A Novel Vesicle Derived Directly from Endoplasmic Reticulum is Involved in the Transport of Vacuolar Storage Proteins in Rice Endosperm

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We found novel vesicles derived from rough endoplasmic reticulum (ER) in rice endosperm. The novel vesicles had characteristic structures different from that of the ER-derived protein body type I and the Golgi-derived dense vesicles. Immunocytochemical analysis revealed that the novel vesicles are derived directly from the aggregates of vacuolar storage proteins in the rough ER. In addition, BiP, an ER-resident molecular chaperone, was localized in the novel vesicles, but also in protein storage vacuoles (PSVs). These results suggest that the novel vesicles mediate transport of vacuolar storage proteins directly from the ER to PSVs in rice endosperm.

Keywords: Endoplasmic reticulum — Endosperm — Protein body — Rice — Seed storage protein — Vesicle transport

Abbreviations: C-ER, cisternal endoplasmic reticulum; DAF, days after flowering; ER, endoplasmic reticulum; PAC vesicle, precursor-accumulating vesicle; PB, protein body; PB-ER, protein body endoplasmic reticulum; PSV, protein storage vacuole.

Plant seeds accumulate storage proteins as a source of amino acids for use during germination and the subsequent growth of seedlings. Storage proteins are protected against uncontrolled premature degradation by membrane-bound organelles called protein bodies (PBs). Most storage proteins, including globulins and some prolamins, have been shown to be transported to protein storage vacuoles (PSVs) via the Golgi apparatus (Chrispeels 1985, Shotwell and Larkins 1988). Chrispeels (1983) reported the presence of electron-dense vesicles close to the Golgi stacks in maturing bean cotyledons. Hohl et al. (1996) demonstrated immunocytochemically in maturing pea cotyledons that electron-dense vesicles with a diameter of ~100 nm associated with any cis, medial and trans cisternae of the Golgi apparatus contain storage proteins. These results suggest that the storage proteins are transported to PSVs via the Golgi-derived dense vesicles.

There are two morphologically distinct PBs found in rice endosperm (Tanaka et al. 1980, Yamagata et al. 1982a). Rice glutelin is stored in protein body type II (PB-II), which is a uniformly electron-dense structure 2–3 µm in diameter. Rice prolamin is deposited in protein body type I (PB-I), which is a spherical proteinaceous particle of lamellar structure 1–2 µm in diameter. The biosynthesis of rice glutelins and prolamins has been studied in detail by several researchers (Yamagata et al. 1982a, Luthe 1983, Wen and Luthe 1985, Krishnan and Okita 1986, Li and Okita 1993). The glutelins are first synthesized as pre-proglutelins (~59 kDa) by endoplasmic reticulum (ER)-bounded polysomes. The pre-proglutelins have N-terminal signal peptides that are responsible for co-translational transport from the cytoplasmic side of the ER into the ER lumen. The signal peptides are co-translationally detached, and the pre-proglutelins are processed into proglutelins (~57 kDa). The proglutelins are then transported via the Golgi apparatus to the vacuole (Krishnan et al. 1986), and are proteolytically processed into acidic subunits of 37–39 kDa and basic subunits of 22–23 kDa (Yamagata et al. 1982a, Yamagata et al. 1982b, Luthe 1983). The glutelin subunits assemble into polymers through intermolecular disulfide bonds (Sugimoto et al. 1986), and the vacuoles, along with their stored glutelin subunits, are finally transformed into PB-II (Tanaka et al. 1980, Yamagata et al. 1982a). The prolamins are also synthesized as precursors with N-terminal signal peptides by ER-bound polysomes. The signal peptides are co-translationally detached, and the prolamins accumulate directly within the ER. The distensions of the ER are finally transformed into PB-I (Yamagata et al. 1982a, Krishnan et al. 1986).

