Aggregation of proteins having Golgi apparatus sorting determinant induces large globular structures derived from the endoplasmic reticulum in plant seed cells

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Abstract Endoplasmic reticulum (ER)-derived compartments are found in many plant species. Although it has been assumed that aggregation induces formation of the ER-derived compartments in plant seed cells, the effect of aggregation on the trafficking from the ER to the Golgi has not yet been elucidated. In this study, we used an aggregated type of red fluorescent protein (DsRED) to investigate the effect of aggregation on sorting in seed cells. DsRED fused to the Golgi sorting determinant was found mainly in large globular structures derived from the ER where ER-resident proteins were excluded. These results indicate that aggregation of the Golgi protein blocks transport from the ER to the Golgi.

Keywords: Aggregation; ER-derived compartment; Seed

1. Introduction

Many plants generate storage compartments derived from the endoplasmic reticulum (ER) to accumulate a large amount of protein [1]. An ER-derived compartment is observed as spindle-shaped bodies in Arabidopsis cotyledon, hypocotyl and root cells that express an ER-targeted green fluorescent protein (GFP) [2]. Another form of ER-derived compartment, precursors accumulating (PAC) vesicles, is found in maturing seeds of pumpkin (Cucurbita maxima), castor bean (Ricinus communis) [3], soybean (Glycine max) [4] and rice (Oryza sativa) [5]. PAC vesicles contain the precursor forms of seed storage proteins. It is assumed that the aggregates in the ER are related to a formation of the ER-derived compartments. However, the effect of the aggregation on the trafficking from the ER to the Golgi apparatus has not been elucidated.

Plant cells contain multiple vacuoles, some of which have storage or digestive function [6]. Maturing seed cells possess protein storage vacuoles containing seed storage proteins [7]. Previously, we developed a transient expression system using maturating soybean seeds in order to characterize sorting determinants required for deposition of these storage proteins [8–11]. This transient expression system is useful for examining the effects of the co-expression of dominant negative mutants of small GTPases and various drugs on the sorting to the vacuole and the Golgi apparatus in seed cells [8]. SAR1 and ARF1, small GTPases, participate in the formation of COPI and COPII-coated vesicles, respectively [12,13]. Co-expression of small GTPases sar1 or arf1 dominant negative mutant inhibits the vacuolar sorting of a reporter protein on maturing soybean seed cells, indicating that COPI and COPII-coated vesicles play an important role on a membrane traffic in plant seed cells [8]. Brefeldin A (BFA) inhibits the guanine nucleotide exchange of ARF1 [14,15]. In the presence of BFA, Golgi proteins redistribute into the ER, resulting in that ER–Golgi apparatus hybrid compartments are formed on maturing soybean seed cells [8].

Recently, various fluorescent proteins have been used as a reporter in living cells. The tetrameric Discosoma sp. red fluorescent protein (DsRED) exhibits bright red fluorescence, but it forms aggregates in cells [16]. Monomeric red fluorescent protein (mRFP) was developed, because an aggregation of DsRed have hindered its use as a genetically encoded fusion tag [17]. mRFP fusion proteins have been used in plant cells as various organelle markers [8,18–20].

Here, we used DsRED and mRFP to investigate an effect of aggregation of the Golgi apparatus protein on the budding from the ER and the sorting to the Golgi apparatus in seed cells. When we transiently expressed DsRED fused to a Golgi apparatus sorting determinant in maturing soybean seed cells, the fusion proteins accumulated in large globular structures. We suggest, by expression of dominant negative mutants of small GTPases and drug treatment experiments, that an aggregation of the Golgi apparatus protein acts as a trigger for a formation of ER-derived compartments and ER-resident proteins were excluded from the ER-derived structures during their formation.

2. Materials and methods

2.1. Constructs

Standard molecular techniques were used as described by Sambrook et al. [21]. Schemes of constructs used in this study are shown in Fig. 1. Expression plasmids for GFP:CT, GFP:HDEL, Xyl::YFP and Xyl::mRFP have been described previously [8,9]. To construct expression plasmids for Xyl::DsRED, a coding region of DsRED in the pDsRED-Express-1 plasmid (Clontech) was used. To generate the

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Abbreviations: BFA, brefeldin A; DsRED, Discosoma sp. red fluorescent protein; ER, endoplasmic reticulum; GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; PAC vesicle, precursor accumulating vesicle; YFP, yellow fluorescent protein

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expression plasmids for Mut-Xyl:DsRED and Mut-Xyl:mRFP, we performed site-directed mutagenesis by PCR. Expression plasmids for the truncated Xyl:DsRED and Xyl:mRFP mutants (Xyl(TM):DsRED and Xyl(TM):mRFP) which contain Xyl:DsRED and Xyl:mRFP coding regions without the cytosolic region of β1,2-xylosyltransferase were also created by PCR. DNAs for BiP, PDI and Sec61 were selected from Arabidopsis mRNA from the RIKEN Bioresource Center (Identification codes; pda12219 for BiP, pda00289 for PDI and pda08639 for Sec61) were used for constructing the expression plasmids for GFP:BiP, GFP:PDI and GFP:Sec61.

