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Molecular Cloning, Expression and Subcellular Localization of a BiP Homolog from Rice Endosperm Tissue

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The ER luminal binding protein, BiP, has been linked to prolamine protein body formation in rice. To obtain further information on the possible role of this chaperone in protein body formation we have cloned and sequenced a BiP cDNA homolog from rice endosperm. The rice sequence is very similar to the maize BiP exhibiting 92% nucleotide identity and 96% deduced amino acid sequence identity in the coding region. Substantial amino acid sequence homology exists between rice BiP and BiP homologs from several other plant and animal species including long stretches of conservation through the amino-terminal ATPase domain. Considerable variation, however, is observed within the putative carboxy-terminal peptide-binding domain between the plant and nonplant BiP sequences. A single band of approximately 2.4 kb was visible when RNA gel blots of total RNA purified from seed tissue were probed with radiolabeled rice BiP cDNA. This band increased in intensity during seed development up to 10 days after flowering, and then decreased gradually until seed maturity. Protein gel blots indicated that BiP polypeptide accumulation parallels that of the prolamine polypeptides throughout seed development. Immunocytochemical analysis demonstrated that BiP is localized in a non-stochastic fashion in the endoplasmic reticulum membrane complex of developing endosperm cells. It is abundant on the periphery of the protein inclusion body but not in the central portion of the protein body or in the cisternal ER membranes connecting the protein bodies. These data support a model which proposes that BiP associates with the newly synthesized prolamine polypeptide to facilitate its folding and assembly into a protein inclusion body, and is then recycled.

Key words: BiP-Endosperm — Prolamine — Protein Body — Rice.

Developing rice endosperm cells accumulate both major types of storage proteins, the glutelins (globulin-like) and the prolamines (Juliano 1972, Muench and Okita 1997), which are readily observed in seed tissue of all plants. Both protein types are synthesized on the ER but are deposited into distinct protein bodies (PBs) via different cellular pathways. The glutelins are deposited into protein storage vacuoles via the Golgi complex, whereas the prolamines are deposited into PBs derived directly from the ER (Krishnan et al. 1986, Yamagata et al. 1986). Electron microscopy reveals two types of rough ER in rice endosperm cells; the cisternal ER (C-ER) and the protein body ER (PB-ER). As shown by biochemical, in vitro hybridization and in situ hybridization studies, prolamine mRNAs are enriched 7 to 10-fold over the glutelin mRNAs on the PB-ER whereas the glutelin mRNAs are enriched more than 2-fold over the prolamine mRNAs on the C-ER (Li et al. 1993a). Therefore, the packaging of the prolamines and glutelins into separate PBs is facilitated by the preferential localization of their transcripts to the PB-ER and C-ER, respectively.

As proposed by Okita et al. (1994) the preferential localization of prolamine and glutelin mRNA on the PB-ER and C-ER respectively, may involve one of a number of possible mechanisms. The first mechanism involves an mRNA targeting signal that is recognized by a cytoskeleton associated factor which then mediates transport to a protein factor that anchors the RNA to the PB-ER. A second model involves a heterogeneous population of signal recognition particles (SRPs) associated with subdomains of the ER allowing different RNAs to dock with specific subdomains via their encoded signal peptides (Mitsukawa and Tanaka 1991). Finally, the enrichment of prolamine mRNA on the PB-ER may result from events involved with the folding and assembly of the prolamine nascent polypeptides in the lumen of the PB-ER.

Other than the general morphological features, very little is known about how prolamines are assembled to form PBs. In maize, a direct assembly model was originally proposed since the major prolamine polypeptide class, α-zein, is composed of a tandemly repeated peptide. These repeated peptides have the potential to form α-helices that fold to form a rod-shape molecule that could be assembled into tightly packed bundles by interchain hydrogen bonding. Protein body formation would be facilitated by the largely hydrophobic character of the repeated peptide in the ER lumen, an environment of high ionic strength

Abbreviations: C-ER, cisternal ER; PB-ER, protein body ER; SRPs, signal recognition particles.

