Planta (2012) 236:1519–1527
DOI 10.1007/s00425-012-1714-y

ORIGINAL ARTICLE

Expression of OsBiP4 and OsBiP5 is highly correlated with the endoplasmic reticulum stress response in rice

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Received: 30 May 2012 / Accepted: 10 July 2012 / Published online: 24 July 2012

Abstract Binding protein (BiP) is a chaperone protein involved in the folding of secretory proteins in the ER lumen. OsBiP1 is constitutively expressed in various tissues, whereas the expression of OsBiP4 and OsBiP5 (OsBiP4&5) is not detected in any tissue under normal conditions. However, expression of OsBiP4&5 was highly and specifically activated under ER stress conditions induced by DTT treatment, *OsBiP1* knockdown, *OsBiP1* overexpression, *OsIRE1* overexpression, or various exogenous recombinant proteins in transgenic rice. In contrast, OsBiP4&5 did not accumulate in *OsIRE1* knockdown transgenic rice even after DTT treatment. When the subcellular localization of OsBiP4&5 was investigated in seed endosperm cells under the ER stress condition, OsBiP4&5 were localized to the ER, but did not participate in ER-derived protein body (PB-I) formation in a different manner to OsBiP1. These results indicate that OsBiP4&5 levels were positively correlated with stress levels in the ER. Taken together, these results suggest that OsBiP4&5 are ER stress-related BiP proteins that are regulated by OsIRE1/OsbZIP50 pathway and that they may have a distinct function from that of OsBiP1 in rice.

Keywords BiP · bZIP50 · Endoplasmic reticulum (ER) · ER stress · IRE1 · *Oryza* · Transgenic rice

Electronic supplementary material The online version of this article (doi:10.1007/s00425-012-1714-y) contains supplementary material, which is available to authorized users.

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Abbreviations

ER	Endoplasmic reticulum
ERAD	ER associated degradation
HSP	Heat shock protein
KD	Knockdown
OE	Overexpression
PB	Protein body
UPR	Unfolded protein response

Introduction

Our previous work generated transgenic rice plants that accumulate health-promoting proteins or peptides in their seeds, which are beneficial for human diets (Takagi et al. 2005; Yasuda et al. 2006a; Wakasa et al. 2011a, b). To create these transgenic plants, it was important to maximize the accumulation of desired recombinant proteins in ER-derived protein bodies through the secretory pathway. However, when certain recombinant proteins or peptides accumulate to high levels in rice endosperm tissue, an aberrant grain phenotype with floury and shrunken features has sometimes been observed (Oono et al. 2010). We showed that this decrease in grain quality correlated with an ER stress response (Oono et al. 2010). The ER stress response is a mechanism to maintain ER homeostasis by balancing the folding capacity and the folding demand imposed on the ER. In yeast and mammal cells, the ER stress response is activated to relieve the load of unfolded protein in the ER lumen and to recover homeostasis, thus the ER stress response is also referred to as the "unfolded protein response" (UPR). At least three distinct intracellular signal transduction pathways are activated by the ER stress response (Chakrabarti et al. 2011). It is thought that

the ER stress response also regulates the folding and removal of misfolded or unfolded proteins by the ER-associated degradation (ERAD) pathway, the inhibition of translation, and apoptosis (Harding et al. 1999; Bertolotti et al. 2000; Okamura et al. 2000; Oyadomari and Mori 2004; Yamaguchi et al. 2008; Chakrabarti et al. 2011).

Avoidance or alleviation of ER stress in transgenic rice grains is expected to lead to higher accumulation of recombinant proteins without a decrease in seed quality. Therefore, understanding the molecular mechanisms underlying the ER stress response will enable optimum production of beneficial recombinant proteins in rice. However, most studies on ER stress or protein quality control mechanisms have been done in yeast and mammals. To control ER stress in rice, a system to monitor the ER stress level with easily identifiable markers in various tissues (especially seed tissue) is required.

BiP is one of the main ER chaperones and belongs to the heat shock protein 70 (HSP70) family. BiP has a signal peptide at the N terminus and an ER retention signal (i.e., His/Lys-Asp-Glu-Leu) at the C terminus. BiP interacts with nascent immature proteins, which are synthesised on polysomes attached to the ER, and is involved in folding and assembling the newly synthesised polypeptides within the ER lumen. Human and rodents BiP (GRP78) or yeast BiP (KAR2) is encoded by a single gene (Fu et al. 2008; Kimata and Kohno 2010, Rose et al. 1989; Ting and Lee 1988; Wooden et al. 1988). In contrast, at least five *OsBiP* genes [*Os02g0115900* (*OsBiP1*), *Os03g0710500* (*OsBiP2*), *Os05g0367800* (*OsBiP3*), *Os05g0428600* (*OsBiP4*) and *Os08g0197700* (*OsBiP5*)] have been identified in rice (Hayashi et al. 2012). Their amino acid sequences share 66–94 % identity to each other (Fig. S1). OsBiP1, one of the major rice BiP proteins, is constitutively expressed in plant tissues and is up-regulated in response to ER stress (Oono et al. 2010; Wakasa et al. 2011c; Hayashi et al. 2012). Overexpression and knock down of OsBiP1 in rice endosperm induces a severe ER stress response and leads to a deterioration of grain properties (Yasuda et al. 2009; Wakasa et al. 2011c). Reduction of *OsBiP1* also resulted in the induction of *OsBiP2*, *OsBiP3*, *OsBiP4*, and *OsBiP5*, which were characterised as novel BiP proteins associated with the ER stress response. Furthermore, expression of these novel BiPs was regulated by OsIRE1-mediated mRNA splicing of the OsbZIP50 transcriptional factor (OsIRE1/OsbZIP50) in a manner similar to the signalling pathways IRE1/HAC1 in yeast and IRE1/XBP1 in mammals (Hayashi et al. 2012).

