glutelin acidic subunit
Glutelin basic subunit
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14kD prolamine
13kD prolamine
10kD prolamine
B
PDIL1-1
glutelin precursor
C
Relative content (%)
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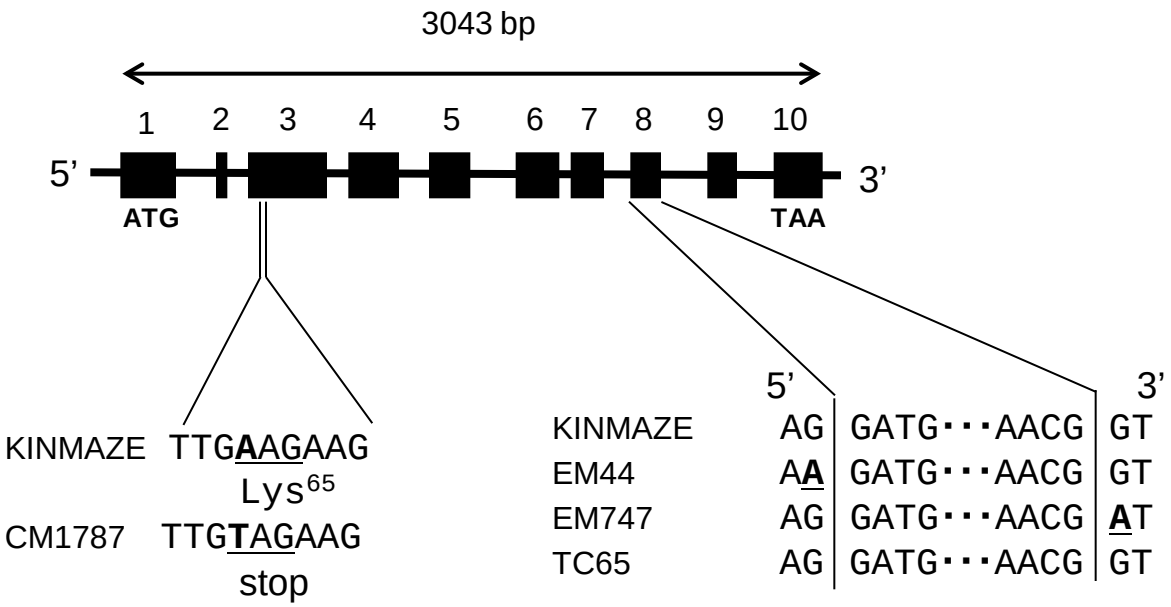
60
40
20
0
+++
++e
+ee
eee
Genotype
PDIL1-1 protein
glutelin precursor
glutelin acidic subunit

Figure 2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2
3
4
5
6
7
8
9
10
5'
3'
ATG
TAA
KINMAZE TTGAAGAAG
Lys65
CM1787 TTGTAGAAG
stop
5'
3'
KINMAZE AG GATG···AACG GT
EM44 AA GATG···AACG GT
EM747 AG GATG···AACG AT
TC65 AG GATG···AACG GT