To construct the plant binary vector encoding Xyl:DsRED for Arabidopsis transformation, the DNA fragment consisting of seed-specific β-conglycinin α' promoter and Xyl:DsRED coding region was inserted between BamHI and KpnI sites of pBI101 (Clontech), whose SacI site had been replaced by a KpnI site by means of inserting a KpnI linker (TaKaRa BIO).

We previously indicated that either mRFP or yellow fluorescent protein (YFP) fused to a transmembrane domain and a cytosolic region of Arabidopsis β1,2-xylosyltransferase (Xyl:mRFP or Xyl:YFP) is sorted to the Golgi apparatus on maturing soybean seed cells [8]. DsRED fused to the transmembrane domain and the cytosolic region of β1,2-xylosyltransferase (Xyl:DsRED) was used for examining effects of an aggregation of the Golgi apparatus protein on the sorting in seed cells (Fig. 2). Xyl:DsRED mainly exhibited a large globular pattern in maturing soybean seed cells and did not overlap with GFP:CT (GFP fused to C-terminal 10 amino acids of soybean seed storage protein β-conglycinin α' subunit; protein storage vacuole marker) and Xyl:YFP (Golgi apparatus marker) (Fig. 2A and B). GFP:HEDEL (GFP fused to a peptide His-Asp-Glu-Leu; ER marker) seemed to localize partially with Xyl:DsRED, but was hardly observed inside globular structures of Xyl:DsRED (Fig. 2C). Although a small part of BiP or PDI, both soluble ER proteins, fused to GFP (GFP:BiP and GFP:PDI) colocalized with globular structures of Xyl:DsRED, they were not detected inside the globular structures similarly to GFP:HEDEL (Fig. 2D and E). Further, we examined colocalization of Sec61, a component of the ER translocation channel, fused to GFP (GFP:Sec61) with Xyl:DsRED (Fig. 2F). Sec61 has a transmembrane domain inserted into the ER membrane [23]. GFP:Sec61 showed reticular-like structures with globular structures overlapped with Xyl:DsRED. These results suggest that a delivery of Xyl:DsRED to the Golgi apparatus is defective, and distinct from those of Xyl:YFP and Xyl:mRFP.

3. Results

3.1. DsRED fused to β1,2-xylosyltransferase is not sorted to the Golgi apparatus

We previously indicated that either mRFP or yellow fluorescent protein (YFP) fused to a transmembrane domain and a cytosolic region of Arabidopsis β1,2-xylosyltransferase (Xyl:mRFP or Xyl:YFP) is sorted to the Golgi apparatus on maturing soybean seed cells [8]. DsRED fused to the transmembrane domain and the cytosolic region of β1,2-xylosyltransferase (Xyl:DsRED) was used for examining effects of an aggregation of the Golgi apparatus protein on the sorting in seed cells (Fig. 2). Xyl:DsRED mainly exhibited a large globular pattern in maturing soybean seed cells and did not overlap with GFP:CT (GFP fused to C-terminal 10 amino acids of soybean seed storage protein β-conglycinin α' subunit; protein storage vacuole marker) and Xyl:YFP (Golgi apparatus marker) (Fig. 2A and B). GFP:HEDEL (GFP fused to a peptide His-Asp-Glu-Leu; ER marker) seemed to localize partially with Xyl:DsRED, but was hardly observed inside globular structures of Xyl:DsRED (Fig. 2C). Although a small part of BiP or PDI, both soluble ER proteins, fused to GFP (GFP:BiP and GFP:PDI) colocalized with globular structures of Xyl:DsRED, they were not detected inside the globular structures similarly to GFP:HEDEL (Fig. 2D and E). Further, we examined colocalization of Sec61, a component of the ER translocation channel, fused to GFP (GFP:Sec61) with Xyl:DsRED (Fig. 2F). Sec61 has a transmembrane domain inserted into the ER membrane [23]. GFP:Sec61 showed reticular-like structures with globular structures overlapped with Xyl:DsRED. These results suggest that a delivery of Xyl:DsRED to the Golgi apparatus is defective, and distinct from those of Xyl:YFP and Xyl:mRFP.