(Argos et al. 1982, Burr and Burr 1976, Pfeffer and Rothman 1987). The capacity of microinjected *Xenopus* oocytes to condense newly synthesized zeins which sediment with the same density as native PBs is also consistent with a self-assembly model (Hurkman et al. 1981). Alternatively, PB formation may involve the role of molecular chaperones, including the lumenal binding protein BiP. Three independent maize mutants, *floury-2*, Defective endosperm *B-30*, and *Mucronate*, have reduced concentrations of zein and an increase in the concentration of b-70, a polypeptide bound to PBs (Boston et al. 1991, Fontes et al. 1991, Marocco et al. 1991, Zhang and Boston 1992). Structural and biochemical analysis of b-70 revealed that it is a homolog of the immunoglobulin binding protein, BiP (Fontes et al. 1991). As the PBs in these mutants have morphologies that are severely disrupted (from small spherical to large irregularly shaped) and the normal pattern of zein aggregation within the PBs is disturbed, the mutation is likely a result of defective folding or assembly of zein polypeptides. Recent sequence analysis of a cDNA encoding the *floury-2* allele indicates that it encodes a variant α-zein containing a point mutation resulting in an amino acid substitution at the carboxy-terminal position of the signal peptide (Coleman et al. 1995). This substitution results in an uncleavable signal peptide that likely causes the protein to be anchored to the PB-ER membrane and thereby prevents its proper folding. Accumulation of unfolded zein polypeptides would provide the signal for transcriptional activation of BiP genes.

Recent evidence has demonstrated that BiP may have a significant role in rice prolamine PB formation. In rice endosperm cells, BiP was readily detected in crude rough microsomal and PB fractions but at different amounts (Li et al. 1993b). The bulk (90%) of the total BiP observed in developing endosperm tissue was found in the PB fraction with less than 10% detected in the rough microsomal fraction enriched for C-ER membranes (Li et al. 1993b). The enriched localization of BiP with the PB fraction suggests that this chaperone is involved in the folding and assembly of newly synthesized prolamine polypeptides. Direct biochemical evidence for this role was obtained by analysis of the interaction of BiP with nascent and intact prolamine polypeptide chains. BiP forms a complex with nascent prolamine polypeptides attached to polysomes in an ATP-insensitive manner, and also forms an ATP-sensitive complex with mature free prolamine polypeptides and those associated with the surface of the protein inclusion body itself (Li et al. 1993b). That these three stable prolamine/BiP complexes are present from the initial prolamine synthesis to its assembly into an inclusion body supports the view that BiP functions to retain prolamine polypeptides within the ER lumen in addition to its role in protein folding and assembly (Li et al. 1993b).

In an effort to better understand the role of BiP in rice prolamine PB formation we have cloned a cDNA encoding rice BiP and demonstrate that the expression of BiP protein parallels that of prolamine protein during seed development. We also provide immunocytochemical evidence that substantiates our earlier observation (Li et al. 1993b) using crude organelle fractionation that rice BiP is not distributed in a stochastic fashion within the ER membrane system but is primarily associated with the prolamine PBs. In addition, we show that BiP is detected only at the periphery of the prolamine protein body. These results are consistent with a BiP-mediated model of prolamine PB formation.

Materials and Methods

cDNA cloning and PCR—A cDNA library was constructed from mRNA isolated from mid-developing rice endosperm and was probed with radiolabelled maize BiP cDNA (Fontes et al. 1991) as described by Kim and Okita (1988). Only partial BiP cDNA clones were isolated and, therefore, 5′-RACE (rapid amplification of cDNA ends) PCR was used to obtain the remaining coding region and a large portion of 5′ untranslated region. For this procedure, total RNA was isolated from mid-developing rice seed as described (Kim and Okita 1988), and an antisense primer was synthesized (Fig. 1). A 5′ RACE kit was used and the conditions for amplification were described by the manufacturer (Gibco BRL).

RNA isolation and RNA gel blots—Total RNA was isolated from rice seed using an established protocol (Reeves et al. 1986). For RNA gel blots, 10 to 15 μg of total RNA was fractionated by electrophoresis through 1.2% formaldehyde gels and then transfered to nitrocellulose membranes by the capillary method. Hybridization was performed as described (Kim and Okita 1988).

Protein extraction and protein gel blots—Total protein was extracted from endosperm tissue, and 50 μg of protein fractionated by SDS-PAGE. Protein gel blots were probed with antisera as described (Li and Okita 1993). Polyacrylamide gels were composed of a 5–15% gradient with a stacking gel and antisera used were rice anti-prolamine, rice anti-glutelin and maize anti-BiP.