Figure 3

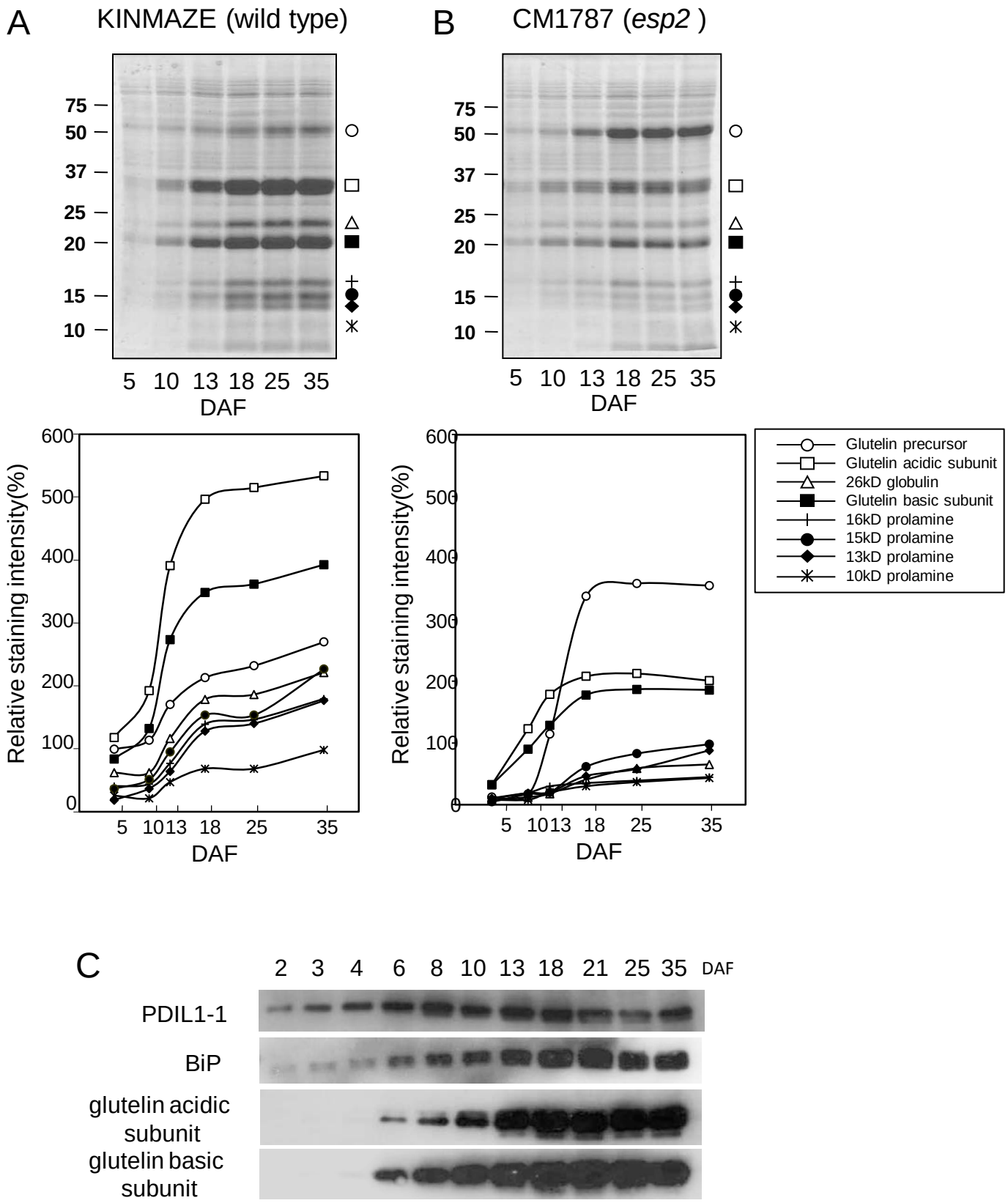