3.2. Xyl:DsRED accumulates in large globular structures in the presence of BFA

We examined an effect of BFA, which has been widely used as a reversible inhibitor of vesicle trafficking in yeast, mammalian and plant cells [14,15]. Previously, we showed that Xyl:mRFP, the Golgi apparatus marker, resides in an ER–Golgi hybrid in the presence of BFA in maturing seed cells [8]. When Xyl:DsRed was expressed in the presence of BFA, the characteristic localization to large globular structures was preserved.
These large globular structures of Xyl:DsRED overlapped with the ER–Golgi hybrids of a Xyl:YFP (Fig. 3A). Conversely, in the presence of BFA, Xyl:DsRed did not colocalize with a globular domain of GFP:CT (Fig. 3B). Previously, we reported that the vacuolar sorting of the reporter protein is inhibited by the presence of BFA and that the repor-
ter protein for the protein storage vacuole condenses into globular structures within the ER–Golgi hybrid structures together with ER residents [8]. The results obtained here together with the previous report suggest that GFP:CT is condensed into

Fig. 3. Effect of brefeldin A on localization of Xyl:DsRED. Xyl:DsRED was co-expressed with Xyl:YFP (Golgi marker) (A) or GFP:CT (protein storage vacuole marker) (B). After introducing expression plasmids, maturing seed cells were incubated on the medium containing brefeldin A. A boundary between cells is shown by dotted lines. Bars, 10 μm.

Fig. 4. Effect of co-expression of dominant negative mutant of SAR1 or ARF1 on large globular structure formation of Xyl:DsRED. Xyl:mRFP or Xyl:DsRED was co-expressed with sar1 dominant negative mutant (sar1[H74L]) (Xyl:mRFP, A; Xyl:DsRED, C) or arf1 dominant negative mutant (arf1[Q71L]) (Xyl:mRFP, B; Xyl:DsRED, D) in maturing seed cells. A boundary between cells is shown by dotted lines. Bars, 10 μm.
globular structures different from the protein storage vacuole observed in Fig. 2A and that Xyl:DsRED and GFP:CT exist separately within the ER–Golgi hybrid compartments.

3.3. Co-expression of sar1 or arf1 dominant negative mutant does not affect the formation of the large globular structure of Xyl:DsRED

Previously, we established a transient assay system for an examination of the effects of Arabidopsis sar1 and arf1 dominant negative mutants (Atsar1[H74L] and Atarf1[Q71L]) in soybean maturing seed cells [8]. Whether SAR1 and ARF1 are involved in sorting of Xyl:mRFP to the Golgi apparatus was examined. Whereas Xyl:mRFP alone shows the dotted pattern [8], Xyl:mRFP co-expressed with Atsar1[H74L] in maturing soybean seed cells exhibited a reticular pattern in all cells exhibiting red fluorescence (Fig. 4A). This indicates that the Golgi apparatus targeting of Xyl:mRFP is clearly inhibited. Co-expression of Atarf1[Q71L] also blocked the sorting of Xyl:mRFP to the Golgi apparatus (Fig. 4B). On the other hand, the co-expression of Atsar1[H74L] or Atarf1[Q71L] did not affect the distribution of Xyl:DsRED, the large globular structures (Fig. 4C and D). These results suggest that Xyl:DsRED is not transported to the Golgi apparatus.

Fig. 5. Role of cytosolic region of Xyl:RFP on large globular structure formation. Confocal images of maturing seed cells expressing mutated Xyl:mRFP or Xyl:DsRED in which the basic amino acids of MSKRNPLI of β1,2-xyllosyltransferase were replaced with non-charged hyrophilic residues (Mut-Xyl:mRFP, A; Mut-Xyl:DsRED, B). Confocal images of mRFP or DsRED fused to only transmembrane domain of β1,2-xyllosyltransferase are shown in (C) (Xyl(TM):mRFP) or (D) (Xyl(TM):DsRED). A boundary between cells is shown by dotted lines. Bars, 10 μm.
3.4. Deletion of a cytosolic region of β1,2-xylosyltransferase in Xyl:DsRED does not affect the formation of large globular structures