Immunocytochemistry—Rice seed fixation, sectioning, immunostaining and electron microscopy were performed as described previously (Li and Franceschi 1990). Ultrathin sections were probed with maize anti-BiP antiserum and the antibody-antigen complex was detected with protein A conjugated with 15 nm gold and visualized on an Hitachi-600 transmission electron microscope.

Results

Isolation of BiP cDNA clones—A lambda ZAP cDNA library constructed from mid-developing rice seed mRNA was probed with a radiolabelled maize BiP cDNA. Several clones were isolated, the longest of which was 1,931 bp in length. DNA sequence analysis revealed the presence of a long single open reading frame that was lacking coding sequences for the amino-terminal end. Efforts to isolate a full-length cDNA clone from the library were not successful and, therefore, a PCR-based method was used to isolate the remaining 5′ end of the cDNA. A 5′ RACE-derived fragment was isolated and this fragment contained

Fig. 1 Nucleotide sequence of cDNAs encoding rice and maize (Wrobel 1996) BiP. Numbers refer to the nucleotide number, dots indicate nucleotide matches and spaces represent gaps between the two sequences. The sequence complimentary to the antisense primer used for PCR amplification is underlined. The translation start and termination codons are underlined.

the remaining 5′ coding region including 120 bp of 5′ untranslated sequence (Fig. 1).

DNA sequence analysis revealed that the rice BiP cDNA was very similar to the maize BiP cDNA sequence. The two sequences share 92% nucleotide identity within the coding region and have an identical open-reading frame length (Fig. 1). The deduced amino acid sequences of the maize and rice clones display 96% sequence identity. Except for the C-terminal end, most of the primary sequence including the signal peptide of the rice and maize amino acid sequences are identical. The few differences involved neutral changes in amino acid residues. Similar to maize and tobacco BiP, rice BiP contains the tetrapeptide HDEL at the C-terminus, a sequence known to function in the ER-retention/retreival of this protein (Pelham 1988). The HDEL tetrapeptide sequence, however, is not universal among plant proteins as other ER-targeted plant proteins possess the KDEL retention signal common to mammalian ER-resident proteins (Akasofu et al. 1989, Hesse et al. 1989).

Amino acid comparison with other BiP sequences— Alignment of the deduced rice BiP polypeptide sequence with BiP homologs from several other species demonstrates a high level of sequence conservation among kingdoms (Fig. 2). Substantial variation between rice BiP and the other BiP sequences occurs in the signal peptide and the