OsbZIP50 is activated by OsIRE1-mediated cytoplasmic splicing of its mRNA. Protein translated from unspliced *OsbZIP50* mRNA is not translocated into the nucleus. Under ER stress conditions, unconventional splicing of *OsbZIP50* mRNA by OsIRE1 causes a frameshift in the C-terminal region of OsbZIP50, which results in the appearance of a nuclear localization signal in the newly translated OsbZIP50 (Hayashi et al. 2012). The *Arabidopsis* AtbZIP60 is also activated by AtIRE1-mediated splicing, similar to the activation of rice OsbZIP50 (Deng et al. 2011; Nagashima et al. 2011).

In this study, in order to address the relationship between the OsIRE1/OsbZIP50 signalling and BiP proteins induced under ER stress conditions, we prepared anti-OsBiP4&5 polyclonal antibody, and demonstrated the potential utility of anti-OsBiP4&5 antibody as a marker to evaluate ER stress levels in several rice tissues. Furthermore, based on expression analysis of OsBiP4 and OsBiP5 in some rice tissues under various ER stress situations using the anti-OsBiP4&5 antibody, the functions of ER stress specific-OsBiPs in the ER stress response are discussed.

Materials and methods

Plant materials

Callus, root, and seed tissues from rice (*Oryza sativa* L. cv. Kitaake or cv. Koshihikari) were used as samples. Seeds were obtained from National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan. Transgenic rice lines included the following: constitutive *OsIRE1*-knock down (KD), constitutive *OsIRE1*-overexpression (OE), endosperm-specific *OsIRE1*-OE, endosperm-specific *OsBiP1*-KD, endosperm-specific *OsBiP1*-OE, and various recombinant proteins, including lactostatin peptide derived from β-lactoglobulin (Nagaoka et al. 2001; Wakasa et al. 2011a), novokinin peptide derived from ovalubumin (Onishi et al. 2004; Wakasa et al. 2011b), APL4 peptide derived from human collagen type II (Wakamatsu et al. 2009), modified GLP-1 (mGLP-1) derived from human glucagon-like protein 1 (Yasuda et al. 2006a, b), human β-amyloid (Oono et al. 2010), and human cathelicidin (Sang and Blecha 2008). These proteins were expressed under the control of rice endosperm-specific promoters and were produced by *Agrobacterium*-mediated transformation. Expression gene cassette constructs are shown in Fig. S2. These transgenic and non-transgenic rice were grown in a closed greenhouse.

RT-PCR analyses of *OsBiP1*, *OsBiP2*, *OsBiP3*, *OsBiP4*, *OsBiP5*, *OsbZIP50*, and *17S ribosomal RNA* were performed as described previously (Hayashi et al. 2012).

Antibody preparation

The MH_2-GGAPEDGNVDDED-OH peptide derived from OsBiP5 (Os08g0197700) was synthesised and used to raise anti-OsBiP5 polyclonal antibody in a rabbit (Scrum Inc.,

Tokyo, Japan). This antibody reacts with OsBiP5 and OsBiP4 (Os05g0428600). OsBiP4 is almost identical to OsBiP5 in amino acid sequence (94 % identity, Fig. S1), molecular weight, and expression pattern (Hayashi et al. 2012). Since the amino acid sequence (MH_2-GGAPED GNVDDED-OH) of the antigen peptide was identified in only OsBiP4 and OsBiP5, the antibody was expected to specifically recognize OsBiP4 and OsBiP5. We confirmed that anti-OsBiP4&5 antibody did not react with OsBiP1, OsBiP2, or OsBiP3 proteins (Fig. S3).

Recombinant proteins of full-length OsBiP1 and the upstream region of the putative transmembrane domain of OsbZIP50 were expressed in *E. coli*, and the purified recombinant proteins were used to raise anti-BiP1 and anti-OsbZIP50 polyclonal antibodies in a rabbit (Scrum Inc.). For the production of rabbit polyclonal antibodies against calnexin (Os04g0402100), lactostatin, novokinin, APL4, mGLP-1, and β-amyloid, specific peptides for these proteins were used to raise polyclonal rabbit antibodies (Scrum Inc.). Anti-cathelicidin polyclonal rabbit antibody was purchased from Abcam plc. (Cambridge, UK).

DTT treatment of rice tissues

Calli were produced from mature rice seeds on N6D medium [4 g/L CHU salt mixture (Wako, Osaka, Japan), 30 g/L sucrose, 2.78 g/L proline, 100 mg/L myo-inositol, 300 mg/L casamino acid, 1 mL/L 1,000× N6-vitamin, 2 mg/L 2,4-D, 0.4 % gelrite, pH 5.8] for 5 weeks at 30 °C (16 h light/8 h dark). DTT was used to induce the ER stress response in calli and in roots of seedlings. Calli were placed on N6D medium containing 2 mM DTT for 0, 0.5, 1, 2, 4, 8, and 24 h at 30 °C (16 h light/8 h dark). Roots of seedlings (1 week after germination) were treated with MS liquid medium [4 g/L MS salt mixture (Wako), 30 g/L sucrose, 1 mL/L 1,000× B5-vitamin, pH 5.8] containing 2 mM DTT under the same time-course as used for calli. After DTT treatment, roots and calli were immediately frozen in liquid N_2 before the extraction of total proteins.