On the other hand, a Golgi-independent transport pathway for storage proteins has also been reported. Levanony et al. (1992) reported that wheat prolamins are transported to the PSVs without any contribution of the Golgi apparatus. The precursor-accumulating (PAC) vesicles of the maturing pumpkin seeds mediate the direct transport of the precursors of storage proteins from the ER into PSVs (Hara-Nishimura et al. 1998). The PAC vesicles have diameters of 200–400 nm and contain an electron-dense core of storage proteins surrounded by an electron-translucent layer; some vesicles also contain small...
vesicle-like structures. The protein cores are derived from numerous aggregates of storage proteins formed within the rough ER. The PAC vesicles have been proposed as a Golgi-independent pathway for the transport of insoluble aggregates of storage proteins directly to PSVs (Hara-Nishimura et al. 1998).

Calreticulin, which contains an ER-resident signal at the C-terminus, is taken back into the ER, when it escapes to the Golgi apparatus (Pelham 1990, Vitale and Denecke 1999). Torres et al. (2001) reported that the ER-resident molecular chaperone accumulated not only in PB-I, but also in PB-II. This result suggests that there is a Golgi-independent pathway to PB-II in rice endosperm, although glutelins have been thought to pass through the Golgi apparatus en route to PB-II. However, there has been no direct evidence for an alternative pathway for glutelin transport in rice endosperm.

PAC vesicles increase from the middle to late stages of pumpkin seed maturation (Hara-Nishimura et al. 1998). Thus, we performed an ultrastructural analysis of rice endosperm at the middle stage of seed maturation. Electron microscopy of rice endosperm cells at 15 days after flowering (DAF) showed numerous vesicles distinct from the Golgi-derived dense vesicles (Krishnan et al. 1986, Krishnan et al. 1992), as indicated by the asterisks in Fig. 1A. With diameters of ~1 µm, these vesicles are much larger than the Golgi-derived dense vesicles, although they have a density similar to that of PB-II (Fig. 1A). They also have a characteristic structure, i.e. an electron-dense core surrounded by an electron-translucent layer (Fig. 1B). In addition, numerous small vesicle-like structures were observed within the electron-translucent layer (Fig. 1B). The novel vesicles were frequently observed close to the rough ER, and were surrounded by ribosomes (Fig. 1B). This finding strongly suggests that the novel vesicles are directly derived from rough ER. In rice, prolamins aggregates that are formed within the ER are converted directly to PB-I (Yamagata et al. 1982a, Krishnan et al. 1986). However, the novel vesicles described here had characteristic structures different from that of PB-I (Fig. 1A). These characteristics of the newly observed vesicles in rice endosperm cells were consistent with those of PAC vesicles of maturing pumpkin seeds (Hara-Nishimura et al. 1998).

The PAC vesicles of maturing pumpkin seeds accumulate storage proteins in their electron-dense cores (Hara-Nishimura et al. 1998). To examine whether the PAC-like vesicles in rice endosperm cells accumulate storage proteins, we performed an immunocytochemical analysis using antibodies raised against three rice storage proteins: prolamin, glutelin and α-globulin. The prolamins were localized in the PB-I, but not in the PAC-like vesicles (Fig. 2A). In contrast, the glutelins and α-globulins were localized in the electron-dense cores of the PAC-like vesicles and PB-II (Fig. 2B, C). These results indicate that the major vacuolar storage proteins are accumulated in the electron-dense cores of the PAC-like vesicles. This raised the question of whether each PAC-like vesicle contains both glutelins and α-globulins or are there PAC-like vesicles that are specific to each storage protein. To answer this question, we performed double immunogold labeling using antibodies raised against glutelin (10 nm gold) and α-globulin (5 nm gold). Double immunogold labeling resulted in gold particles of both 5 and 10 nm in the electron-dense area of each PAC-like vesicle, as shown in Fig. 2D. This result suggests that each PAC-like vesicle accumulates both of the two major vacuolar storage proteins in rice endosperm.

