Quantitative Analysis of ER Body Morphology in an Arabidopsis Mutant

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Although fluorescence microscopy screening has proven useful in the identification of genes involved in plant organelle biogenesis and integrity, the quantitative and statistical study of the geometric phenotype is highly limited. This situation could generate unconscious bias in the understanding and presentation of a mutant phenotype. Therefore, we have developed an automated quantification system for green fluorescent protein (GFP) images, which enabled us to easily obtain quantitative data on ER bodies (an endoplasmic reticulum-derived organelle). We isolated an ER body morphology mutant of Arabidopsis thaliana, leb-1 (long ER body). The leb-1 mutant had significantly fewer and larger ER bodies than the wild-type. An amino acid substitution of Cys29 with tyrosine (C29Y) on PYK10, a major component protein of ER bodies, was found in leb-1. Non-reducing SDS–PAGE revealed that the electrophoretic mobility of PYK10 in the leb-1 mutant was clearly different from that in the wild type. This difference suggests that the C29Y amino acid substitution caused a tertiary structural change of the PYK10 protein. While the bglu21-1 and pyk10-1 single mutations slightly affected the number and morphology of the ER bodies, a bglu21-1 pyk10-1 double mutant had fewer and larger ER bodies than the wild type. The quantitative ER body phenotypes of leb-1 were similar to those of bglu21-1 pyk10-1 and bglu21-1 leb-1, suggesting that the leb-1 mutation allele acts dominantly to the BGLU21 wild-type allele. The leb-1 type PYK10 protein, which has an abnormal structure, may competitively inhibit interactions between the wild-type BGLU21/PYK10 protein and an unknown partner.

**Keywords:** Arabidopsis thaliana • β-glucosidase • ER body • Image quantification • PYK10.

**Abbreviations:** BGLU, β-glucosidase; EMS, ethyl methanesulfonate; ER, endoplasmic reticulum; GFP, green fluorescent protein; ROI, region of interest; RT–PCR, reverse transcription–PCR.

**Introduction**

Endoplasmic reticulum (ER), which is the most multitalented and adaptable compartment in plant cells (Staehelin 1997), exhibits a variety of shapes and movements within cells. The ER membrane surface is composed of an extensive network of tubules and cisternae, and occupies nearly half of the total membrane area in a cell. Plant cells can generate various types of compartments from the ER in response to the active synthesis of a distinct protein(s); these compartments range in size from 0.1 to 10 µm (Hara-Nishimura and Matsushima 2003).

Green fluorescent protein (GFP) allows real-time visualization of various organelles in living cells. GFP fusion proteins have provided new insights into novel subcellular compartments and their dynamic changes. For in vivo observations of the ER, GFP should be fused with a signal peptide at the N-terminus and with an ER retention signal ([H(K)DEL, Lys/His-Asp-Glu-Leu]) at the C-terminus. When the ER-targeted GFP is expressed in Arabidopsis, numerous bright fluorescent spindle-shaped organelles (~1 µm diameter × ~10 µm long) are conspicuous against the background of a green ER network in cotyledon epidermal cells (Haseloff et al. 1997,
Kohler 1998, Ridge et al. 1999). These organelles are referred to as proplastids in the widely used textbook ‘Essential Cell Biology’ (Alberts et al. 1998) and were described as ‘mystery organelles’ by Gunning (1998). In 2001, immunogold analysis with anti-GFP antibodies (Hayashi et al. 2001) showed that the unidentified GFP-fluorescing organelles correspond to the dilated cisternae of the ER, which were first observed in radish root cells by electron microscopy in 1965 (Bonnett and Newcomb 1965). This indicated that the GFP-fluorescing organelles are derived from the ER, and led to their designation as ER bodies (Hayashi et al. 2001).

ER bodies are observed in seedlings and mature roots (Matsushima et al. 2002). Rosette leaves have few ER bodies under normal conditions. However, wounding rosette leaves or treating them with exogenous methyl jasmonate (a plant hormone that induces a defense response) induces many spindle-shaped structures that resemble ER bodies. These observations suggest that ER bodies may mediate plant defenses against herbivores and/or pathogens. In other Brassicaceae plants, structures like ER bodies were described as dilated cisternae (Bonnett and Newcomb 1965, Iversen 1970, Behnke and Eschlebeck 1978, Bones et al. 1989). ER bodies in roots accumulate copious amounts of the β-glucosidase PYK10/BGLU23 (Matsushima et al. 2003, Matsushima et al. 2004, Xu et al. 2004). Glycoside hydrolase family 1, β-glucosidase (BGLU), is widely distributed in prokaryotes and eukaryotes (Xu et al. 2004), and one of its major roles in plants is chemical defense (Rask et al. 2000, Lipka et al. 2005). Forty-seven BGLUs have been reported in Arabidopsis thaliana (Xu et al. 2004).

