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RESEARCH PAPER

A green fluorescent protein fused to rice prolamin forms protein body-like structures in transgenic rice

Yuhi Saito[1], Koichi Kishida[1], Kenji Takata[1], Hideyuki Takahashi[1], Takeaki Shimada[1], Kunisuke Tanaka[1], Shigeto Morita[1,2], Shigeru Satoh[1,2] and Takehiro Masumura[1,2,*]

[1] Laboratory of Genetic Engineering, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Shimogamo, Kyoto 606-8522, Japan
[2] Kyoto Prefectural Institute of Agricultural Biotechnology, Kitainayazuma, Seika-cho, Soraku-gun, Kyoto 619-0244, Japan

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Abstract

Prolamins, a group of rice (*Oryza sativa*) seed storage proteins, are synthesized on the rough endoplasmic reticulum (ER) and deposited in ER-derived type I protein bodies (PB-Is) in rice endosperm cells. The accumulation mechanism of prolamins, which do not possess the well-known ER retention signal, remains unclear. In order to elucidate whether the accumulation of prolamin in the ER requires seed-specific factors, the subcellular localization of the constitutively expressed green fluorescent protein fused to prolamin (prolamin–GFP) was examined in seeds, leaves, and roots of transgenic rice plants. The prolamin–GFP fusion proteins accumulated not only in the seeds but also in the leaves and roots. Microscopic observation of GFP fluorescence and immunocytochemical analysis revealed that prolamin–GFP fusion proteins specifically accumulated in PB-Is in the endosperm, whereas they were deposited in the electron-dense structures in the leaves and roots. The ER chaperone BiP was detected in the structures in the leaves and roots. The results show that the aggregation of prolamin–GFP fusion proteins does not depend on the tissues, suggesting that the prolamin–GFP fusion proteins accumulate in the ER by forming into aggregates. The findings bear out the importance of the assembly of prolamin molecules and the interaction of prolamin with BiP in the formation of ER-derived PBs.

Key words: Endoplasmic reticulum, *Oryza sativa*, prolamin, protein body, storage protein, transgenic rice.

Introduction

Plants generate a specialized compartment derived from the endoplasmic reticulum (ER) to accumulate the enormous quantities of proteins that are actively synthesized on the ER (Chrispeels and Herman, 2000). ER-derived structures are found in both vegetative and storage organs of plants (Hara-Nishimura *et al.*, 2004). ER bodies of *Arabidopsis thaliana*, KDEL vesicles of black gram (*Vigna mungo*), and ricinosomes of castor bean (*Ricinus communis*) are examples of ER-derived structures that are found in vegetative organs (Schmid *et al.*, 1998; Toyooka *et al.*, 2000; Hayashi *et al.*, 2001). All of these structures have been shown to accumulate a hydrolytic enzyme with a well-known ER retention signal (KDEL) at the C-terminus (Schmid *et al.*, 1998; Toyooka *et al.*, 2000; Matsushima *et al.*, 2003). Protein bodies (PBs) of maize (*Zea mays*) and precursor-accumulating (PAC) vesicles of pumpkin (*Cucurbita maxima*) are among the ER-derived structures that are found in storage organs (Hara-Nishimura *et al.*, 1998; Herman and Larkins, 1999). These structures are responsible for the accumulation of seed storage proteins, which do not possess the KDEL signal, in the ER (Hara-Nishimura *et al.*, 1998; Herman and Larkins, 1999).

In rice endosperm, there are two types of PBs (Tanaka *et al.*, 1980; Yamagata *et al.*, 1982) and these are responsible for storing prolamins and glutelins, the major

* To whom correspondence should be addressed. E-mail: masumura@kpu.ac.jp

seed storage proteins of rice (Bechtel and Juliano, 1980; Oparka and Harris, 1982; Krishnan *et al.*, 1986; Yamagata and Tanaka, 1986). Although both prolamins and glutelins are synthesized on the rough ER, they are transported by different pathways (Tanaka *et al.*, 1980; Yamagata *et al.*, 1982). Because of their hydrophobicity, prolamins are stored in spherical intracisternal inclusion granules, referred to as type I PBs (PB-Is), within the ER lumen (Tanaka *et al.*, 1980). In contrast, glutelins are introduced into the vesicular transporting system, resulting in accumulation in protein storage vacuoles (PSVs) that are electron-dense and irregularly shaped, referred to as type II PBs (PB-IIs) (Tanaka *et al.*, 1980; Krishnan *et al.*, 1986).

The specific subcellular localization of storage proteins in rice endosperm is a unique process involving seed-specific events. First, the rice storage protein mRNAs are localized to distinct subdomains of the ER. Prolamin mRNAs are targeted to ER that surrounds the PB-I (PB-ER), whereas glutelin mRNAs are localized to the cisternal ER (Crofts *et al.*, 2004). The rice storage proteins are synthesized on each ER membrane, and translocated into the ER lumen. Secondly, chaperone proteins in the ER lumen are crucial for the sorting of storage proteins. The rice prolamin does not contain a KDEL/HDEL ER retention sequence, but its deposition has been shown to be assisted by the binding protein (BiP) (Li *et al.*, 1993). BiP is highly enriched on the periphery of PB-Is (Muench *et al.*, 1997) and has been proposed to be necessary to maintain the prolamin in a competent state for subsequent assembly in the ER. Another molecular chaperone, protein disulphide isomerase, is required for the segregation of prolamins and proglutelins in the ER lumen (Takemoto *et al.*, 2002).

Several recent reports have investigated the intracellular localization of recombinant proteins expressed in seeds, revealing that such proteins can be deposited in unexpected places (Wright *et al.*, 2001; Chikwamba *et al.*, 2003; Arcalis *et al.*, 2004; Petruccelli *et al.*, 2006). Drakakaki *et al.* (2006) reported that the recombinant phytase was secreted from leaf cells of rice, as expected, whereas it was retained in PB-Is and PB-IIs in the endosperm cells. They concluded that the storage function of rice endosperm may determine whether or not the recombinant phytase is secreted.

Recently, the sorting mechanisms of rice storage proteins have been investigated, although the mechanisms by which prolamins assemble into PB-Is are still poorly understood. To investigate whether ER-derived PB-I formation of prolamin is specific to the highly specialized endosperm, the stability and the subcellular deposition site of the constitutively expressed green fluorescent protein fused to prolamin (prolamin–GFP) in the seeds, leaves, and roots of transgenic rice plants were examined. The results demonstrate that prolamin–GFP fusion proteins are stable not only in the seeds but also in the leaves and roots of transgenic rice. Furthermore, it is shown that prolamin–GFP forms the PB-like structures in leaves and roots of transgenic rice

Materials and methods

Plant materials

Rice (*Oryza sativa* L. cv Nipponbare) was used in all experiments. The transgenic rice plants were grown with soil in a naturally illuminated temperature-controlled (28 °C) greenhouse at the experimental field of the Kyoto Prefectural Institute of Agricultural Biotechnology. The seedlings were grown at 28 °C under a continuous light condition (30 μmol photons m^{-2} s^{-1}) in an incubation room.

Plasmid constructions and transformation of rice plants

The *p35S:GFP* plasmid vector (Takahashi *et al.*, 2004) containing the 35S promoter of the cauliflower mosaic virus (CaMV), the first 5′-untranslated region (UTR) intron of the superoxide dismutase (SOD) gene (*sodCc2*) (Sakamoto *et al.*, 1995), the coding sequence of sGFP(S65T) (Chiu *et al.*, 1996), and the polyadenylation signal of the nopaline synthase (NOS) gene was used.