It has been reported that basic residues in the cytosolic regions of glycosyltransferase and prolyl 4-hydroxylase, type II membrane proteins, play an important role in the exit from the ER [24,25]. The sequence of a putative cytosolic region of Xyl:mRFP is MSKRNPKI. We expressed a mutated form of Xyl:mRFP (Mut-Xyl:mRFP) in which the basic amino acids of MSKRNPKI were replaced with non-charged hydrophilic residues (MSTTNPLT; Bold indicates the mutated residues). Mut-Xyl:mRFP showed mainly a punctate pattern (Fig. 5A), whereas the mutated Xyl:DsRED (Mut-Xyl:DsRED) showed large globular structures (Fig. 5B). These suggest that the basic amino acids of MSKRNPKI are not essential for the exit from the ER, because the reticular pattern of the ER was not observed within the cells expressing Mut-Xyl:mRFP. On the other hand, mRFP fused to the transmembrane domain of β1,2-xylosyltransferase (Xyl(TM):mRFP) did not show a punctate pattern and most of them was secreted to the plasma membrane and/or the intercellular space (Fig. 5C), whereas Xyl(TM):DsRED still showed the large globular patterns (Fig. 5D). Therefore, the cytosolic region of Xyl:mRFP plays an important role in sorting and retention to the Golgi apparatus, but it is not related to the large globular structure formation of Xyl:DsRED. Further, these results are consistent with our data in Figs. 2–4 suggesting that Xyl:DsRED is not transported to the Golgi apparatus.

3.5. Xyl:dsRED forms large globular structures in transgenic Arabidopsis seeds

To examine whether Xyl:DsRED accumulates in large globular structures in transgenic plants, we generated transgenic Arabidopsis plants expressing Xyl:DsRED under a control of a seed-specific promoter. We investigated both dry and maturing seeds by confocal microscopy. Globular structures of red fluorescence were detected in both dry and maturing seeds by confocal microscopy. Globular structures of red fluorescence were detected in both dry and maturing Arabidopsis seeds similarly to the transient expression experiments in maturing soybean seed cells (Fig. 6A–C). Therefore, we propose that Xyl:DsRED accumulates in globular structures during normal seed development.

4. Discussion

In this study, we report that tetrameric red fluorescent protein, DsRED, fused to a Golgi apparatus sorting determinant (Xyl:DsRED) accumulated in large globular structures in seed cells (Fig. 2). Even in the presence of BFA, which blocks transport between the ER and the Golgi apparatus, Xyl:DsRED was deposited in the large globular structures (Fig. 3). Further,
co-expression of sar1 and arfl dominant negative mutants did not affect the formation of the large globular structures (Fig. 4). Deletion of the cytosolic region, which plays an important role in the targeting to the Golgi apparatus, did not affect the formation of the large globular structures (Fig. 5). These results suggest that Xyl:DsRED is not transported to the Golgi apparatus and imply that these large globular structures are derived from the ER in seed cells (Fig. 7A). The ER contains functionally distinct subdomains. Cargo molecules to the Golgi apparatus are packaged into transport carriers, COPII vesicles, in the ER export site, one of functional subdomains in the ER [26–28]. In the presence of BFA, soluble proteins (GFP:CT and GFP:HDEL) accumulated in a globular domain exhibiting bright green fluorescence within the ER–Golgi hybrid (Figs. 3 and 7B [8]). The globular domain containing soluble proteins (GFP:CT and GFP:HDEL) is considered to be derived from an ER export site (Fig. 7B). Thus, Xyl:DsRED may accumulate in and bud from an ER subdomain distinct from the ER export sites. An export from the ER exit site might be blocked by the aggregation of Xyl:DsRED. Interestingly, soluble ER-resident proteins (GFP:HDEL, GFP:BiP and GFP:PDI) were hardly observed inside globular structures of Xyl:DsRED (Fig. 2C–E), whereas Sec61γ, the component of the ER translocation channel, fused to GFP (GFP:Sec61) showed reticular-like structures with globular structures overlapped with Xyl:DsRED (Fig. 2F). These results indicate that the globular structures of Xyl:DsRED are derived from the ER and that soluble ER-resident proteins are excluded from the globular structure of Xyl:DsRED during their formation. Further studies are required to determine the accumulation and budding mechanisms of Xyl:DsRED in the ER.

Previously, we examined maturing soybean seed by electron microscopy and showed that ER-derived compartments were scarcely observed in a normal variety of soybean [4]. Further, a comparison of electron microscopic data among many soybean varieties lacking specific seed proteins indicates that a large amount of seed storage proteins having a low solubility is necessary to form ER-derived compartment in soybean seeds [4]. We used seeds of the normal soybean variety in all experiments. Therefore, we consider that the globular structures were induced by an expression of Xyl:DsRED and did not contain seed storage proteins.

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