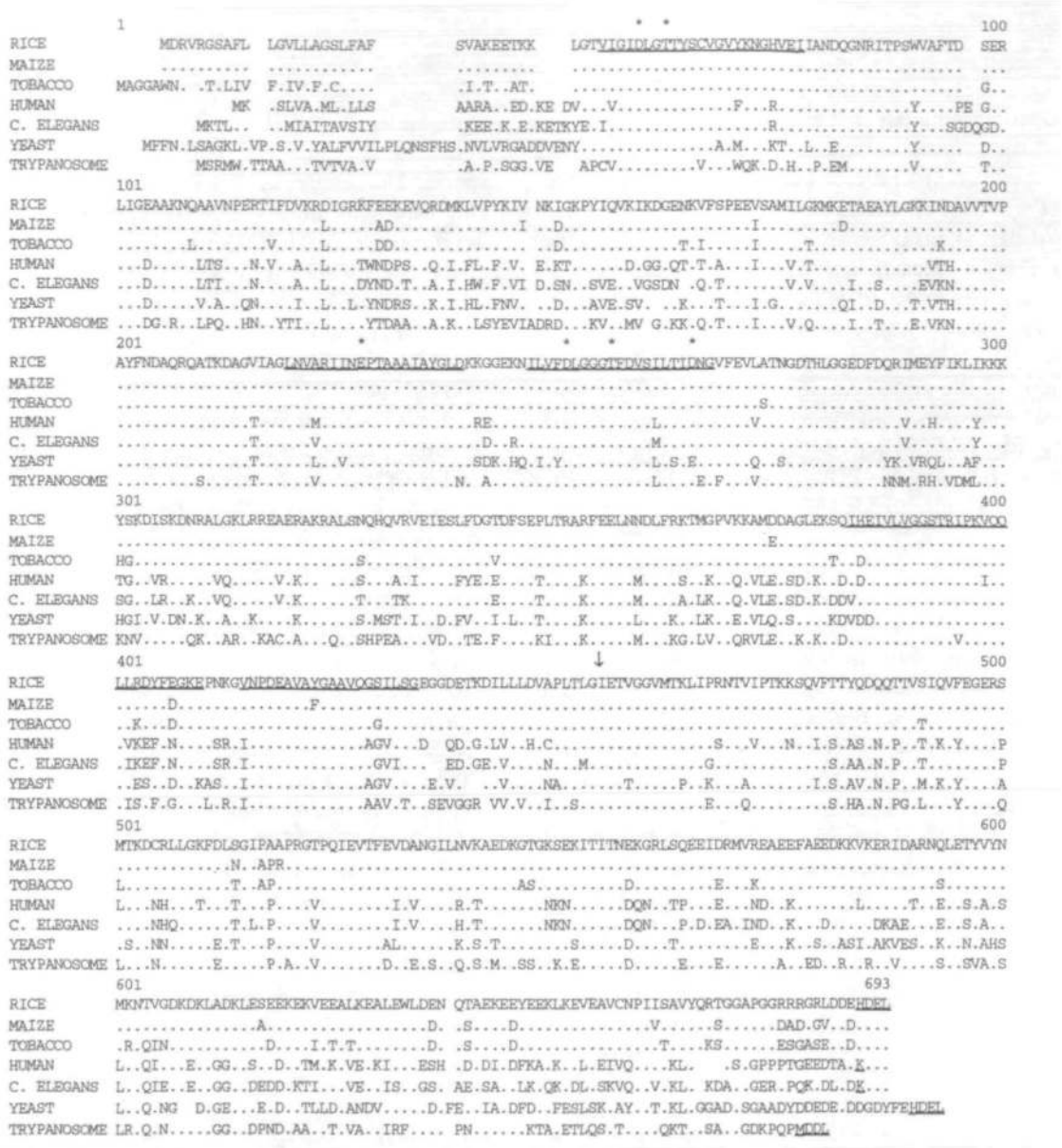

Fig. 2 Comparison of the deduced amino acid sequence of rice BiP with homologs from maize (Wrobel 1996), tobacco (BLP-4, Denecke et al. 1991), human (C. Chao, genbank accession #X87949), *C. elegans* (hsp 3, Heschl and Baillie 1989), yeast (KAR-2, Rose et al. 1989) and trypanosome (Bangs et al. 1993). Numbers, dots and gaps are as in Fig. 1. The carboxy-terminal ER-retention signals are underlined as are the conserved peptide regions within the ATPase domain. The arrow indicates the cleavage site dividing the ATPase domain from the peptide-binding domain. The functional residues important for ATP-binding as determined by site-specific mutagenesis are marked above with an asterisk (*).

carboxy-terminal region, although regions from amino acid 100 to 200 and 300 to 385 also show many differences. Compared to the rice BiP sequence, many of the residues that are variant in one non-plant sequence are also variant in the other non-plant sequences as well.

BiP and other hsp 70 homologs possess two active domains, an amino-terminal domain (about 45 kDa) which contains stretches of high conservation and possesses an ATPase activity, and a carboxy-terminal domain which displays more variation and is believed to be the peptide-binding domain (Hartl 1996). Amino-terminal proteolytic fragments of approximately 44 kDa from several hsp 70 homologs retain ATPase activity. Thus, the ATP activity can be uncoupled from the carboxy-terminal peptide-binding domain activity (Gething and Sambrook 1992). The amino acid that separates the amino-terminal ATPase do-

main from the carboxy-terminal peptide-binding domain corresponds to residue 454 in Fig. 2. Conserved regions important for the amino-terminal ATPase activity have been identified in mammalian BiP and hsp 70, as well as functionally diverse proteins such as actin and several sugar kinases (Bork et al. 1992, Gaut and Hendershot 1993, Wilbanks et al. 1994). Based on the optimal superposition of these different proteins and on sequence conservation from members of each family, a pattern of conserved and invariant residues was distributed into five sequence motifs (Bork et al. 1992). These five motifs are also highly conserved in the plant BiP sequences (Fig. 2). Site-specific mutagenesis of the motifs has identified several important amino acid residues within these motifs which are required for maximal ATPase activity and/or substrate affinity (Gaut and Hendershot 1993, Wilbanks et al. 1994). Each of these residues is conserved in the BiP sequences as shown in Fig. 2.