To generate a fusion gene, the coding region for rice 13 kDa prolamin (*λRM1*) (Mitsukawa *et al.*, 1999) was amplified by PCR using the following primer set: forward, 5′-GCG-GAGGGATCCATGAAGATCATTTTCGTATTTGCTC-3′ containing a *Bam*HI site; and reverse, 5′-GCGGAGGGA-TCCGCCGCCGCCGTACCAGACACCACCAACGG-3′ containing a *Bam*HI site. The amplified PCR fragment was digested with *Bam*HI, then inserted into the *Bam*HI site of the N-terminal region of the sGFP(S65T)-coding sequence of the plasmid vector (Takahashi *et al.*, 2004), and the resulting plasmid was named *p35S:Pro–GFP*.

The expression cassettes of *p35S:GFP* and *p35S:Pro-GFP* were transferred into the cloning site of the pIG121Hm plasmid (Ohta *et al.*, 1990). The resulting binary vectors were introduced into rice, using an *Agrobacterium*-mediated method described previously (Hiei *et al.*, 1994).

RT-PCR analysis

Total RNA was extracted from leaves and roots of 9-day-old seedlings and 12 days after flowering (DAF) developing seeds of wild type (WT) and 35S:Pro-GFP plants with an RNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA (0.5 μg) was converted into cDNA by ReverTra Ace (MMLV Reverse Transcriptase RnaseH-; Toyobo, Osaka, Japan) with oligo(dT) as primer, yielding 20 μl of cDNA solution. PCR was performed in a 10 μl reaction volume containing 1 μl of cDNA solution, 1× PCR buffer, 0.25 μM dNTPs, 1.0 μM of each primer, and 0.5 U of *Taq* polymerase (Takara, Otsu, Japan). The following primers were used: *sGFP* (GFP-F, 5′-TATA-TCATGGCCGACAAGCA-3′; and GFP-R, 5′-GAACTC-CAGCAGGACCATGT-3′) and *actin* (actin-F, 5′-TACA-ACTCCATCATGAAGTGCG-3′; and actin-R, 5′-AGAA-GCATTTCCTGTGCACAAT-3′). The PCR program consisted of 25 repetitive cycles with a denaturation step at 94 °C for 30 s, an annealing step at 57 °C for 30 s, and an elongation step at 72 °C for 1 min. The PCR cycles

were preceded by an extra denaturation step at 94 °C for 4 min and ended with an extra elongation step of at 72 °C for 7 min.

Protein extraction and subcellular fractionation

For the extraction of total proteins, the leaves and roots of the 15-day-old seedling and mature seeds were homogenized in SDS sample buffer A [62.5 mM TRIS-HCl (pH 6.8), 4 M urea, 2% (w/v) SDS] supplemented with 5% (v/v) 2-mercaptoethanol (2-ME). The homogenates were centrifuged at 15 000 *g* for 30 min to obtain the protein extracts as supernatant solutions and then heated (100 °C). An *RC DC* Protein Assay Kit (Bio-Rad, Hercules, CA, USA) was used for measurement of total proteins according to the manufacturer's instructions. The 10 μg aliquots of proteins were analysed by SDS–PAGE and immunoblot.

For subcellular fractionation, leaf and root cell fractions were prepared according to the method of Tamura *et al.* (2003). Leaves and roots of the 21-day-old seedlings were chopped with a razor blade on ice in HEPES buffer [50 mM HEPES-KOH (pH 7.5), 0.4 M sucrose, and protease inhibitors (Complete; Roche, Basel, Switzerland)]. The homogenates were filtered through Miracloth (Calbiochem, La Jolla, CA, USA). Filtrates were centrifuged at 15 000 *g* and 4 °C for 20 min. The pellets were added to HEPES buffer (the P15 fractions), and the supernatants were centrifuged at 100 000 *g* and 4 °C for 20 min. The pellets were added in HEPES buffer (the P100 fractions), and the supernatants (the S100 fractions) were concentrated by using a Microcon YM-10 centrifugal filter device (Millipore, Billerica, MA, USA). Each of the fractions P15, P100, and S100 was analysed by SDS–PAGE and immunoblot.

For the extraction of proteins under non-reducing conditions, the powder of mature seeds was extracted with the SDS sample buffer A. Leaves were chopped on ice with a razor blade in the HEPES buffer. The homogenates were filtered through Miracloth (Calbiochem). Filtrates were centrifuged at 15 000 *g* and 4 °C for 20 min. The pellets were added in 1× SDS sample buffer B [62.5 mM TRIS-HCl (pH 7.5), 10% (v/v) glycerol, 2% (w/v) SDS] (the P15 fractions), and the supernatants (the S15 fractions) were concentrated by using a Microcon YM-10 centrifugal filter devices (Millipore). The supernatants were mixed with an equal volume of 2× SDS sample buffer B. For extraction under reducing conditions, the SDS sample buffers A and B were supplemented with 5% (v/v) 2-ME. Each of the fractions P15 and S15 was analysed by SDS–PAGE and immunoblot.

After SDS–PAGE analysis, separated proteins were electrotransferred to an Immun-Blot PVDF Membrane (Bio-Rad), revealed using anti-GFP antibodies (dilution 1:2000; Medical & Biological Laboratories, Nagoya, Japan) and anti-13 kDa prolamin antibodies (1:1000; Furukawa *et al.*, 2003), and detected with the alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (dilution 1:10 000; Promega, Madison, WI, USA). Proteins were stained with 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (BCIP/NBT Color Development Substrate; Promega) according to the manufacturer's instructions.

Fluorescence microscopic analysis

Frozen sections of mature rice seeds were prepared by a method described previously (Saito *et al.*, 2008). Mature seeds were vacuum infiltrated overnight with 2% (w/v) carboxymethyl cellulose (CMC) gel. The samples were embedded in 2% (w/v) CMC gel and frozen in cooled hexane (–94 °C). Seed sections were generated using a Cryofilm transfer kit (FINETEC, Tokyo, Japan) and cryostat (Microm Model 500 M; Global Medical Instrumentation, Inc., Ramsey, MN, USA). The 2 μm thick cryostat sections were incubated with 0.1 μM rhodamine B hexyl ester, the ER membrane stainer, in phosphate-buffered saline (PBS)/methanol (50:50) to label the PB-I membrane. After being washed in PBS, the seed sections were inspected with a fluorescence microscope (BX51; Olympus, Tokyo, Japan). Images were analysed with an Aquacosmos system (Hamamatsu Photonics, Hamamatsu, Japan).

The leaves and roots of 18-day-old seedlings of WT, 35S:GFP, and 35S:Pro-GFP were inspected with an E600 microscope and C1si confocal laser scanning microscope system (Nikon, Tokyo, Japan). GFP was excited at a laser wavelength of 488 nm and detected through a filter for a fluorescence wavelength of 500–530 nm.

Immunoelectron microscopy

Small pieces of 12 DAF developing seeds were vacuum infiltrated for 10 min with a fixative that consisted of 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) and treated for another 3 h at room temperature with the fixative. After washing with the same buffer, the seeds were dehydrated in a graded ethanol series and then embedded in LR White resin (London Resin Co. Ltd, Hampshire, UK). Blocks were polymerized at 55 °C for 48 h. Ultrathin sections were cut with a diamond knife using a Leica Ultracut UCT (Leica, Wetzlar, Germany) and mounted on nickel grids.