Protein extraction, SDS-PAGE, and immunoblotting

The total proteins were prepared using extraction buffer (50 mM Tris–HCl pH 6.8, 8 M urea, 4 % SDS, 20 % glycerol, 5 % 2-mercaptoethanol, 0.01 % bromophenol blue). Extraction buffer was added to the roots, calli, and seed powder and then vortexed for more than 1 h at room temperature. The mixture was centrifuged at 12,000*g* for 20 min at room temperature, and the crude soluble protein sample was decanted into a new tube. Total proteins were subjected to immunoblot analysis using polyclonal rabbit antibody after electrophoresis on 12 % SDS-PAGE.

Confocal immunohistochemical analysis

Immature wild-type and transgenic rice seeds were collected at 15 days after flowering (DAF) and used for immunocytochemical analysis using the anti-OsBiP1, anti-OsBiP4&5 and anti-calnexin rabbit polyclonal antibody as described by Yasuda et al. (2006b). Briefly, 200 µm of section sample was treated with 3.7 % formaldehyde in phosphate buffer saline (PBS) for 1 h. Then, the sample was incubated with cell wall digestion solution (1 % cellulose and 0.1 % pectolyase in PBS) at 30 °C for 10 min. Primary antibodies (anti-OsBiP1, anti-OsBiP4&5 and anti-calnexin antibodies) were used at a 1:300 dilution. Alexa488-conjugated goat anti-rabbit IgG (Invitrogen) was used at a 1:500 dilution as the secondary antibody. Rhodamine B was used to stain ER-derived protein bodies (PB-I). The samples were observed through a confocal laser scanning microscope (FLUOVIEW; Olympus).

Results

Expression of *OsBiP2*, *OsBiP3*, *OsBiP4*, and *OsBiP5* is regulated by the ER stress signalling pathway through OsIRE1-mediated splicing of *OsbZIP50* mRNA (Hayashi et al. 2012). As shown in Fig. 1, expression of these *OsBiP*s was exclusively detected in ER-stressed cells, such as roots treated with DTT (Fig. 1a) and *OsBiP1*-KD seeds (Fig. 1b). Furthermore, the spliced form of *OsbZIP50* was detected only in ER-stressed cells (Fig. 1). On the other hand, expression and splicing of *OsbZIP50* mRNA were strongly suppressed in OsIRE1-KD rice, even during stress induction with DTT treatment (Fig. 1a).

We prepared antibodies against these proteins, and an antibody that specifically recognized OsBiP4 and OsBiP5 was successfully produced (anti-OsBiP4&5 antibody). We used this antibody as a marker to investigate if the accumulation levels of OsBiP4 and OsBiP5 were correlated with stress levels in the ER lumen of various rice tissues.

Calli and roots were treated with 2 mM DTT as an ER stress-inducing agent, and proteins were extracted and then subjected to immunoblot analysis using the anti-OsBiP4&5 antibody (Fig. 2). OsBiP4&5 proteins were clearly detected at 4 h after DTT treatment for both tissues. OsbZIP50 protein was detected at 1 h after DTT treatment and approximately 3 h before the appearance of OsBiP4&5 (Fig. 2), suggesting that the induced *OsbZIP50* mRNA was smoothly spliced by OsIRE1 in these experiments. Furthermore, it is notable that the production of OsBiP4&5 is dependent on ER stress, and levels increased markedly in response to longer treatments with DTT. In contrast, OsBiP1 showed constitutive expression in both roots and calli, and levels increased in response to DTT (Fig. 2),

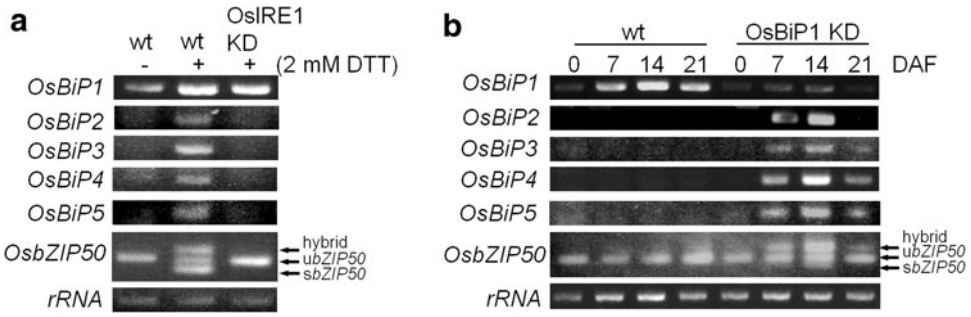

Fig. 1 Expression of *OsBiP1*, *OsBiP2*, *OsBiP3*, *OsBiP4*, *OsBiP5*, and *OsbZIP50* in ER-stressed cells. **a** RT-PCR analysis of DTT-treated root mRNA. Wt, wild type; *OsIRE1*-KD, *OsIRE1* knock down transgenic plant; −, no-DTT treatment; +, DTT treatment; ubZIP50, unspliced *OsbZIP50*; sbZIP50, spliced *OsbZIP50*; hybrid, annealing products of sbZIP50 and ubZIP50. **b** RT-PCR analysis of *OsBiP1*-KD seed mRNA. *DAF* days after flowering, *OsBiP1*-KD seed-specific *OsBiP1* knock down transgenic plant