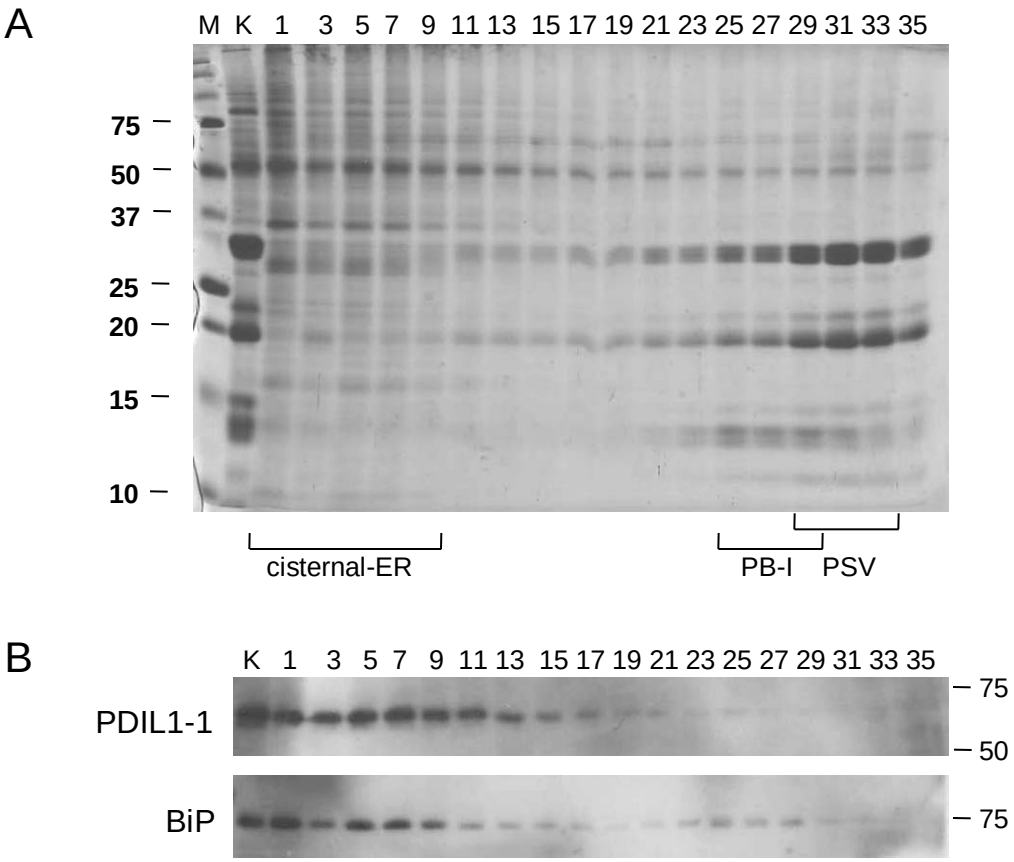