For high pressure freezing and freeze substitution, leaves and roots of 12-day-old seedlings were frozen with a high pressure freezing machine (model HPM010; Bal-Tec, Balzers, Liechtenstein). The frozen samples were treated with acetone for 2 d at –80 °C and warmed at –20 °C for 3 h, 4 °C for 2 h, and room temperature for 2 h. Fixed samples were dehydrated in graded ethanol and embedded in LR White resin.

Ultrathin sections were treated with blocking solution of 1% (w/v) bovine serum albumin in 0.1 M sodium phosphate buffer (pH 7.2) for 30 min at room temperature. The sections were then incubated with anti-GFP antibodies (dilution 1:100; Medical & Biological Laboratories) overnight at 4 °C and with anti-pumpkin BiP antibodies (1:100; Hara-Nishimura *et al.*, 1998) for 1 h at room temperature. After washing with 0.1 M sodium phosphate buffer (pH 7.2), the sections were incubated with a solution of 10 nm

gold-labelled goat anti-rabbit IgG antibodies (1:50; GE Healthcare, Buckinghamshire, UK) in the blocking solution for 1 h at room temperature. The sections were washed with distilled water and then stained with 2% (w/v) uranyl acetate. After staining, the sections were examined with a transmission electron microscope (JEM-1220; JEM, Tokyo, Japan) at 100 kV.

Results

Production of transgenic rice constitutively expressing prolamin–GFP

Prolamins are encoded by a multigene family, and separated into three major groups according to their apparent molecular sizes of 10, 13, and 16 kDa (Ogawa *et al.*, 1987). The 13 kDa prolamins are the most abundant group of prolamins in rice (Horikoshi *et al.*, 1991). A chimeric gene encoding a fusion protein of 13 kDa prolamin (λRM1) and GFP was constructed. The prolamin-coding sequence was fused to the region upstream of GFP. GFP is a soluble protein that is secreted efficiently when introduced into plant ER via a signal peptide (Batoko *et al.*, 2000; Frigerio *et al.*, 2001). This chimeric gene was driven by the CaMV 35S promoter (35S:Pro-GFP; Fig. 1). As a control, the GFP gene driven by the CaMV 35S promoter, without the prolamin-coding sequence, was used (35S:GFP; Fig. 1). The first intron of the superoxide dismutase *sodCc2* gene (Sakamoto *et al.*, 1995) was inserted between the promoter and the GFP gene to enhance promoter activity. This construct was transferred into rice (*O. sativa* L. cv. Nipponbare) by an *Agrobacterium*-mediated transformation method (Hiei *et al.*, 1994). RT-PCR was used to analyse the expression of the prolamin–GFP gene in different tissues of 35S:Pro-GFP plants (Fig. 2). As expected, the transcripts of the prolamin–GFP gene were detected in all tissues analysed, reflecting the activity of the CaMV 35S promoter. The expression of *GFP* was not detected in tissues of WT plants.

Accumulation of prolamin–GFP fusion proteins in seeds, leaves, and roots of transgenic rice

To investigate the accumulation of the prolamin–GFP fusion proteins in different tissues of the transgenic rice plant, immunoblot analysis of protein extracts from seeds, leaves, and roots was performed with anti-GFP antibodies (Fig. 3). Bands with apparent molecular masses of ~27 kDa and ~40 kDa were detected in the 35S:GFP and 35S:Pro-GFP in all tissues, respectively (Fig. 3). The 40 kDa mass is consistent with the predicted molecular size of prolamin–GFP fusion proteins, because the molecular sizes of prolamin and GFP are 13 kDa and 27 kDa, respectively. The ~40 kDa polypeptides also reacted with anti-13 kDa prolamin antibodies (Fig. 3). These results suggested that the prolamin–GFP fusion proteins accumulated stably not only in the seeds but also in the leaves and roots. The bands of 13, ~23, and ~27 kDa were also detected in the seeds of all plants by anti-13 kDa prolamin antibodies. The 13 kDa proteins are endogenous 13 kDa prolamin. The bands of 23 kDa and 27 kDa are non-specific signals. In addition, the processed form of the prolamin–GFP fusion proteins was detected in the leaves and roots of 35S:Pro-GFP plants (Fig. 3).

Localization of prolamin–GFP fusion proteins in the PB-Is in starchy endosperm cells

The subcellular localization of prolamin–GFP fusion proteins in the endosperm cells was next investigated. Fluorescence of prolamin–GFP was detected in the starchy endosperm cells of 35S:Pro-GFP plants, and fluorescence signals were also detected in aleurone cells (Fig. 4A, B). However, the particles emitting fluorescence signals in the aleurone layer were smaller than those in the starchy endosperm cells. In the starchy endosperm cells, prolamin–GFP was primarily found in spherical organelles, but it was not observed in starch granules or the intracellular space (Fig. 4B). The GFP fluorescence was not detected in the endosperm cells of the WT (Fig. 4C, D). The co-localization of PB-I and GFP fluorescence was analysed in the endosperm cells of WT, 35S:GFP, and 35S:Pro-GFP plants. When the seed sections were stained with the rhodamine B hexyl ester, which preferentially labels PB-Is (Choi *et al.*, 2000), the fluorescence of prolamin–GFP was localized mainly to the PB-Is (Fig. 4G). In contrast, GFP fluorescence was not detected in the WT endosperm (Fig. 4E), and GFP fluorescence was detected in the cytosol in the 35S:GFP endosperm (Fig. 4F). Immunogold labelling of prolamin–GFP fusion proteins in the starchy endosperm cells of a 35S:Pro-GFP plant showed that the gold particles were distributed in the PB-Is, but not in PB-IIs (Fig. 4H). The

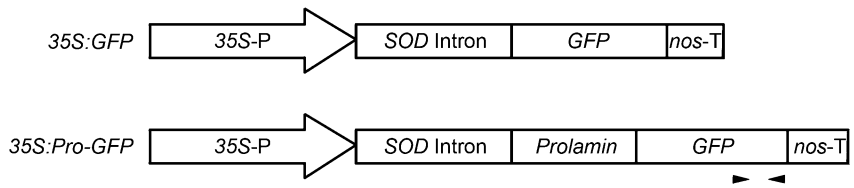

Fig. 1. Constructs of 35S:GFP and 35S:Pro-GFP that were expressed in transgenic rice plants. *35S*-P, the 35S promoter of cauliflower mosaic virus; *SOD* Intron, the first intron of rice *sodCc2*; *Prolamin*, the coding sequence for the 13 kDa prolamin gene (*λRM1*); *GFP*, the modified green fluorescent protein gene; *nos*-T, terminator of the nopaline synthase gene. The arrowheads indicate the RT-PCR primers used for the experiments in Fig. 2.

gold particles were frequently detected in the core of mature PB-Is (Fig. 4I). Some of the PB-Is in 35S:Pro-GFP endosperm cells had a cracked structure and were larger than in WT and 35S:GFP endosperm cells. It is possible that the structure of PB-Is is influenced by the accumulation of prolamin–GFP fusion proteins. In the endosperm cells of the WT, the gold particles were not detected (data not shown). Without an additional sequence, GFP proteins accumulate diffusely in the cytoplasm and nucleus (Chiu *et al.*, 1996). No significant signals of the gold particles were detected in the endosperm cells of 35S:GFP plants (data not shown); it is more difficult to detect diffuse proteins than aggregated proteins by immunocytochemistry. Immunogold labelling of prolamin–GFP in the aleurone cells of a 35S:Pro-GFP plant showed that the gold particles were detected in novel electron-dense structures with a diameter of ~300 nm (Fig. 4J, K). This result showed that prolamin–GFP fusion proteins are capable of forming the protein aggregates in the alurone cells.