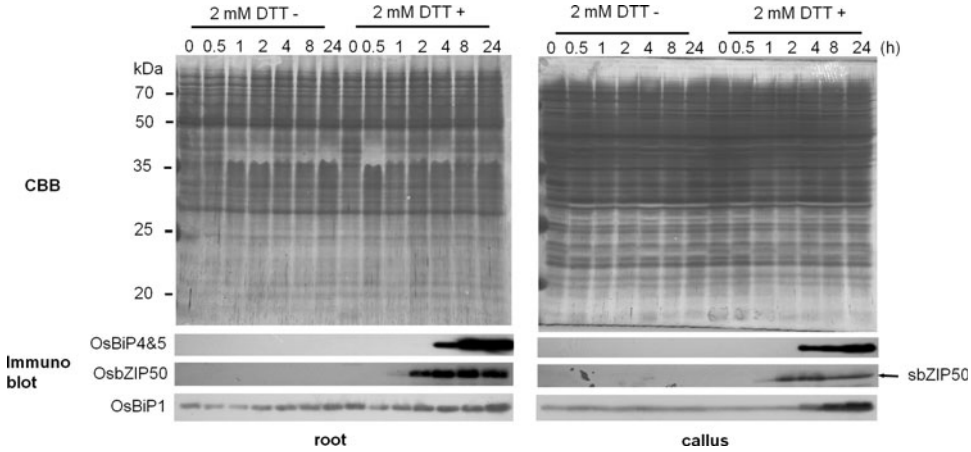

Fig. 2 Induction of ER stress by DTT treatment in callus and root. Calli (*left panels*) and roots (*right panels*) of seedlings were treated with 2 mM DTT for 0, 0.5, 1, 2, 4, 8, and 24 h. SDS-PAGE and immunoblot analyses using anti-OsBiP4&5, anti-OsbZIP50, and anti-OsBiP1 antibodies are shown. −, no-DTT treatment; +, 2 mM DTT treatment; sbZIP50, spliced OsbZIP50

however, changes in the OsBiP4&5 levels in callus and root were more pronounced than that of OsBiP1 after DTT treatment.

Next, we examined the accumulation of OsBiP4&5 in the roots of constitutive OsIRE1-KD transgenic rice subjected to DTT treatment and in the roots of *OsIRE1*-OE transgenic rice without DTT treatment (Fig. 3). Overexpression of IRE1 induces an ER stress response in animal and plant tissues (Hayashi et al. 2012; Yoshida et al. 2001), therefore, we examined whether OsbZIP50 was translocated to the nuclei of *OsIRE1*-OE transgenic rice seed cells (Fig. S4). In roots of the *OsIRE1*-KD line, OsBiP4&5 and OsbZIP50 were not detected even under stress-induction treatments with DTT. In contrast, roots of the *OsIRE1*-OE line accumulated OsBiP4&5 constitutively, even without DTT treatment. OsbZIP50 proteins in the *OsIRE1*-OE line were detected as two bands, representing inactive (unspliced) and active (spliced) forms of OsbZIP50, respectively. In non-stressed cells, the results suggested that OsBiP4&5 is primarily regulated by the OsIRE1/OsbZIP50 signalling pathway because OsBiP4&5 accumulation depended on the accumulation of the active (spliced) form of OsbZIP50. However, the amount of active OsbZIP50 that accumulated in *OsIRE1*-OE roots was much lower than that in roots treated with DTT, whereas BiP4&5 levels were much higher in *OsIRE1*-OE roots. This lack of correlation between OsBiP4&5 levels and the active form of OsbZIP50 may be explained by the fact that DTT treatment induces only a short period of ER stress; this is in contrast to the constitutive activation of the ER stress signalling pathway caused by overexpression of OsIRE1, which results in greater accumulation of OsBiP4&5.

Next, we investigated the relationship between OsBiP4&5 levels and the ER stress response in seed tissue. As DTT treatments cannot be applied to seed tissue, rice seeds harvested from transgenic plants in which ER stress was induced by the overexpression or suppression of *OsBiP1* or high accumulation of exogenous recombinant proteins were used for these experiments. OsBiP1, the major BiP protein in rice, is constitutively expressed in various rice tissues. Transgenic rice seeds in which *OsBiP1* is suppressed (*OsBiP1*-KD) exhibited severe ER stress (Wakasa et al. 2011c). On the other hand, very high expression of OsBiP1 (*OsBiP1*-OE) also induces the ER stress response in transgenic rice seeds (Yasuda et al. 2009; Wakasa et al. 2011c). *OsBiP1*-KD and *OsBiP1*-OE transgenic rice seeds

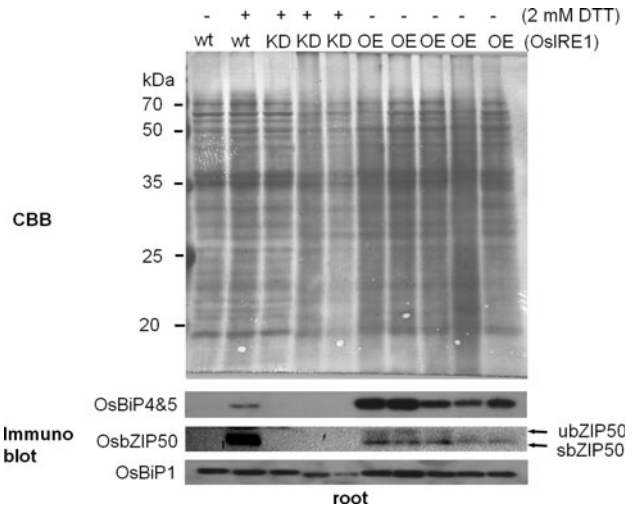

Fig. 3 The relationship between OsBiP4&5 accumulation and OsbZIP50 in *OsIRE1*-KD and *OsIRE1*-OE lines. Root tissues of wild-type and independent transgenic rice lines with or without DTT treatment are used as samples. SDS-PAGE and immunoblot analyses using anti-OsBiP4&5, anti-OsbZIP50, and anti-OsBiP1 antibodies are shown. Wt, wild type; KD, *OsIRE1*-KD; OE, *OsIRE1*-OE; −, no-DTT treatment; +, DTT treatment for 8 h; ubZIP50, unspliced *OsbZIP50*; sbZIP50, spliced *OsbZIP50*