We previously isolated an Arabidopsis mutant that lacks ER bodies and designated it as nai1 (nai means ‘nothing’ in Japanese). The NAI1/At2g22770 gene encodes a basic helix-loop-helix-type transcription factor (Matsushima et al. 2004). NAI1 regulates the expression of NAI2, PYK10, JAL22/At2g39310, JAL23/At2g39330, JAL31/At3g16430, JAL33/At3g16450, PBP1/JAL30/At3g16420, GLL23/At1g54010 and GLL25/At1g54030 (Nagano et al. 2008). PBP1 localizes to the cytosol, while PYK10 localizes to the ER body. Recently, we found that PYK10 forms a large complex with JALs and GLLs in disrupted cells. JALs and GLLs regulate the size of the PYK10 complex and may regulate its substrate specificity. Another mutant nai2 also has no ability to form ER bodies (Yamada et al. 2008). NAI2 may regulate unknown factors that are responsible for ER body formation. These results suggest that ER body formation is regulated by several factors.

Identifying the molecular mechanisms underlying organelles biogenesis should provide new insights into their physiological functions and membrane dynamics. Microscopy screening has proven useful in the identification of genes involved in plant organelle biogenesis and integrity (Avila et al. 2003, Tamura et al. 2005, Tamura et al. 2007, Boulaflous et al. 2008). However, the quantitative and statistical evaluation of the geometric phenotype is less common, in part because the quantitative approach is not essential for isolating mutants.

A quantitative analysis is necessary to avoid unconscious bias in the understanding and presentation of a mutant phenotype. Recently, several user-friendly image analysis software programs have become freely available (Hamilton 2009). Computer-assisted quantification will not only help remove bias, but will also reduce the burden on the researcher. In contrast to analyzing particulate organelles, ERs with amorphous shapes and/or complex structures are rarely analyzed. In this study, we show a quantitative analysis of an ER body morphology mutant as a model case.

Results

Characterization of a long ER body mutant (leb-1) by an image quantification system

We isolated an ER body morphology mutant, leb-1 (long ER body), by manual fluorescent microscopy screening. The mutant had fewer and larger ER bodies than those of the wild type (Fig. 2A). To characterize the phenotypes quantitatively, we developed and used an automated system (Fig. 1) that quantified various ER body characteristics (e.g. number, area, length and signal intensity) from fluorescence microscopic images. In quantifying the ER body morphology,
we first intended to discard the stomatal region from the raw images because the guard cell edges had strong GFP signals and become an impediment for quantification. To perform this pre-process automatically, we trained the classifier to recognize the stomatal region by the machine learning method. We used random forests (Breiman 2001). As training data, we manually segmented 147 stomata-containing regions of interest (ROIs) and 346 epidermal cell-containing ROIs from 347 stack images (512 × 512 pixels). Using 186 features, including statistical geometric features (SGFs) (Chen et al. 1995), we finally obtained a classifier for stomata recognition. The classifier, combined with random ROI segmentation, effectively discarded the stomatal region (accuracy by cross-validation: 98.4%). All ROI sizes were fixed at 100 × 100 square pixels.

The pre-processed images were used to quantify the ER body morphology. We treated objects with strong GFP signals as ER bodies. Morphological parameters (area, major length, maximum signal intensity, etc.) of each ER body in the images were measured. Because GFP signals derived from the ER network are detected as small particles with weak signal intensities, we ignored objects whose areas were <1 µm² or whose maximum intensities were <1,500.

The results of quantification were summarized in Table 1. The leb-1 mutant was statistically analyzed by using Welch’s t-test (an adaptation of the Student’s t-test intended for use with two samples having possibly unequal variances) and Mann–Whitney U-test (a non-parametric test for assessing whether two independent samples of observations come from the same distribution). The leb-1 mutant had significantly smaller numbers of ER bodies than the wild type (Fig. 2B, Welch’s t-test: $P < 1.34 \times 10^{-7}$), and the mean area of an ER body in the leb-1 mutant was significantly larger than that in the wild type (Fig. 2B, Welch’s t-test: $P < 2.46 \times 10^{-7}$). The proportion of large ER bodies was increased in the leb-1 mutant (Fig. 2C, Mann–Whitney U-test: $P < 2.2 \times 10^{-16}$).

A single amino acid change of PYK10 changes the ER body morphology

To identify causal mutation(s) of the leb-1 mutant, we performed map-based cloning (Fig. 3A). A leb-1 mutant (Col-0 background) was crossed with a wild-type Ler. From the F₂ population, 233 homozygous plants were identified and subsequently scored by a series of genetic markers. Our initial mapping of the leb-1 mutation located it in the long arm of chromosome 3. For fine mapping, we developed 10 molecular markers, CRE455843, CRE464712, CRE469714, CRE469740, CRE470642, CRE441692, CRE441693, CRE470258, CRE475546 and CRE464937. Analysis of 466 chromosomes revealed that the leb-1 mutation was located in the 196 kb region between CRE441693 and CRE475546 (Fig. 3A). The PYK10 locus and 68 other loci (from At3g09140 to At3g09770) are located in this region.