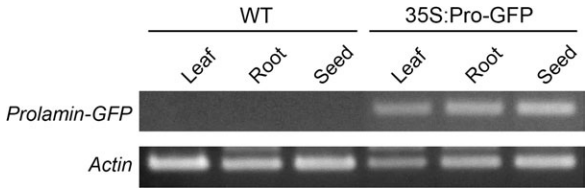

Fig. 2. Expression of prolamin–GFP mRNA in different tissues of transgenic plants. RT-PCR analysis of the *prolamin–GFP* transcript in seeds, leaves, and roots of wild type (WT) and 35S:Pro-GFP plants. The bottom panel shows the expression of the *actin* genes as a control.

Formation of aggregates of prolamin–GFP fusion proteins in the leaves and roots

To investigate the subcellular location of prolamin–GFP in leaves and roots, subcellular fractionation was performed according to the method of Tamura *et al.* (2003). The homogenates from leaves or roots from the seedlings were separated into three subcellular fractions: a 15 000 *g* pellet (P15), a 100 000 *g* pellet (P100), and a 100 000 *g* supernatant (S100). P100 and S100 contain the microsomal proteins and the mixed cytosolic and vacuolar soluble proteins, respectively. The immunoblots of each fraction with anti-GFP antibodies showed that most of the prolamin–GFP was detected in the P15 fractions of leaves and the P15 and P100 fractions of roots (Fig. 5A, B). In the 35S:GFP plants, the GFP proteins were detected specifically in the S100 fractions. The high concentration of prolamin–GFP in the pellet fractions (P15 and P100) suggested that the aggregated prolamin–GFP fusion proteins are retained in the ER lumen.

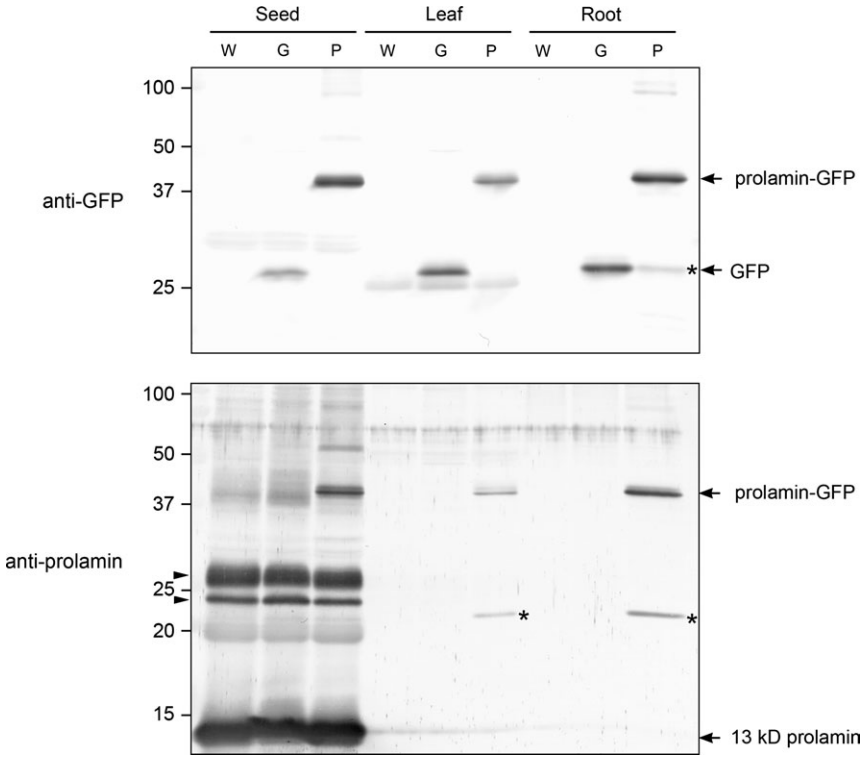

Fig. 3. Accumulation of prolamin–GFP fusion proteins in different tissues of transgenic plants. Seeds, leaves, and roots of WT (W), 35S:GFP (G), and 35S:Pro-GFP (P) plants were subjected to SDS–PAGE followed by immunoblot with anti-GFP and anti-13 kDa prolamin antibodies. The upper and lower bands correspond to prolamin–GFP and GFP, respectively. The truncated prolamin–GFP was observed in the leaf and root tissues of 35S:Pro-GFP plants (asterisks). The arrowheads indicate the non-specific signal. The molecular masses are given on the left in kDa.