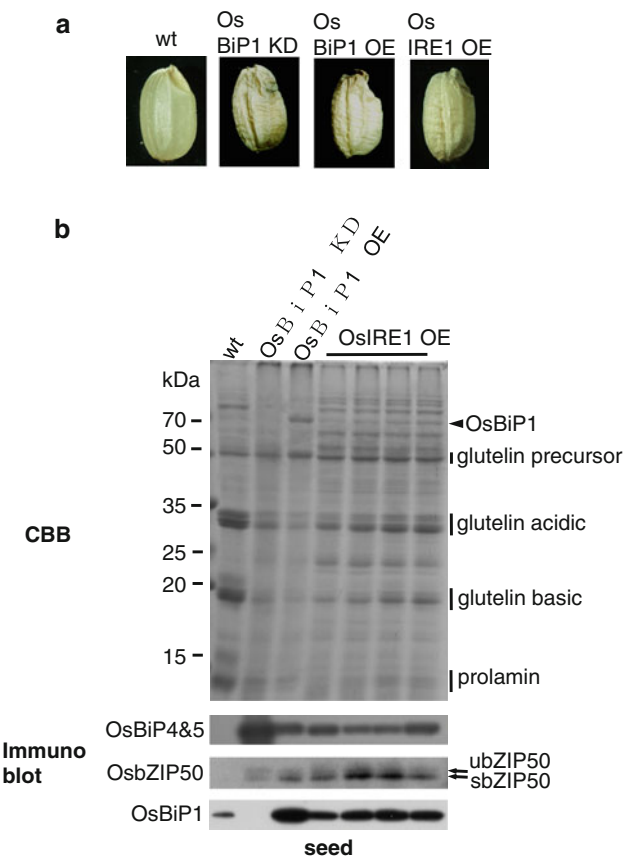

Fig. 4 OsBiP4&5 accumulation in seed tissues under ER stress conditions. Rice seeds from wild-type, *OsBiP1*-KD, *OsBiP1*-OE, and *OsIRE1*-OE transgenic lines are used as samples. SDS-PAGE and immunoblot analyses using anti-OsBiP4&5, anti-OsbZIP50, and anti-OsBiP1 antibodies are shown. The positions of OsBiP1, glutelin precursor, glutelin acidic or basic, globulin, and prolamin on the SDS-PAGE gel are indicated on the *right side* of the panel. ubZIP50, unspliced *OsbZIP50*; sbZIP50, spliced *OsbZIP50*

display a severely abnormal phenotype with floury and shrunken features (Yasuda et al. 2009; Wakasa et al. 2011c). Furthermore, both transgenic lines suppressed the accumulation of seed storage proteins compared with non-transgenic rice seed. Thus, these abnormal grain phenotypes may be useful as an indicator of ER stress. We used *OsBiP1*-KD, *OsBiP1*-OE, and *OsIRE1*-OE transgenic seeds for the evaluation of the OsBiP4&5 antibody in seed tissues. As shown in Fig. 4a, wild-type seed displayed a normal phenotype, whereas aberrant phenotypes such as floury and shrunken features were observed in the transgenic seeds. Immuno-blot analysis shows that inactive OsbZIP50, active OsbZIP50, and OsBiP4&5 proteins were detected in the transgenic rice seeds, but these proteins were not detected in wild-type seed (Fig. 4b).

The accumulation of OsBiP4&5 was investigated in transgenic rice seeds expressing various recombinant proteins. We generated a number of transgenic rice lines expressing different recombinant proteins or bioactive peptides to provide greater nutritional qualities and human health-promoting functions to wild-type rice. We investigated the relationship between the seed phenotype and OsBiP4&5 levels in transgenic rice expressing lactostatin (Wakasa et al. 2011a), novokinin (Onishi et al. 2004; Wakasa et al. 2011b), APL4 (one of the analogue peptides of the type II collagen-reactive T cell epitope) (Wakamatsu et al. 2009), mGLP-1 (Yasuda et al. 2006a, b), β-amyloid (Oono et al. 2010), and cathelicidin (Sang and Blecha 2008). Lactostatin, novokinin, and APL4 were expressed as fusion proteins with the seed storage protein glutelin, whereas mGLP-1, β-amyloid, and cathelicidin were linked to the ER retention signal peptide (Lys-Asp-Glu-Leu) at their C termini and were directly expressed as secretory proteins. When lactostatin and APL4 were produced as fusion proteins in transgenic rice seeds, the resulting phenotypes were not significantly different to those of the wild-type seeds. Transgenic seeds accumulating novokinin displayed a slightly chalky phenotype. However, rice seeds accumulating mGLP-1, β-amyloid, and cathelicidin resulted in severely abnormal phenotypes (Fig. 5a), suggesting that they were under strong ER stress conditions compared with the wild-type and other transgenic rice seeds. Importantly, OsBiP4&5 accumulation levels were closely correlated with the observed deterioration of seed phenotypes. A very faint level of OsBiP4&5 was detected in transgenic rice seeds expressing lactostatin or APL4 (Fig. 5b), which exhibited normal phenotypes. A weak