When compared with the other BiP sequences, rice, maize and tobacco BiP have a considerable amount of variation at the carboxy terminus, especially in the last 100 amino acid residues (Fig. 2). It has been proposed that the carboxy-terminal domain is divided into two parts, a 16 kDa portion containing the peptide-binding site (corresponding approximately to residues 454 to 598 in Fig. 2) and a more variable 10 kDa segment comprised of the terminal part of the protein (Chappell et al. 1987, Hartl 1996). The plant sequences, however, do not show considerable differences even though the tobacco BiP sequence shown in Fig. 2 was isolated from style and stigma tissue, whereas rice and maize cDNAs were from endosperm tissue.

Temporal expression of BiP in developing rice endosperm tissue—As rice is the only species where BiP is shown to have a direct role in the deposition of storage proteins on to the PB (Li et al. 1993b), we were interested in determining its expression pattern in developing rice endosperm. The relative abundance of rice BiP mRNA and protein was determined at 5 day intervals throughout seed development. BiP mRNA was visible as a single band of approximately 2.4 kb on RNA gel blots when equal amounts of total mRNA were loaded and probed with the rice BiP cDNA (Fig. 3). BiP mRNA accumulated maximally at 10 days after flowering, and declined steadily until it was barely visible at 25 days after flowering. No hybridization was visible in root and leaf tissue, even upon longer exposures. As BiP is an essential protein in the folding and/or assembly of proteins localized within or transported through the endomembrane system of eukaryotic cells, the lack of observed hybridization signal suggests that it is expressed at a level below the detection limits of our analysis or that tissue-specific forms are expressed. Antisera raised to maize BiP and rice prolamine and glutelin were used to probe protein gel blots (Fig. 4). When equal amounts of total protein were loaded in each lane, BiP accumulated was parallel with prolamine throughout development, as it peaked at day 20 and dropped slightly at 25 days after flowering. No detectable band was visible in leaf or root tissue.

Immunolocalization of BiP in rice endosperm tissue—Previous studies of crude organelle fractions have shown that a majority of BiP in the rice endosperm cell is associated with the PB fraction (Li et al. 1993b). Subsequent experiments have shown that this crude PB fraction was signifi-

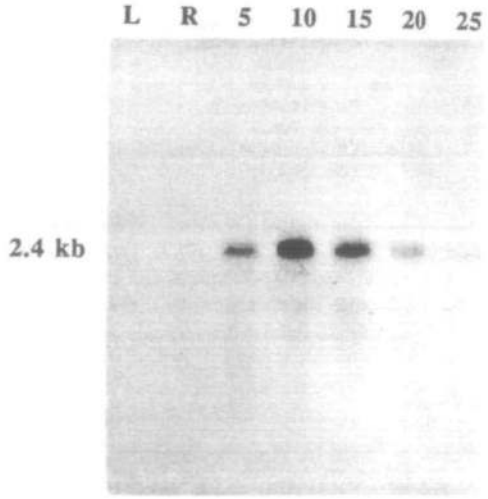

Fig. 3 Developmental analysis of BiP mRNA from rice endosperm. Total RNA was isolated from endosperm tissue at 5, 10, 15, 20 and 25 days after flowering, as well as from total root (R) and leaf (L). The RNA was fractionated by electrophoresis, blotted to nitrocellulose and probed with the rice BiP cDNA. The number in the margin refers to size in kilobases.