A
KINMAZE (wild type)
B
CM1787 (esp2)
75
50
37
25
20
15
10
5 10 13 18 25 35
DAF
Relative staining intensity(%)
600
500
400
300
200
100
0
Glutelin precursor
Glutelin acidic subunit
26kD globulin
Glutelin basic subunit
16kD prolamine
15kD prolamine
13kD prolamine
10kD prolamine
C
2 3 4 6 8 10 13 18 21 25 35 DAF
PDIL1-1
BiP
glutelin acidic subunit
glutelin basic subunit

Figure 4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50
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15
10
cisternal-ER
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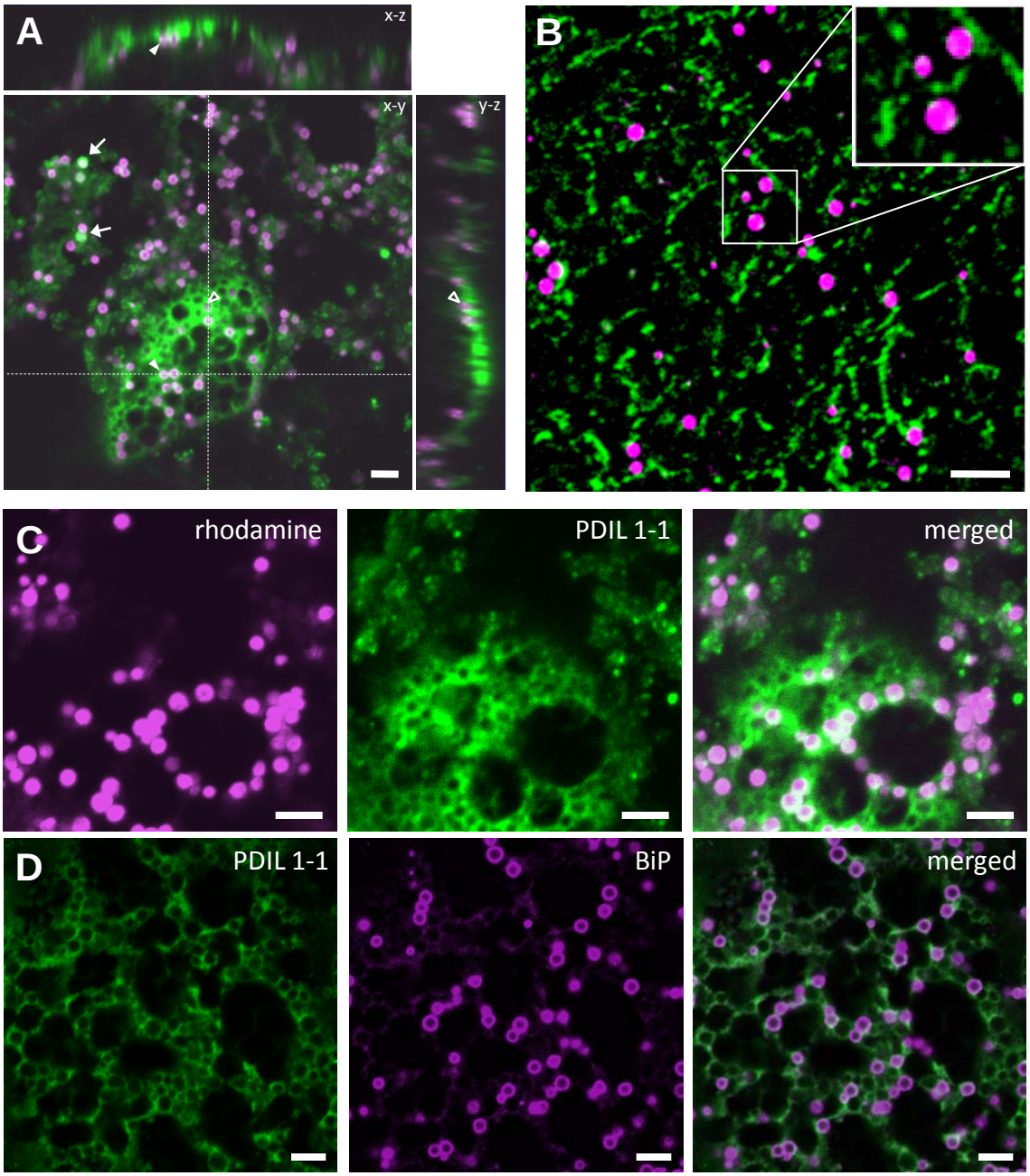
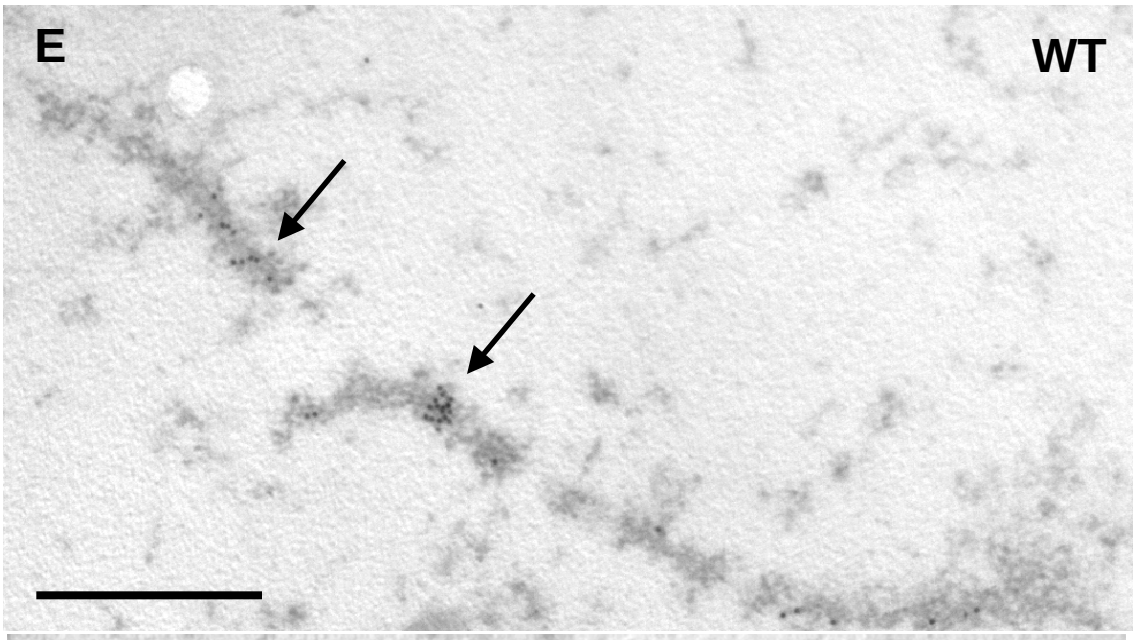
PDIL1-1
75
50
BiP
75