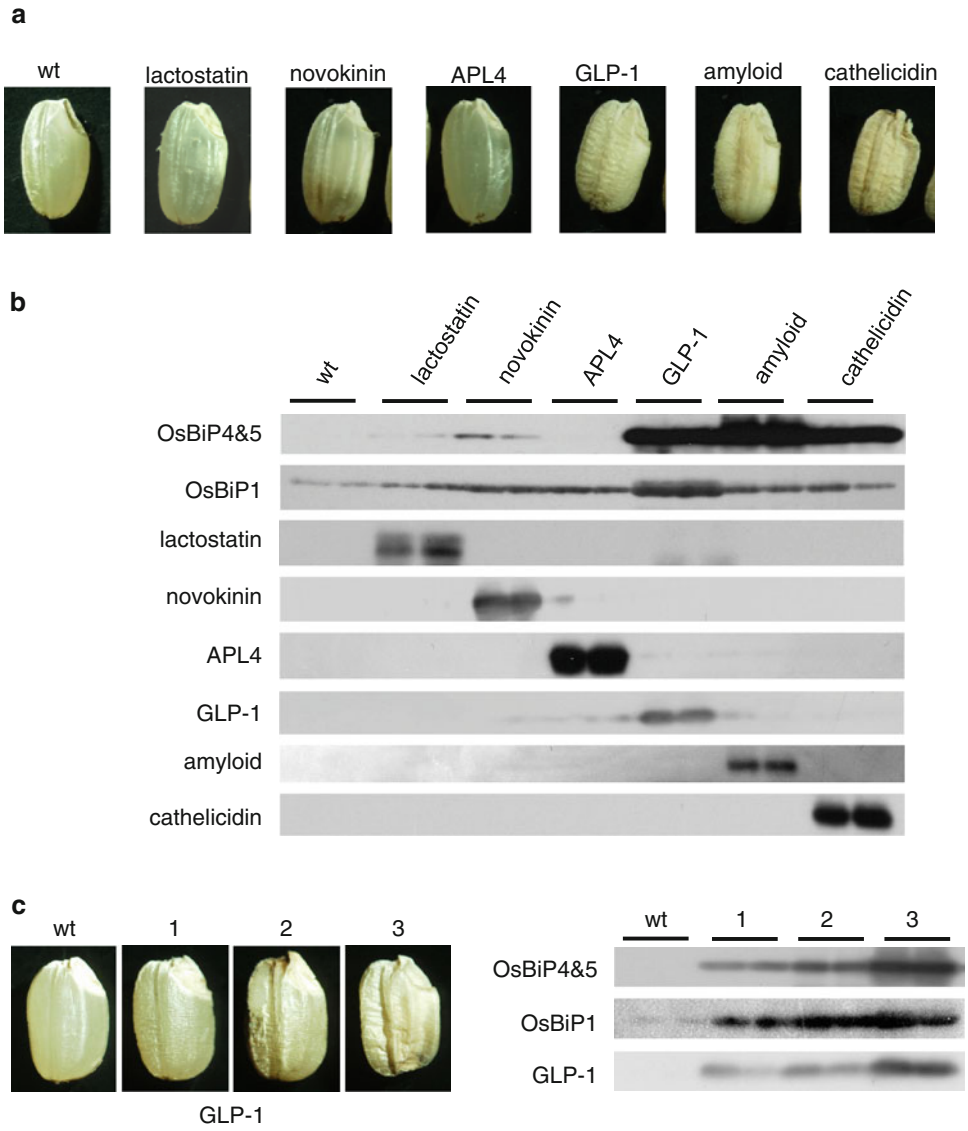

Fig. 5 OsBiP4&5 accumulation in transgenic rice seeds expressing various recombinant proteins. **a** Phenotype of wild-type and transgenic seeds accumulating various recombinant proteins. **b** Immunoblot analysis of seeds in **a**. *Wt* wild type, *lactostatin* pentapeptide derived from bovine milk β-lactoglobulin with hypocholesterolemic activity, *novokinin* a new potent anti-hypertensive peptide based on the sequence of novokinin (2–7) derived from ovalbumin, *APL4* one of the altered peptide ligands that controls type II collagen-reactive T cells, *GLP-1* a 30-amino acid peptide hormone involved in insulin stimulation, *β-amyloid* dimers of mature 42-amino acid β-amyloid peptide ($A\beta_{1-42}$), *cathelicidin* antimicrobial peptide derived from human. **c** Phenotypes of transgenic rice seeds accumulating mGLP-1 at various levels, and immunoblot analysis of these seeds

level of BiP4&5 was detected in transgenic rice seed expressing novokinin. However, remarkably high levels of OsBiP4&5 were detected in transgenic seeds expressing mGLP-1, β-amyloid, or cathelicidin, which exhibited severely abnormal phenotypes (Fig. 5a, b). These results indicate that ER stress and severely aberrant seed phenotypes are strongly correlated with OsBiP4&5 accumulation levels induced by ER stress in seeds. Notably, the highest level of OsBiP1 was observed in transgenic rice expressing mGLP-1; relatively higher levels of OsBiP1 accumulation compared to wild-type rice seed were observed in these transgenic rice seeds. However, the severe seed phenotype observed in transgenic lines expressing mGLP-1, β-amyloid, or cathelicidin could not be caused by the accumulation of OsBiP1, because OsBiP1 levels were not significantly different among the lines expressing lactostatin, novokinin, APL4, β-amyloid, and cathelicidin, but the grain phenotypes were quite different. The relationship between seed quality and concentration of OsBiP4&5 was further examined for transgenic rice accumulating mGLP-1 (Fig. 5c). The results indicate that increases in OsBiP4&5 levels were dependent on the level of mGLP-1 accumulation, leading to the severe grain phenotypes with floury and shrunken features.

Finally, to examine the function of ER stress-associated BiP, such as OsBiP4 and OsBiP5, the subcellular localization of OsBiP4&5 in IRE1-OE endosperm cells was investigated by indirect histochemical analysis using confocal microscopy (Fig. 6). ER-derived protein body-I (PB-I) was stained by rhodamine, and OsBiP1 and OsBiP4&5 were detected using anti-BiP1 and anti-BiP4&5 primary antibodies and Alexa 488-conjugated anti-rabbit IgG secondary antibody. Furthermore, calnexin protein was used as a marker protein localized to the ER (Gupta and Tuteja 2012; Takahashi et al. 2012) (Fig. 6m–o).