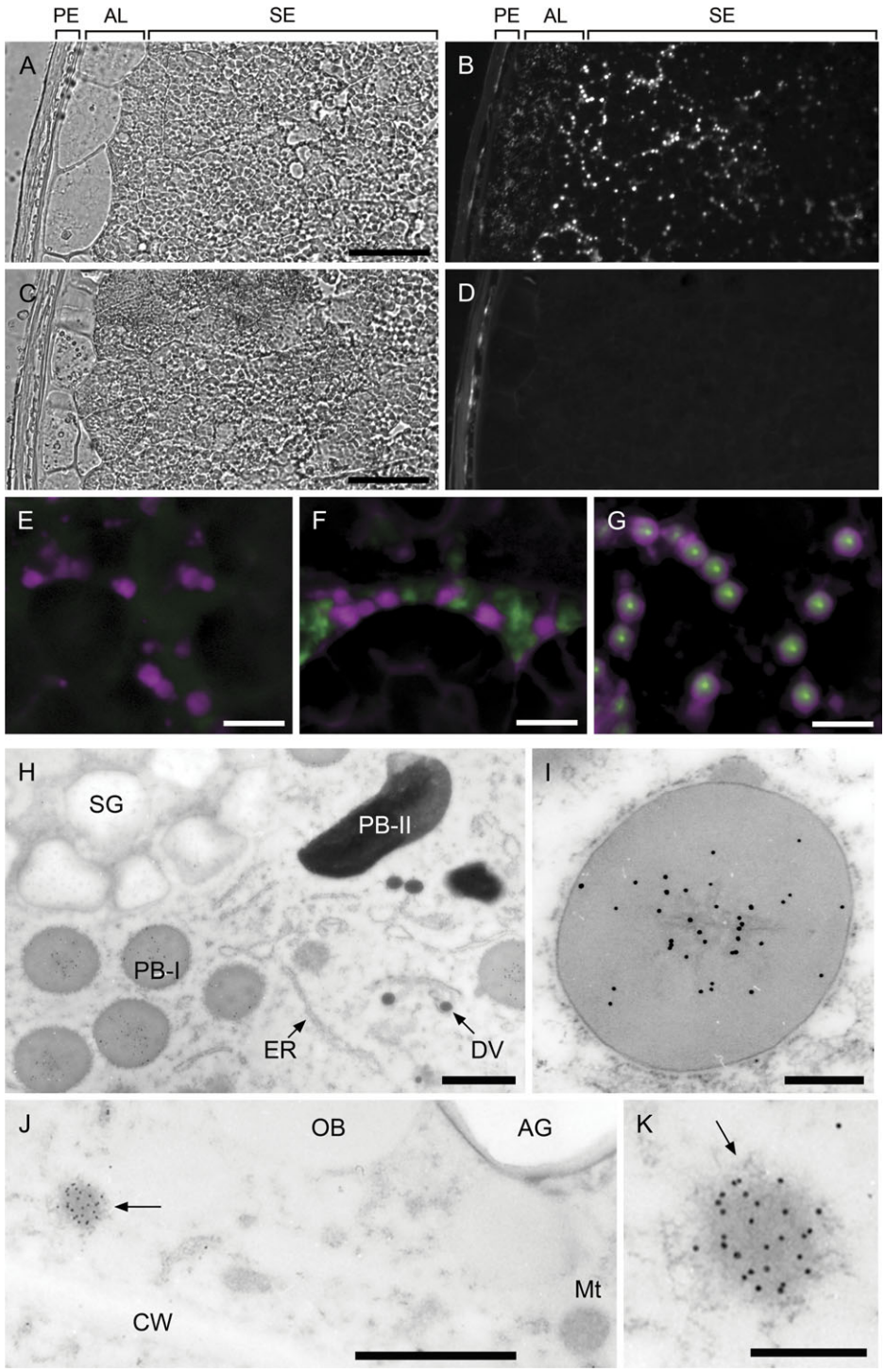

Fig. 4. Localization of prolamin–GFP fusion proteins in PB-Is in the endosperm cells. Light transmission (A and C) and GFP fluorescence (B and D) images of the mature seed section of a 35S:Pro-GFP plant (A and B) and the WT (C and D) are shown. PE, pericarp; AL, aleurone layer; SE, starchy endosperm. Bars in A and A and C = 50 μm. (E–G) Mature seed sections of WT (E), 35S:GFP (F), and 35S:Pro-GFP (G) were stained with rhodamine B hexyl ester, which specifically binds to the ER membrane and ER-derived PB-I membrane. Green and magenta fluorescence indicate GFP and PB-I, respectively. Bars in E–G=5 μm. (H–K) Immunogold labelling of prolamin–GFP with anti-GFP antibodies in the developing starchy endosperm cells (H and I) and aleurone cells (J and K) of 35S:Pro-GFP plants. (G) Magnified image of the PB-I. Gold particles are detected in the electron-dense structures in the aleurone cells (J and K, arrows). PB-I, type I protein body; PB-II, type II protein body; SG, starch granule; ER, endoplasmic reticulum; DV, dense vesicle; OB, oil body; AG, aleurone grain; CW, cell wall; Mt, mitochondrion. bar in H and J=1 μm; bar in I and K=250 nm.

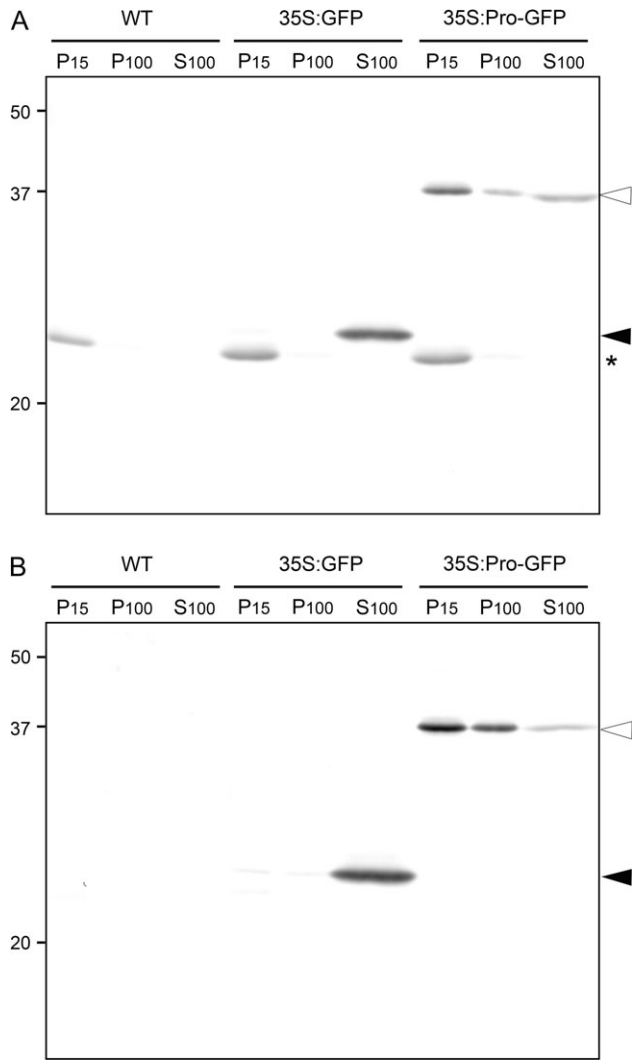

Fig. 5. Subcellular distribution of prolamin–GFP fusion proteins in leaves and roots of transgenic plants. Homogenates from leaves (A) and roots (B) of WT, 35S:GFP, and 35S:Pro-GFP plants were subjected to differential centrifugation to obtain the 15 000 *g* pellet (P15), the 100 000 *g* pellet (P100), and the 100 000 *g* supernatant (S100). Each fraction was subjected to immunoblotting with anti-GFP antibodies. Bands of ~40 kDa (open arrowheads) correspond to prolamin–GFP, while bands of ~27 kDa (filled arrowheads) correspond to the truncated form. An asterisk indicates the non-specific signal. Molecular masses are given on the left in kDa.

The subcellular distribution of prolamin–GFP in leaf and root cells was analysed by fluorescence microscopy (Fig. 6). In the leaf and root cells of the 35S:GFP plants, the fluorescence of GFP was detected in the nucleus and cytosol (Fig. 6B, E). The fluorescence of prolamin–GFP showed a bright punctate pattern in both the leaf and root cells of the 35S:Pro-GFP plants, and did not show the network pattern of the ER (Fig. 6C, F). The fluorescence was not detected within the vacuole in these cells. In addition, the fluorescence signals of prolamin–GFP in root cells appeared to be slightly weaker than those in leaf cells (Fig. 6C, F).

Formation of PB-like structures containing prolamin–GFP fusion proteins in leaf and root cells

To characterize these punctate structures of prolamin–GFP in the leaf and root cells, an ultrastructural analysis was performed with an electron microscope using a high pressure frozen/freeze substitution technique for the preparation of the thin sections. Immunogold labelling of prolamin–GFP on these thin-sectioned samples showed that the gold particles were localized in electron-dense structures in the leaf and root cells of 35S:Pro-GFP plants (Fig. 7). Most of the structures containing prolamin–GFP were spherical, with diameters of 50–500 nm in the leaf cells, but some of them were irregular structures (Fig. 7B) or spherical structures with a diameter of 2 μm (Fig. 7C). In addition, these structures in leaf cells were often surrounded by the membrane (Fig. 7D, arrowheads). Some of these structures were also associated with the membrane (Fig. 7E). These structures labelled with anti-GFP antibodies were found in all types of leaf cells, including mesophyll and vascular cells. The novel structures were also found in the root cells (Fig. 7F, G). The characteristics of these structures in the leaves and roots looked similar to those of PB in the endosperms. Examination of several leaf and root sections of WT, 35S:GFP, and 35S:Pro-GFP plants showed that these PB-like structures labelled with anti-GFP antibodies were found only in 35S:Pro-GFP plants and were not present in WT and 35S:GFP plants (data not shown).

Association of the ER-resident molecular chaperone, BiP, with PB-like structures in the leaf and root cells

The PB-like structures in the leaf cells were often observed in close proximity to the membrane (Fig. 7D, E). To determine whether the PB-like structures were derived from the ER, an immunoelectron microscopy analysis was performed with antibodies raised against pumpkin BiP, an ER-resident molecular chaperone. The anti-BiP antibodies were localized in the peripheral region of PB-Is in WT rice endosperm (Takahashi *et al.*, 2005). Immunogold labelling of BiP revealed the presence of gold particles within PB-Is in the endosperm of the 35S:Pro-GFP plants (Fig. 8A). In the leaf and root cells, the gold particles were detected in electron-dense structures (Fig. 8B, C), and the size and shape of these structures were highly similar to those of the PB-like structures observed in Fig. 7. Figure 8B shows the presence of electron-dense structures on the membrane, causing the area to appear swollen. Gold particles were also detected within the continuous ER membrane region (Fig. 8B). The electron-dense structures labelled with anti-BiP antibodies were not present in WT and 35S:GFP plants (data not shown). These results showed that the PB-like structures are derived from rough ER.