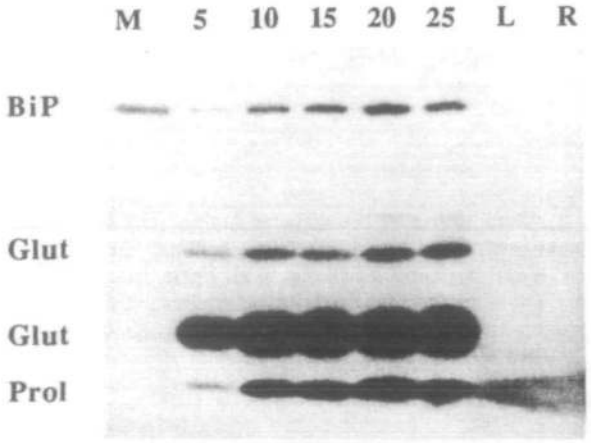

Fig. 4 Developmental accumulation of BiP polypeptide from rice endosperm. Fifty μg of each crude protein extract was fractionated by electrophoresis, blotted to nitrocellulose and probed with anti-BiP antisera from maize and anti-prolamine (Prol) and anti-glutelin (Glut) antisera from rice. The large (upper) and small (lower) glutelin subunits are labeled. Lane designations are the same as in Figure 3, except that M denotes 2 μg of maize BiP loaded as a control.

cantly contaminated with C-ER membranes (Y. Wu and T.W. Okita, unpubl.) and therefore the proportion of BiP associated with the PBs may differ from what was earlier reported. In order to more clearly define the location of BiP in the endosperm cell, ultrathin sections of mid-developing rice endosperm were probed with anti-BiP antiserum and visualized with gold particles (Fig. 5). BiP was primarily located at the periphery of the prolamine PBs and was excluded from the interior of the protein aggregate (Fig. 5a). A direct continuity of the C-ER membrane with the prolamine PB is seen (Fig. 5a), which is consistent with the C-ER origin of the prolamine PBs. However, very little labeling was evident on either the C-ER membranes or the glutelin protein bodies (Fig. 5a, b). BiP was observed at all