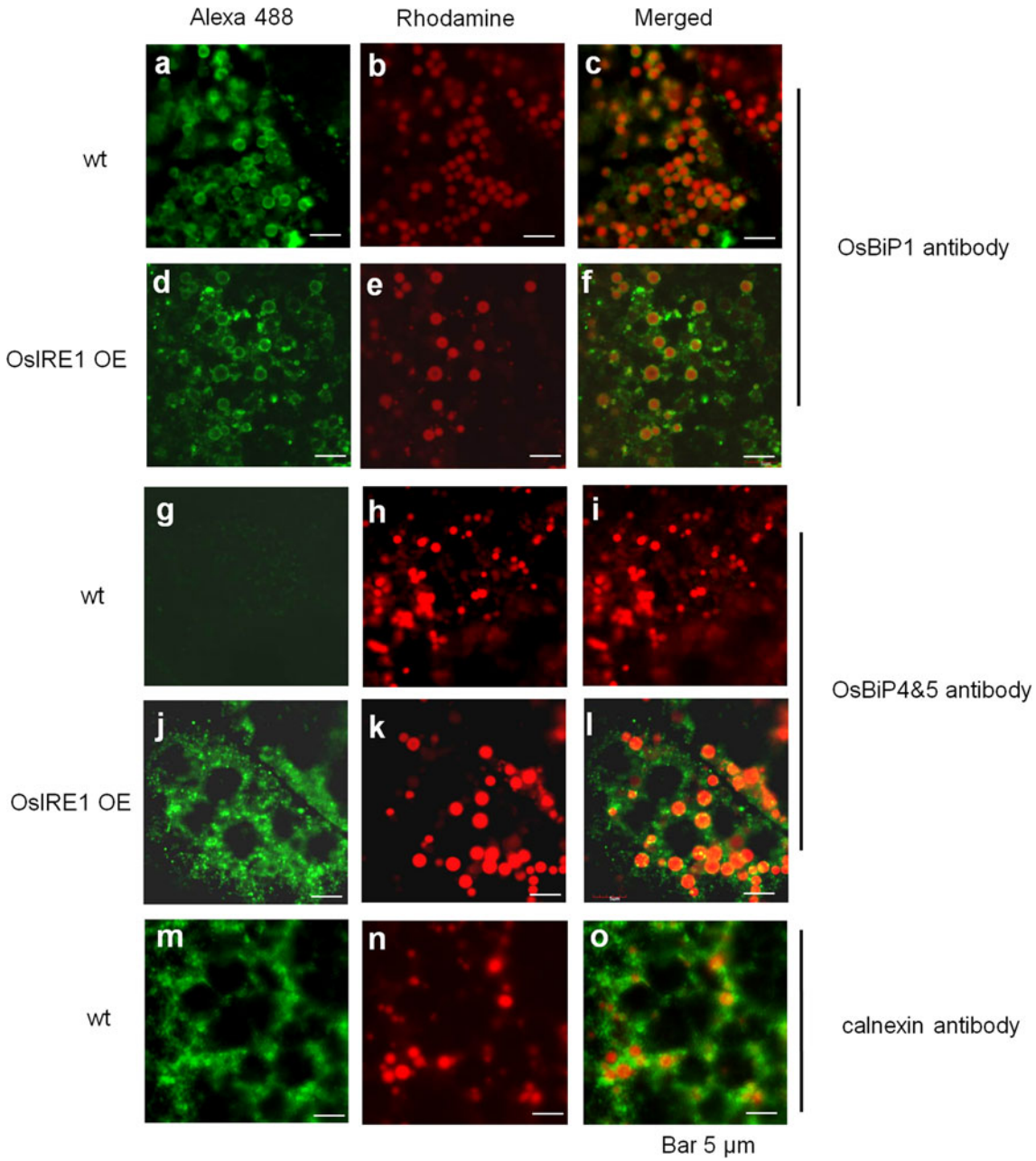

Fig. 6 Indirect immunohistochemical analysis of OsBiP1 and OsBiP4&5 in rice cells. *Left panels* show localization of OsBiPs (*green*), *middle panels* show localization of PB-I (*red*), and *right panels* show the merged images. **a–c** Localization of OsBiP1 in wild-type seeds. **d–f** Localization of OsBiP1 in *OsIRE1*-OE transgenic rice seeds. **g–i** Localization of OsBiP4&5 in wild-type seeds. **j–l** Localization of OsBiP4&5 in *OsIRE1*-OE transgenic rice seeds. **m–o** Localization of calnexin, an ER marker protein, in the wild type

OsBiP1 was localized predominantly to the ER and periphery of PB-I in wild-type seeds as described in a previous report (Yasuda et al. 2009), and similar localization patterns were observed in transgenic *OsIRE1*-OE seeds (Fig. 6a–f). Although OsBiP4&5 levels were very low in wild-type seeds (Fig. 6g), they were primarily detected in transgenic *OsIRE1*-OE seeds, namely ER-stressed seeds (Fig. 6j). As the localization of OsBiP4&5 displayed a very similar fluorescent pattern to that of calnexin (Fig. 6m), OsBiP4&5 was considered to be localized to the ER, with very little localization to the periphery of PB-I (Fig. 6j).