2-ME increases the solubility of prolamin–GFP fusion proteins

The rice 13 kDa prolamin encoded by *λRM1* contains four cysteine residues. Because little is known about which

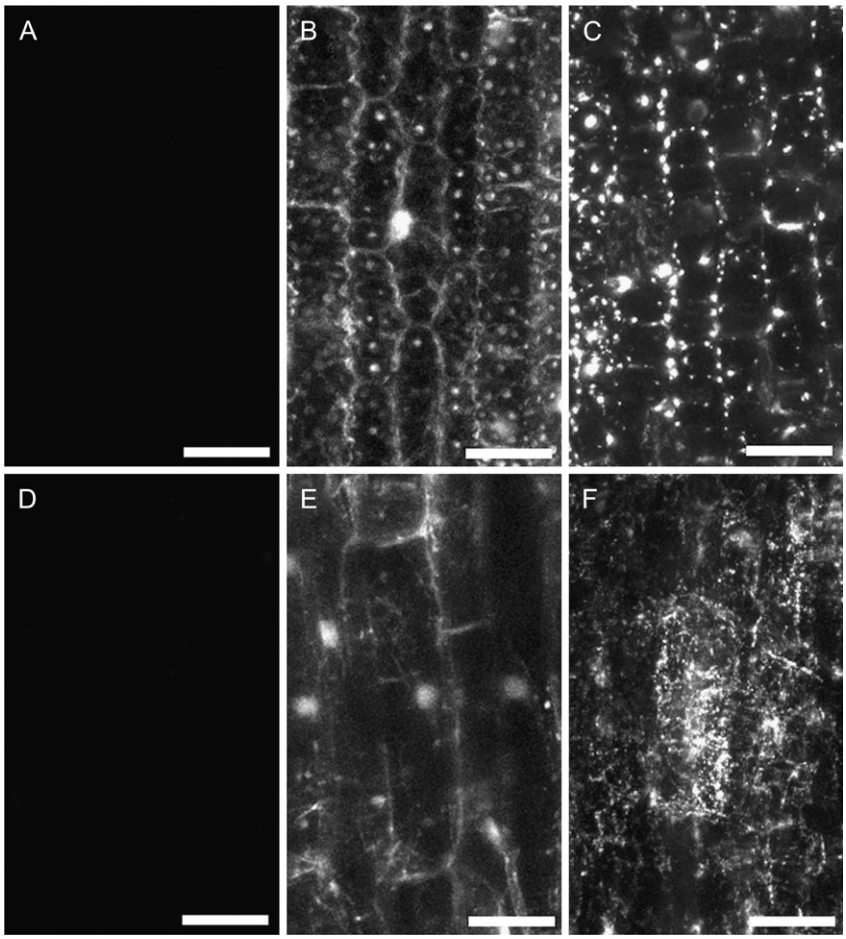

Fig. 6. Fluorescence images of prolamin–GFP in the leaf and root cells. Fluorescence images of the leaves (A–C) and roots (D–F) of WT (A and D), 35S:GFP (B and E), and 35S:Pro-GFP (C and F) plants taken using a confocal laser scanning microscope are shown. Dot-like structures, presumed to be prolamin–GFP proteins, are visible in the leaf cells (C) and the root cells (F) of 35S:Pro-GFP plants. Bars = 20 μm.

cysteine residues in rice prolamins engage in intermolecular and/or intramolecular disulphide bonds, an investigation was carried out to determine whether cysteine residues of prolamin–GFP fusion proteins could form intermolecular disulphide bonds. In the seeds of 35S:Pro-GFP plants, the addition of 4% 2-ME to the extraction buffer reduced the number of disulphide bonds, leading to prolamin–GFP solubilization (Fig. 9A, lane 3, open arrowhead). When proteins were extracted from seeds of 35S:Pro-GFP plants in the absence of 2-ME, prolamin–GFP was not solubilized (Fig. 9A, lane 6). The presence or absence of 2-ME did not affect the GFP solubilization in protein extracts from the seeds of 35S:GFP plants (Fig. 9A, lanes 2 and 5, closed arrowhead). These results suggest that the cysteine residues of prolamin–GFP formed intermolecular disulphide bonds with prolamin–GFP in the endosperm cells. To investigate whether the cysteine residues of prolamin–GFP expressed in the leaves could form intermolecular disulphide bonds with prolamin–GFP molecules, the influence of 2-ME on the solubility of prolamin–GFP fusion proteins in the leaves was examined. The homogenates from leaves were separated into two subcellular fractions: a 15 000 *g* pellet (P15; the PB fractions) and a 15 000 *g* supernatant (S15). When the proteins in the P15 fractions from 35S:Pro-GFP plants were extracted in the presence or absence of 2-ME, prolamin–GFP was solubilized under reducing conditions, whereas the prolamin–GFP was only partially solubilized under non-reducing conditions (Fig. 9B, lanes 3 and 6, open arrowhead). In contrast, GFP was not affected by the absence of 2-ME in the S15 fractions from 35S:GFP plants (Fig. 9B, lanes 2 and 4, closed arrowhead). These results indicate that the prolamin–GFP fusion proteins in the PB-like structures form the intermolecular disulphide bonds.

Discussion

The aim of this study was to determine whether accumulation of prolamin in the ER is specific to endosperm tissue. The principal findings of this study are as follows: (i) the prolamin–GFP fusion proteins accumulated not only in the storage organs but also in the vegetative organs of transgenic rice plants; (ii) the protein aggregates containing the prolamin–GFP were observed in the leaf and the root cells; (iii) some of these structures were surrounded by the membrane; (iv) the ER-resident molecular chaperone BiP proteins accumulated in these structures; and (v) when the leaves were homogenized without 2-ME, the prolamin–GFP