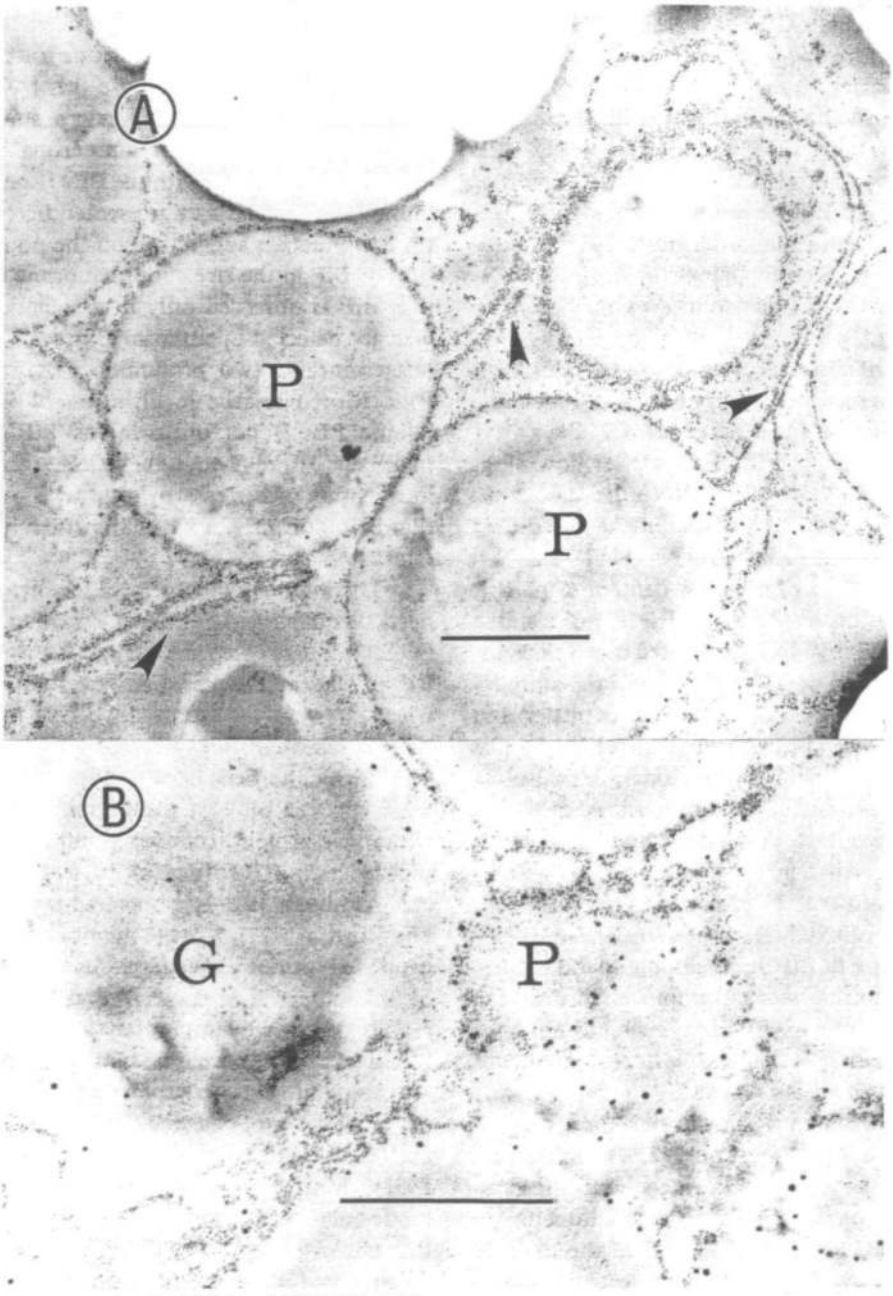

Fig. 5 Ultrathin sections of rice endosperm demonstrating the immunolocalization of BiP to prolamine protein bodies. A Section showing the specificity of the BiP antibody to the periphery of the prolamine protein body (P) but not to the connnecting rough C-ER (arrowheads). B Section showing the limited binding of label on the glutelin (G) protein body and significant binding to smaller, younger prolamine PBs. Bar equals 0.5 μm.

stages of PB development, although more intense labeling was associated with smaller PBs (Fig. 5a, b) that would be more actively synthesizing prolamine than the larger prolamine PBs.

Discussion

We have isolated a rice seed cDNA containing the entire coding region of BiP by screening an lambda cDNA library and by PCR. This cDNA is 2,417 nucleotides in length and codes for a deduced protein composed of 664 amino acids. The primary sequence of the rice BiP is very similar to the maize BiP with the bulk of the differences between the two sequences at the C-terminal end. Rice BiP also displays substantial amino acid conservation to other plant, fungal and animal BiP homologs. Very strong conservation exists within the amino-terminal ATPase domain, which includes residues important for ATPase activity within five functional peptide motifs as proposed by Bork et al. (1993; Fig. 2).

Protein gel blots of equal loadings of crude endosperm protein show that rice seed BiP accumulation parallels the accumulation of prolamine and is maximal at 20 days after flowering and decreases slightly at 25 days after flowering. RNA gel blot analysis shows that BiP mRNA decreases substantially in the latter half of seed development and differs from the continual increase in BiP polypeptide levels. The difference in BiP mRNA accumulation compared to BiP polypeptide accumulation suggests that BiP is quite stable in vivo, and/or that BiP mRNA is translated more efficiently in the latter half of seed development. Since the stability of BiP has been shown in other organisms (Knittler and Haas 1992, Pedrazzini et al. 1994), it is also likely to be quite stable in rice seed. The proposed function of BiP as having an essential role in the folding and assembly of prolamine would necessitate that its relative abundance parallel prolamine levels throughout development. This correlation appears to hold true as prolamine and BiP levels appear to increase coordinately during endosperm development. The concentration of BiP in developing soybean cotyledons also increases during seed development (Kalinski et al. 1995). Although the role of BiP in soybean has not been established, it presumably facilitates the translocation of the nascent storage protein across the ER membrane and folding of the polypeptide as shown in other systems (Nguyen et al. 1991, Sanders et al. 1992, Nicchitta and Blobel 1993). Unlike the situation in rice where BiP is involved in the assembly of the prolamines into a lumenal inclusion granule, BiP likely mediates folding of the soybean storage proteins into a transport competent state where it is sorted through the endomembrane system into a storage vacuole compartment. In view of the initial steps that are common in protein body formation in soybean and rice, i.e. ER translocation and polypeptide folding, it is understandable why the accumulation patterns of BiP do not differ dramatically between these plant species, although the concentration and spatial location of BiP within the ER membrane complex between these two species may differ (see below).