Discussion

We previously reported the expression of novel ER stress responsive *BiP* genes (*OsBiP2–OsBiP5*) in roots and cultured cells treated with the ER stress agents DTT and

tunicamycin (Hayashi et al. 2012). The present study identified a positive correlation between ER stress levels and the accumulation of two BiP proteins, OsBiP4 and OsBiP5, in callus, roots (Figs. 2, 3) and maturing rice seed (Figs. 4, 5) by immunoblot analysis with an anti-OsBiP4&5 antibody. Expression of the OsBiP4&5 genes was very sensitive to various ER stress conditions but was hard to detect under normal conditions. This result is in marked contrast with the finding that OsBiP1 was constitutively expressed in various tissues under normal conditions, although expression was upregulated by ER stress. ER stress-specific induction of OsBiP4&5 is under the control of the OsIRE1/OsbZIP50 signalling pathway; thus, *OsBiP4&5* expression is strongly suppressed not only in *OsIRE1*-KD (Fig. 3) but also in *OsbZIP50*-KD (Hayashi et al. 2012). By contrast, OsBiP1 is mainly regulated by the other signal transduction pathway via OsbZIP39 (Takahashi et al. 2012).

It is notable that the phenotypic deterioration in rice grains, caused by the production of foreign recombinant proteins, was more correlated with high levels of OsBiP4&5 accumulation than with OsBiP1 levels. However, the levels of OsBiP1, OsBiP4&5, OsbZIP50 and OsIRE1 were somewhat contradictory, although the OsBiP4&5 level was regulated by OsIRE1/OsbZIP50 signalling. As shown in Fig. 3, the level of OsBiP4&5 in *OsIRE1*-OE roots (constitutive ER stress) was higher than that in roots treated with DTT (transient induction of ER stress), whereas the amount of active OsbZIP50 in *OsIRE1*-OE roots was much lower than that in roots treated with DTT. Furthermore, the OsBiP4&5 level in *OsBiP1*-KD seeds was much higher than that in *OsIRE1*-OE seeds, although the level of active OsbZIP50 remained low (Fig. 4). In the case of ER stress induced by DTT treatment or in *OsIRE1*-OE, differences in ER stress levels, treatment times and ER stress signalling pathways may explain why there was only a weak relationship between OsBiP50 and OsBiP4&5 levels. The high abundance of OsBiP4&5 in *OsBiP1*-KD seeds might be because OsBiP4&5 must complement the low amount of OsBiP1. *OsBiP1*-KD probably triggers severe ER stress in the absence of the BiP chaperone (Wakasa et al. 2011c). However, the present data cannot address why OsbZIP50 is less abundant in cells lacking OsBiP1 in spite of its high abundance in OsBiP4&5. Another explanation might be that since OsbZIP50 is a transcriptional factor, it is not as stable as BiP proteins, considering that OsBiP1 stably accumulates even in mature rice seed. In the stable transgenic plants, such as *OsIRE1*-OE and *OsBiP1*-KD, OsBiP4&5 may accumulate in stressed tissues, whereas the OsbZIP50 level may be strictly regulated by the stress level and undergo prompt turnover. Thus, the difference in stability between OsbZIP50 and OsBiP4&5 might also explain the non-parallel relationship of these accumulation levels. Further work is required to understand the correlation between OsBiP1, OsBiP4&5 and OsbZIP50 in response to ER stress in rice.

We demonstrated that differences in the intracellular localization of OsBiP1 and OsBiP4&5 were observed in wild type and *OsIRE1*-OE endosperm cells (Fig. 5). OsBiP1 localised at the periphery of PB-Is and the ER, whereas OsBiP4&5 was predominantly distributed within the ER. These results indicate that *OsBiP* genes may show different functions under different types of ER or environmental stress, and during developmental processes. Indeed, differential expression of *BiP* genes has been reported in *Arabidopsis* (Iwata et al. 2008). *AtBiP3* shows a similar expression pattern to *OsBiP4&5* (Chen and Brandizzi 2012; Deng et al. 2011; Nagashima et al. 2011; Hayashi et al. 2012), suggesting that *OsBiP4&5* may be a counterpart of *AtBiP3*. Plant *BiP* is encoded by multiple genes (five in rice and three in *Arabidopsis*), thus, plant *BiP* genes are thought to have become functionally differentiated throughout evolution. By contrast, mammalian *BiP* (*GRP78*) and yeast *BiP* (*KAR2*) are encoded by a single gene (Ting and Lee 1988; Wooden et al. 1988; Rose et al. 1989; Fu et al. 2008; Kimata and Kohno 2010). GRP78 plays a critical role as a chaperone during protein quality control, irrespective of ER stress conditions. Knock out of *GRP78* in mice results in early embryonic lethality (Luo et al. 2006). Deletion of the *KAR2* gene in yeast also causes a recessive lethal mutation (Rose et al. 1989). Thus, difference in gene copy number between plants and animals may explain the difference in responses to various stresses. Since plants cannot move, they have developed/evolved multiple defence mechanisms and protein quality control systems to protect themselves against stresses, including ER stress.

The results of the present study indicate that the level of OsBiP4&5 is regulated by the OsbZIP50/OsIRE1 signalling pathway, which is responsible for the major ER stress response in various rice tissues. OsBiP4&5 accumulation is a valuable marker for evaluating ER stress levels, which can be easily measured by immunoblot analysis using the anti-OsBiP4&5 antibody. Monitoring ER stress levels using this identifiable marker will be useful for basic research investigating the molecular mechanisms underlying the ER stress response, and for the production of transgenic rice seeds that accumulate recombinant proteins.

Further studies on the function of OsBiP4 and OsBiP5 in rice cells under ER stress conditions are required. On-going work aims to produce constitutive or seed-specific *OsBiP4&5*-KD and *OsBiP4&5*-OE lines and to examine the function of these transgenic rice lines.

Acknowledgments The authors thank Ms. Y. Ikemoto, K. Miyashita, Y. Suzuki, M. Utsuno, and Y. Yajima for technical assistance. This research was supported by the research grant, "Genomics and Agricultural Innovation, GMC0003", from the Ministry of Agriculture, Forestry and Fisheries of Japan to F. Takaiwa.

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