Figure 5

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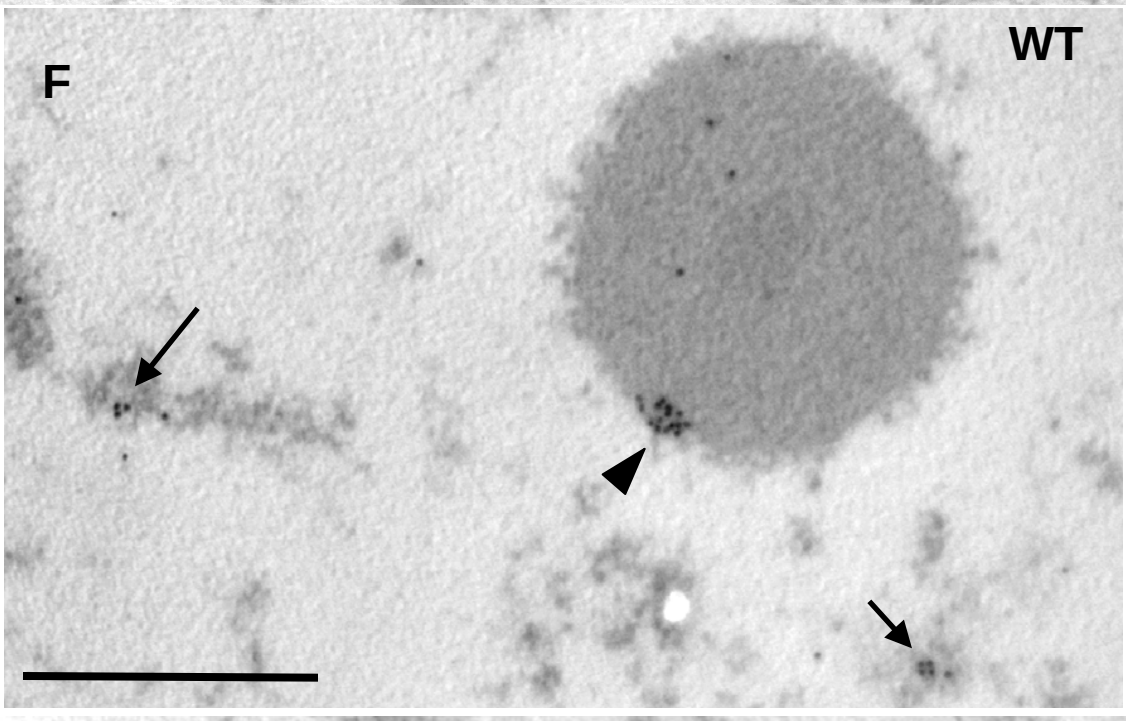
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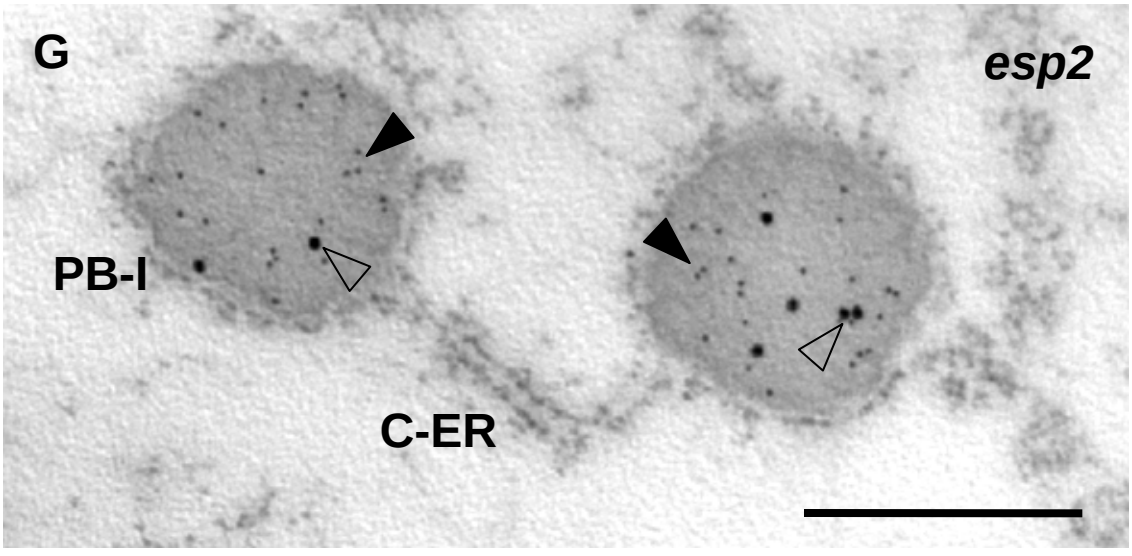
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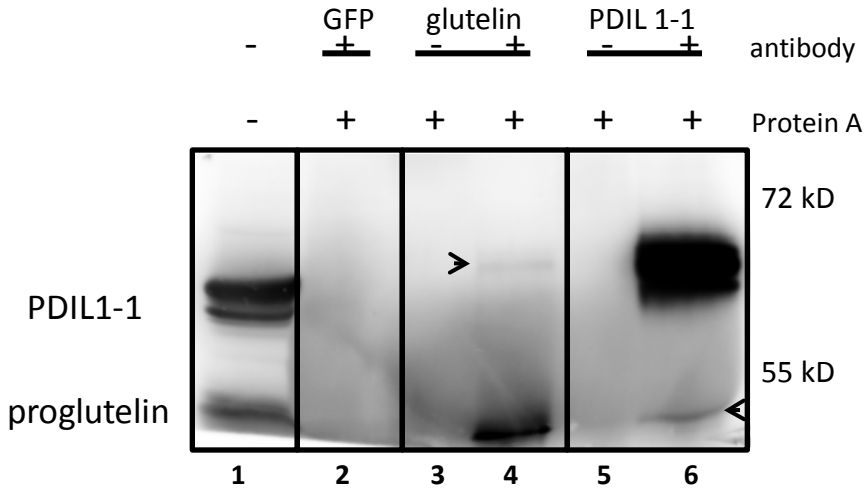
esp2
PB-I
C-ER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glutelin
PDIL 1-1
antibody
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PDIL1-1
proglutelin
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2
3
4
5
6