Using subcellular fractionation techniques, Li et al. (1993b) showed that most of the BiP was found in a crude PB fraction (5,000 × *g* pellet) with only about 10% of the total BiP associated with a microsomal membranes (30,000 × *g* pellet). It was unclear whether this enrichment of BiP in the PB fraction was due to their specific localization to PBs or simply because this fraction contains most of the ER membranes, as microscopic observations indicated the presence of numerous large cisternal ER complexes associated with the PBs (Li et al. 1993a). The immunocytochemical data presented here, however, substantiates our earlier suggestion on the non-stochastic localization of BiP in the rice endomembrane system. Here we see that BiP is observed only in association with the PBs. It was not detected in cisternal ER (even ER membranes that interconnected two prolamine PBs) or with the glutelin PBs. Moreover, the localization of BiP within the prolamine PBs is not uniform, as BiP is restricted to the periphery of the protein inclusion granule. This peripheral localization of BiP is consistent with our earlier hypothesis that BiP is directly involved in prolamine PB formation (Li et al. 1993b).

The immunocytochemical localization of BiP has also been demonstrated in wheat and maize. Levanony et al. (1992) and Rubin et al. (1992) have shown the presence of BiP in wheat PBs. The distribution and location of BiP in wheat, however, substantially differs from that observed in rice. In wheat, BiP is observed mainly within the PB itself and not at the periphery of the inclusion body. It is not clear why the bulk of the immunoreactive BiP is located within the protein inclusion granule itself, but may reflect differences in PB formation between wheat and rice.

Although BiP is associated with maize PBs (Zhang and Boston 1991), its role may not be identical to the one proposed for rice as suggested by the difference in the levels of BiP observed in these two plants. In rice, BiP can be readily detected by immunocytochemistry (Fig. 5) and easily observed by Coomassie blue staining of polyacrylamide gels containing crude PB extracts (Li et al. 1993b). In contrast, BiP was not detected by immunocytochemistry in normal developing maize endosperm (Zhang and Boston 1992). Moreover, when maize immunoblots containing crude endosperm extracts were probed with the same maize BiP antisera used here, very little signal was visible, even when a 10-fold greater amount of protein was loaded on the gel (Boston et al. 1991). In contrast to normal developing maize endosperm, BiP was readily detected in several maize mutants that have reduced zein concentration and elevated BiP expression (Boston et al. 1991, Zhang and

Boston 1992). The increase in BiP concentration in these mutants is likely a response to an increase in malfolded zein storage proteins as supported by the recent evidence of the *fluory-2* mutation (Coleman et al. 1995). This mutation results in an amino acid residue replacement within the signal peptide of a α-zein resulting its inability to be proteolytically processed. The unprocessed α-zein polypeptide was shown to anchor to microsomal membranes in in vitro translation experiments, suggesting that the signal peptide is capable of anchoring the *floury-2* α-zein protein to the ER membrane in maize endosperm (Gillikin et al. 1997). If this is the case, the abnormal folding and packaging of the membrane-bound α-zein likely accounts for the dramatic elevation of BiP levels and its observed localization to the periphery of the abnormal appearing PBs.

The apparently higher BiP levels in rice PBs compared to wild-type maize PBs may be a reflection of the greater role that BiP may have in rice prolamine PB formation. Prolamine PB formation in maize may be more complex than in rice, involving temporal and spatial differences in deposition of the α, β and γ zeins (Lending and Larkins 1989). The differences in the biogenesis of rice and maize prolamine PBs may result in a differential demand for BiP during their formation. The immunocytochemical data of wheat, maize and rice, as discussed above, demonstrates that rice is the only species in which BiP is shown to be conclusively associated with the periphery of the PB.

The preceding data, i.e., temporal expression at the protein level and localization to the periphery of the PB, are consistent with a model for the role of BiP in PB formation in rice endosperm as proposed by Li et al. (1993b). In this model BiP, in addition to functioning in nascent polypeptide translocation across the ER membrane, functions in folding and assembly of prolamine into protein bodies. Upon deposition of prolamine, BiP is released at the expense of ATP and is then recycled. The coordinated expression of BiP with prolamine throughout development would allow for constant deposition of prolamine to the protein aggregate. In addition, the peripheral location of BiP in the protein aggregate is consistent with the recycling of BiP after deposition, as opposed to BiP being deposited in the interior of the protein aggregate and being sequestered there. This relatively long-lived interaction between BiP and prolamine may have an important role in the enrichment of prolamine polysomes on the PB-ER, although other possible mechanisms may also contribute to localization of the storage protein mRNAs on the rice ER membranes.

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