The PAC-like vesicles in rice endosperm cells were frequently observed close to the rough ER, and were surrounded by ribosomes (Fig. 1B). To confirm that the PAC-like vesicles are derived directly from ER, we performed an immunocytochemical analysis using antibodies raised against pumpkin BiP, an ER-resident molecular chaperone. If BiP were detected in the vesicles, it would suggest that BiP is trapped in the protein aggregates of ER and then transported to the vesicles, as discussed by Levanony et al. (1992). The anti-pumpkin BiP antibodies were localized in the peripheral region of PB-I (Fig. 3). This localization is coincident with the rice BiP localization previously reported (Muench et al. 1997). BiP was also localized in the electron-dense cores of the PAC-like vesicles in rice endosperm cells (Fig. 3A). This result supports the notion that the electron-dense cores of the PAC-like vesicles are derived directly from the aggregates in the ER. In addition, the gold particles were observed in the peripheral region of the vesicle-
A novel protein transport vesicle in rice seeds

like structures in the PAC-like vesicles (Fig. 3A, arrow). This localization is similar to the BiP localization in PB-I. This result suggests the possibility that the vesicle-like structures in the PAC-like vesicles are derived from unknown protein aggregates in the ER.

BiP belongs to a group of ER-resident proteins containing a specific C-terminal tetra-amino acid signal that prevents its transport from the ER via the Golgi apparatus (Munro and Pelham 1987, Pelham 1990). Therefore, BiP is not expected to be present in protein storage vacuoles if it is routed to vacuoles via the Golgi apparatus (Kim et al. 1988). However, immunocytochemical labeling of rice endosperm cells at 15 DAF with the anti-BiP antibodies also resulted in labeling of PB-II with gold particles (Fig. 3B, arrow). These results suggest that BiP is trapped in the electron-dense cores of the PAC-like vesicles and is transported to PB-II in rice endosperm cells, by-passing the Golgi apparatus.

Previously, ultrastructural studies have shown that BiP can also be found in PSVs of developing wheat endosperm (Levanony et al. 1992), maturing pea (Robinson et al. 1995) and pumpkin (Hara-Nishimura et al. 1998) seeds. Wheat prolamins and pea and pumpkin globulins are known to pass through the Golgi apparatus, but the presence of BiP in the resulting PSVs has led to suggestions that there may be an
alternative pathway by-passing the Golgi apparatus. Torres et al. (2001) have reported that calreticulin, another ER-resident molecular chaperone, accumulated in ER-derived prolamin protein bodies, but also in glutelin protein storage vacuoles, even though glutelins are known to pass through the Golgi apparatus en route to the glutelin storage sites in rice endosperm. Despite these reports, there has been no direct evidence of an alternative pathway for glutelin transport in rice endosperm, as described for pea (Robinson et al. 1995) and pumpkin (Hara-Nishimura et al. 1998) seeds. We proposed that the PAC-like vesicles may mediate an alternative pathway by-passing the Golgi in transport of glutelin to rice endosperm.

The PAC-like vesicles might have been derived directly from the ER, similar to the way PB-I is derived in rice endosperm cells. However, PB-I and the PAC-like vesicles contain different types of storage proteins; PB-I contains prolamins and the PAC-like vesicles contain glutelins and α-globulins. To confirm whether the PAC-like vesicles and PB-I are formed within a continuous ER or there are discrete parts of the ER that are specific to the PAC-like vesicles and PB-I, we performed an ultrastructural analysis of rice endosperm cells at the middle stage of seed maturation. Electron microscopy of the rice endosperm cells at 15 DAF showed a direct continuity of the ER membrane between the PAC-like vesicles and PB-I (Fig. 4, arrowheads). Immunocytochemical analysis with α-globulin antibodies demonstrated that α-globulin was localized in the electron-dense core of the PAC-like vesicle, but not in the PB-I (Fig. 4). However, BiP was localized in both the PAC-like vesicles and PB-I, as shown in Fig. 3A. These results suggest that the storage proteins are sorted into separate compartments within the continuous ER.