Fig6

Figure 7

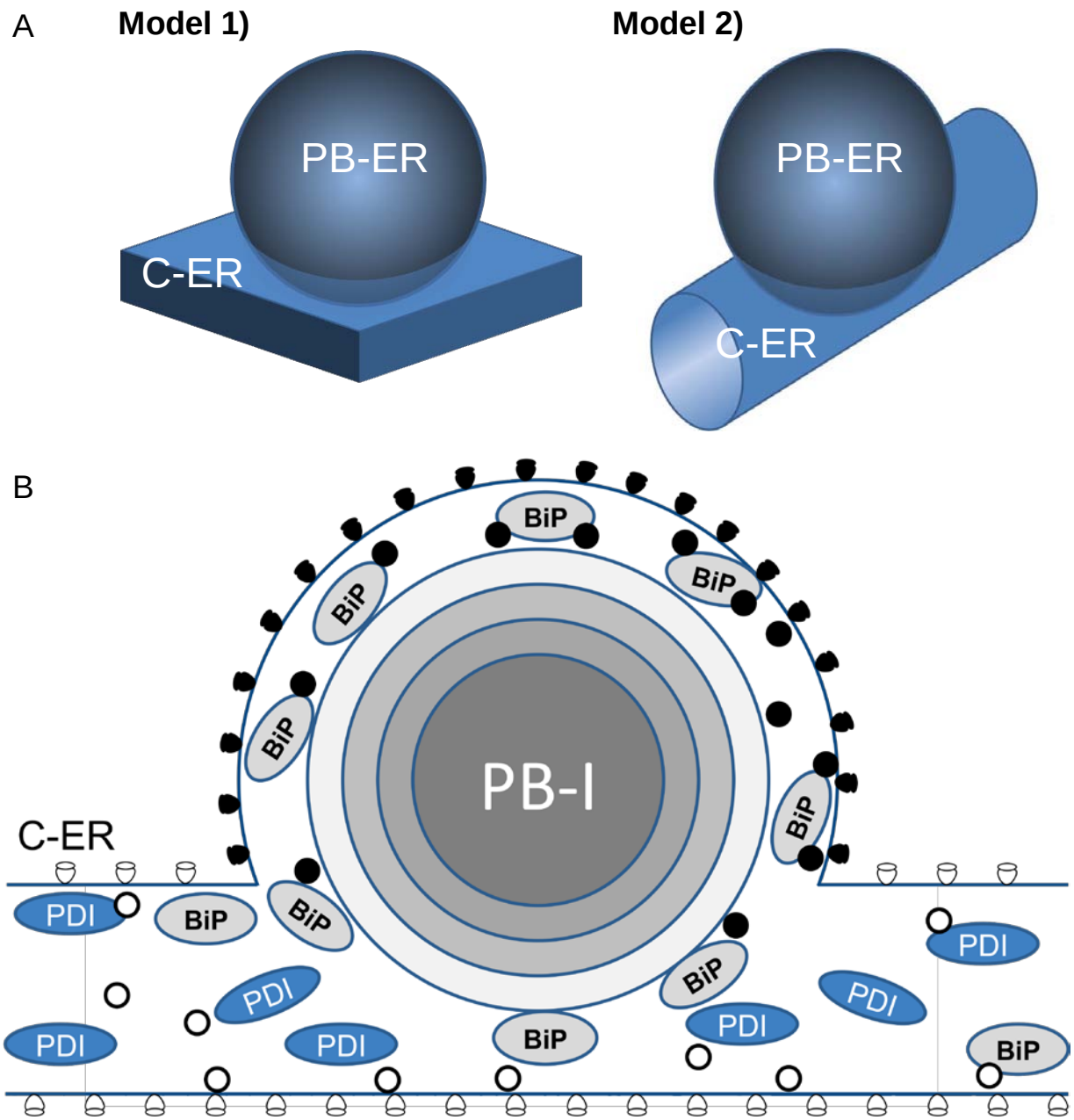
A
Model 1)
Model 2)
PB-ER
C-ER
PB-ER
C-ER
B
BiP
BiP
BiP
BiP
BiP
PB-I
C-ER
PDI
BiP
BiP
PDI
PDI
BiP
PDI
PDI
PDI
PDI
BiP
BiP

Table 1. Segregation mode of *esp2* type and PDI deficiency in F_2 seeds crosses between an *esp2* mutant line, EM44, and the wild type, Kinmaze.

Cross comb.	Segregation in F_2 seeds				Total	X^2 (3:1)
	+		*esp2*			
	PDIL1-1+	PDIL1-1-	PDIL1-1+	PDIL1-1-		
EM44 x Kinmaze	69	0	0	21	90	0.133
	85	0	0	31	116	0.084
Total	154	0	0	52	206	0.006

Table 2. Segregation mode of PDIL1-1 RFLP in *esp2* homozygous F_2 plants from a cross between *esp2* mutant, CM1787 and *indica* cultivar, Kasalath.

	Number of plants		
	PDIL1-1 ^{kasa} *PDIL1-1* ^{kasa}	*PDIL1-1* ^{kasa} *PDIL1-1* ^{esp2}	*PDIL1-1* ^{esp2} *PDIL1-1* ^{esp2}
	0	0	77
	0	0	26
Total	0	0	103