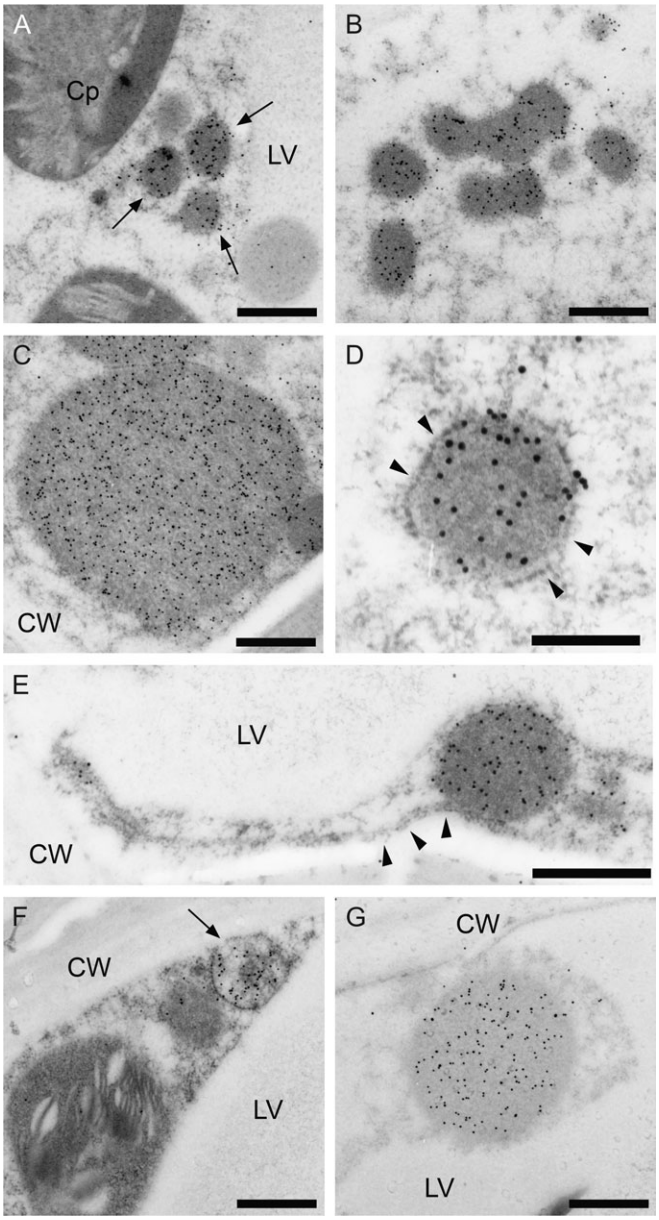

Fig. 7. PB-like structures containing prolamin–GFP fusion proteins in the vegetative tissues. (A–E) Immunogold labelling of prolamin–GFP with anti-GFP antibodies in the leaf cells of 35S:Pro-GFP plants as determined using a high pressure frozen/freeze substitution technique. Numerous electron-dense structures labelled with anti-GFP antibodies were visible in the leaf cells (A, arrows). These structures had different shapes and sizes (A–E), and some of them were in close proximity to the rough ER (D and E, arrowheads). (F and G) Immunogold labelling of prolamin–GFP in the root cells of 35S:Pro-GFP plants. The PB-like structures were also visible in the root cells (F and G). Cp, chloroplast; LV, lytic vacuole; CW, cell wall. Bars in A–C, E, and F = 500 nm; bar in D = 200 nm.

fusion proteins were only partially solubilized. The findings indicate that the seed-specific factors are not essential for the aggregation of prolamin–GFP fusion proteins, suggesting that the assembly of prolamin molecules and interaction of prolamin–GFP with BiP results in the formation of PB.

Prolamin–GFP fusion proteins specifically localize in PB-Is in endosperm cells

In rice endosperm cells, prolamins are deposited within the ER and then form PB-Is (Tanaka *et al.*, 1980). In the endosperm cells of 35S:Pro-GFP plants in the present study, the prolamin–GFP fusion proteins localized in PB-Is (Fig. 4G–I). Kawagoe *et al.* (2005) showed that GFP fused to the α-globulin signal peptide was partitioned primarily into the lumen, rather than into PB-Is, in the ER. They pointed out that GFP itself does not have structural characteristics that promote protein integration into PB-Is. These results indicate that the coding sequence of prolamin contains the determinants required for protein localization in the PB-Is in rice endosperm. Hamada *et al.* (2003) reported that the prolamin RNA transport pathway to the PB-ER requires two partially redundant *cis*-elements, one located in the coding sequence and a second residing in the 3′-UTR. The 35S:Pro-GFP construct used in this study contains the coding sequence of prolamin, but lacks the 3′-UTR (Fig. 1). Further studies are needed to determine whether the prolamin–GFP mRNA is targeted to the PB-ER in starchy endosperm cells.

Accumulation of prolamin–GFP fusion proteins in leaves and roots

The immunoblot analysis revealed that the prolamin–GFP fusion proteins accumulated not only in the seeds but also in the leaves and roots of 35S:Pro-GFP plants (Fig. 3). The subcellular fractionation in leaves and roots revealed that prolamin–GFP fusion proteins were present in the 15 000 *g* pellet (the PB fraction) and the 100 000 *g* pellet (the microsomal fraction) (Fig. 5). These results suggest that the prolamin–GFP was aggregated in the leaves and roots. On the other hand, the processed forms of prolamin–GFP were also detected in the leaves and roots (Fig. 3). Moreover, a portion of the prolamin–GFP expressed in leaves and roots was detected in the 100 000 *g* supernatant (mixed cytosolic and vacuolar soluble proteins fraction) (Fig. 5). The endogenous ER residents are transported constitutively to the vacuoles by bypassing the Golgi complex, and are then degraded (Tamura *et al.*, 2004). It is possible that, in these ways, some prolamin–GFP fusion proteins are transported to the vacuoles in the leaves and roots. Tamura *et al.* (2003) succeeded in stabilizing GFP fluorescence within the acidic vacuoles of *Arabidopsis* plants by incubating them in darkness. In the 35S:Pro-GFP plants grown in darkness, however, the fluorescence of prolamin–GFP was not detected in the vacuoles (data not shown).

Prolamin–GFP fusion proteins form the PB-like structures in leaves and roots

The fluorescence of prolamin–GFP showed a bright punctate pattern in both the leaf and the root cells of the 35S:Pro-GFP plants, and did not show the network pattern of the ER (Fig. 6C, F). Immunoelectron microscopy analysis showed that the prolamin–GFP accumulates in the electron-dense

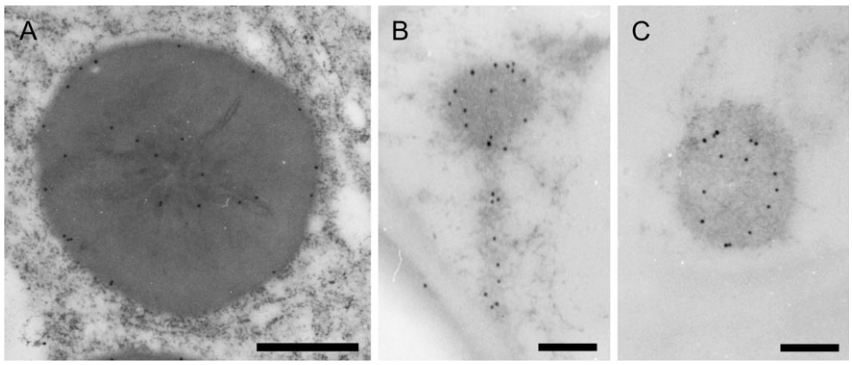

Fig. 8. Association of an ER-resident molecular chaperone, BiP, with PB-I and PB-like structures of 35S:Pro-GFP plants. Immunoelectron micrographs of PB-Is in starchy endosperm cells of seeds (A) and PB-like structures in leaf cells (B) and root cells (C) of 35S:Pro-GFP plants obtained using anti-pumpkin BiP antibodies. Bar in A = 500 nm; bars in B and C = 200 nm.

structures surrounded by the membrane in the aleurone layer (Fig. 4) and the leaves and roots (Fig. 7). The ER-resident protein BiP was observed in the PB-like structures (Fig. 8B, C). These results suggest that the PB-like structures develop inside the rough ER. The ER-targeted GFP fusion protein, which contains an N-terminal signal peptide and the C-terminal amino acid HDEL, has been shown to form a characteristic reticulate network in transgenic *Arabidopsis* plants (Haseloff *et al.*, 1997). These results show that the addition of a prolamin molecule is responsible for the formation of PB-like structures.