Developing rice endosperm cells display two distinct ERs, cisternal ER (C-ER) and protein body ER (PB-ER), the latter delimiting the prolamine PBs. It has been demonstrated that prolamin transcripts are localized on the developing PB-ER, while glutelin transcripts predominate on the C-ER (Li et al. 1993, Choi et al. 2000). These results indicate that the ER may be composed of subdomains that specialize in the synthesis of proteins directed to different compartments of the plant endomembrane system. The identification of novel vesicles derived from the ER in rice endosperm cells is an interesting finding that could add to our understanding of the functional ER subdomain.

Rice (Oryza sativa L. cv Nipponbare) was grown with soil in a greenhouse at the experimental farm of the Kyoto Prefectural Institute of Agricultural Biotechnology. Rice seeds freshly harvested at 15 DAF were used as middle stage samples for the experiments.

To prepare polyclonal antibodies against rice prolamin, we prepared the PB-I fraction from matured rice seeds, and then extracted prolamin polypeptides from the PB-I fraction by previously described methods (Mitsukawa et al. 1999). The rabbit polyclonal antibodies against the prolamin polypeptides were produced by Takara (Otsu, Japan).

To prepare polyclonal antibodies against rice glutelin, we extracted total proteins from matured rice seeds in an extraction buffer [62.5 mM Tris–HCl, pH 7.0, 4 M urea, 2% (w/v) SDS and 5% (w/v) β-mercaptoethanol]. The total proteins were subjected to SDS–PAGE with subsequent staining with Coomassie brilliant blue R-250. The bands corresponding to the 23 kDa glutelin basic subunits were cut from the gel and subjected to electroelution. The rabbit polyclonal antibodies against the 23 kDa glutelin basic subunits were produced by QIAGEN (Hilden, Germany).

Mouse polyclonal antibodies against rice α-globulin (Nakase et al. 1996) were kindly provided by Dr. T. Matsuda of Nagoya University. Rabbit polyclonal antibodies against pumpkin BiP (Hatano et al. 1997) were kindly provided by Dr. I. Hara-Nishimura of Kyoto University.

For immunocytochemical analysis, developing rice seeds were vacuum infiltrated for 10 min with a fixative that consisted of 4% (w/v) paraformaldehyde in 0.1 M 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 cut into slices. The samples were then embedded in LR White resin (London Resin Co., Ltd, Hampshire, U.K.). Blocks were polymerized at 55°C for 48 h. Ultrathin sections were cut with a diamond knife using a LEICA ULTRACUT UCT (Leica, Heidelberg, Germany) and mounted on nickel grids.

The sections were treated with blocking solution of 1% bovine serum albumin in 0.1 M phosphate buffer (pH 7.2) for

Fig. 4 Immunoelectron microscopy of rice endosperm at 15 DAF with α-globulin antibodies. The immunoelectron microscopy shows that the PAC-like vesicle labeled with the gold particles for α-globulin was on the continuous ER membrane of PB-I (arrowheads). The asterisk indicates the PAC-like vesicle in rice endosperm. PB-I, protein body type I.
30 min at room temperature. The sections were then incubated with antibodies raised against prolamin (diluted 1:20,000), glutelin (1:20,000) and α-globulin (1:5,000) in the blocking solution for 1 h at room temperature, and BiP (1:100) in the blocking solution overnight at 4°C. After washing with 0.1 M phosphate buffer (pH 7.2), the sections were incubated with a solution of gold-labeled goat anti-rabbit or mouse IgG antibodies (5 or 10 nm; Amersham Biosciences, Buckinghamshire, U.K.) diluted 1:50 in the blocking solution for 1 h at room temperature. The sections were washed with distilled water and then stained with 2% uranyl acetate. After staining, all sections were examined with a transmission electron microscope (JEM-1220; JEM, Tokyo, Japan) at 100 kV.

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