Zeins, the storage proteins of maize seeds, belong to the prolamin class of storage proteins and are deposited in ER-derived PBs (Larkins and Hurkman, 1978). Zeins consist of several types of polypeptides, the α-, β-, γ-, and δ-zeins, which are structurally distinct (Thompson and Larkins, 1989). The γ-zein, β-zein, and δ-zein have been expressed individually in the leaves of transgenic *Arabidopsis* (γ-zein) and tobacco (β-zein and δ-zein) plants by using the CaMV 35S promoter, and, in all cases, the proteins were retained in the ER-derived PBs (Geli *et al.*, 1994; Bagga *et al.*, 1995, 1997). Furthermore, the N-terminal domains of γ-zein can confer the ability to form ER-derived PBs when fused to the bean vacuolar storage phaseolin in the chimeric protein zeolin (Mainieri *et al.*, 2004). The results agree with the experiments on PB formation of the γ-zein, β-zein, and δ-zein in the leaves of transgenic plants. Meanwhile, the α-zein protein does not accumulate to measurable levels in transgenic plants (Coleman *et al.*, 1996). When co-expressed with β- and γ-zein, the stability of α-zein increased (Coleman *et al.*, 1996, 2004). These results suggest that not only the hydrophobic nature of the prolamins, which is a common characteristic of prolamins, but also additional factors are involved in the ER-derived PB formation.

Retention mechanism of prolamin–GFP fusion proteins in the ER

Although the prolamins of rice and maize are deposited inside ER-derived PBs, they lack the C-terminal ER retention signal KDEL/HDEL. Other than the fact that both are rich in proline residues and overall hydrophobicity, there is a low degree of similarity between the rice prolamins and zeins. The expression of γ-zein in transgenic *Arabidopsis* and *Xenopus* oocytes and γ-gliadin in *Xenopus* oocytes has been employed to determine whether other retention signals exist (Altschuler *et al.*, 1993; Geli *et al.*, 1994; Torrent *et al.*, 1994). These studies indicated that an N-terminal region containing a tandem repeat of PPPVHL (γ-zein) and a tandem repeat of PQQPFPQ (γ-gliadin) is responsible for the localization of these prolamins within the ER. However, rice prolamins lack such tandem repeats.

Prolamin mRNA is transported to the PB-ER in the endosperm cells (Crofts *et al.*, 2004). Hamada *et al.* (2003) demonstrated that prolamin–GFP hybrid RNAs which lack the 3′-UTR are partially targeted to the PB-ER in the endosperm cells. The prolamin–GFP mRNA expressed in leaf and root cells may be segregated on the ER membrane in a similar manner. There is a possibility that the targeting of prolamin–GFP mRNA is responsible for concentration of prolamin–GFP proteins in the PB-like structures.

The role of disulphide bonds in the sorting of storage proteins in rice endosperm cells has been investigated. Glutelins are separated into two groups according to the number of cysteine residues: a high molecular weight and low molecular weight type (Sugimoto *et al.*, 1986). In endosperm of the *esp2* rice mutant, which lacks the protein disulphide isomerase, glutelin precursors were deposited with prolamin polypeptides in the ER (Takemoto *et al.*, 2002). α-Globulin is a monomeric storage protein and forms intramolecular disulphide bonds (Kawagoe *et al.*, 2005). The disulphide bonds formed at the dicysteine residues in the CCxQL motif of α-globulin play a critical role in protein sorting in rice endosperm (Kawagoe *et al.*, 2005). These results suggest that it is important to form the appropriate disulphide bonds for the accumulation of each rice storage protein in the appropriate location. In this study, it was shown that the prolamin–GFP fusion proteins expressed in the seeds and leaves required 2-ME to be completely solubilized (Fig. 9). This suggests that prolamin–GFP fusion proteins form the intermolecular and/or intramolecular disulphide bonds in PB-like structures. Mitsukawa *et al.* (1999) indicated that prolamins are polymerized in

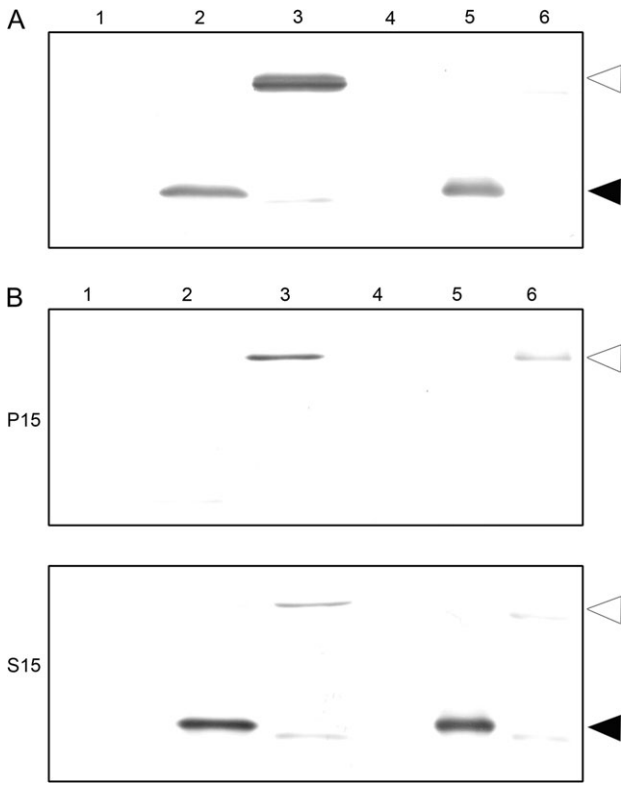

Fig. 9. Influence of 2-ME on prolamin–GFP fusion protein solubilization. In the experiment shown in A, proteins from seeds of WT (lanes 1 and 4), 35S:GFP (lanes 2 and 5), and 35S:Pro-GFP (lanes 3 and 6) plants were extracted with 5% (v/v) 2-ME (lanes 1–3) or without 2-ME (lanes 4–6). In B, homogenates from leaves of WT (lanes 1 and 4), 35S:GFP (lanes 2 and 5), and 35S:Pro-GFP (lanes 3 and 6) plants were subjected to centrifugation to obtain the 15 000 *g* pellet (P15) and the 15 000 *g* supernatant (S15). Proteins in each fraction were extracted with 5% (v/v) 2-ME (lanes 1–3) or without 2-ME (lanes 4–6). The proteins were separated by SDS–PAGE and immunoblotted with anti-GFP antibodies. The upper bands (open arrowheads) and lower bands (filled arrowheads) correspond to prolamin–GFP and GFP, respectively.

PB-Is by intermolecular disulphide bonds. Kawagoe *et al.* (2005) suggested that Cys135 engages in the polymerization of the epitope-tagged prolamin (λRM1) *in vitro*. It is possible that rice prolamins form intermolecular bonds and are polymerized in the ER.

BiP is a major chaperone of the ER (Vitale and Denecke, 1999). BiP transiently binds many newly synthesized secretory proteins and probably prevents unspecific aggregation by interacting with regions rich in hydrophobic amino acids. However, the interaction of BiP and prolamins is peculiar. BiP interacts with rice prolamins during their co-translational translocation into the ER lumen and has been detected at the periphery of rice PBs (Li *et al.*, 1993; Muench *et al.*, 1997). In 35S:Pro-GFP leaves and roots in the present study, BiP was localized in PB-like structures (Fig. 8B, C). The results support the hypothesis that BiP retains prolamins in the ER lumen by facilitating their folding and assembly into PB-Is in rice endosperm (Li *et al.*, 1993; Muench *et al.*, 1997).

In conclusion, the results show that the addition of a single prolamin polypeptide to GFP causes the formation of PB-like structures. The findings bear out the importance of the assembly of prolamin molecules and the interaction of prolamin with BiP in the formation of ER-derived PBs.

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