Because PYK10 is a major component of the ER body, it is a strong causal gene candidate. Comparing the genomic sequences of the PYK10 gene of wild-type Col-0 and the leb-1 mutant revealed a single G to A transition in the first exon of the PYK10 gene (Fig. 3B). This G to A transition is consistent with the mode of action of ethyl methanesulfonate (EMS), the mutagen that was used to generate the mutagenized population from which the leb-1 mutant was isolated. This mutation was expected to cause a single substitution of cysteine into tyrosine in the 29th amino acid position.

The leb-1 mutation alters the amount and structure of the PYK10 protein

To reveal how PYK10 proteins are changed by the leb-1 mutation, an immunoblot analysis with anti-PYK10 antibodies was performed using leb-1 and Col-0 root extracts. The amounts of the PYK10 protein were decreased in the leb-1 mutant (Fig. 4A). RT–PCR analysis revealed that PYK10

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**Table 1** Summary of quantification results

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n#</th>
<th>Number#</th>
<th>Area (µm²)</th>
<th>Major (µm)</th>
<th>Minor (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0</td>
<td>27</td>
<td>48.1 (19.2)*</td>
<td>3.80 (0.98)</td>
<td>3.90 (0.96)</td>
<td>1.23 (0.08)</td>
</tr>
<tr>
<td>leb-1</td>
<td>22</td>
<td>20.9** (10.1)</td>
<td>6.67** (1.84)</td>
<td>6.02** (1.33)</td>
<td>1.35* (0.08)</td>
</tr>
<tr>
<td>pyk10−1</td>
<td>5</td>
<td>26.6* (7.2)</td>
<td>5.01 (0.25)</td>
<td>4.80 (0.26)</td>
<td>1.31 (0.07)</td>
</tr>
<tr>
<td>bglu21−1</td>
<td>6</td>
<td>25.7* (11.1)</td>
<td>3.94 (0.37)</td>
<td>4.01 (0.28)</td>
<td>1.25 (0.15)</td>
</tr>
<tr>
<td>bglu21−1 pyk10−1</td>
<td>7</td>
<td>16.3** (8.3)</td>
<td>8.31** (2.03)</td>
<td>6.17** (1.03)</td>
<td>1.58* (0.13)</td>
</tr>
<tr>
<td>bglu21−1 leb-1</td>
<td>25</td>
<td>18.3** (8.1)</td>
<td>5.89** (2.15)</td>
<td>5.34** (1.04)</td>
<td>1.33 (0.22)</td>
</tr>
</tbody>
</table>

#Number of investigated seedlings.
#Number of ER bodies per image.
#Area of the ER body.
#Major axis length of the ER body.
#Minor axis length of the ER body.
#Mean and SD (in parentheses).

*P < 0.05, **P < 0.001 significantly different from Col-0.
mRNA accumulated in the leb-1 mutant to the same level as in the wild type (Fig. 4B). These results indicate that the leb-1 mutation reduces the stability or the translation efficiency of the PYK10 protein.

The PYK10 protein forms a homodimer joined by a disulfide bond (Nagano et al. 2005). In addition, the leb-1 mutation is expected to alter a cysteine of the PYK10 protein to a tyrosine (Fig. 3B). These data raise the possibility of a tertiary structural change of the PYK10 protein in the leb-1 mutant. We performed SDS–PAGE without 2-mercaptoethanol and a subsequent immunoblot analysis using root extracts of the wild type and the leb-1 mutant. The electrophoretic mobility of PYK10 in the leb-1 mutant was clearly different from that in the wild type (Fig. 4C, open and filled arrowheads), supporting the possibility of a tertiary structural change of the PYK10 protein in the leb-1 mutant.

The mutants leb-1, bglu21-1 pyk10-1 and bglu21-1 leb-1 exhibit similar phenotypes

pyk10-1 is a null mutant with a T-DNA insertion (Nagano et al. 2008). A pyk10-1 mutant was crossed to GFP-h and their ER bodies were measured by the quantification system (Table 1). The pyk10-1 mutant tended to have fewer and larger ER bodies than the wild type, although these tendencies were not statistically significant (Fig. 5A, B, Supplementary Fig. S2). BGLU21 and BGLU22, the two closest homologs of PYK10, also have signal peptides and a putative ER retention signal (-RDEL, -KDEL). Thus, there is a possibility that mutants of these genes may also have ER body defects. To clarify the effects of bglu21 and bglu22 mutations in ER bodies, we attempted to identify T-DNA insertion mutations in the BGLU21 or BGLU22 gene. Although two null mutants of the BGLU21 gene were isolated, unfortunately no null mutant of the BGLU22 gene could be isolated (Supplementary Fig. S1). A bglu21-1 mutant was also crossed to GFP-h and their ER bodies were measured by the quantification system (Table 1). Similar to pyk10-1, the bglu21-1 mutant tended to have fewer and larger ER bodies than the wild type, although these tendencies were not statistically significant (Fig. 5A, B, Supplementary Fig. S2).

Next, we performed a quantitative analysis using two double mutants, bglu21-1 pyk10-1 and bglu21-1 leb-1. ER bodies in bglu21-1 pyk10-1 and bglu21-1 leb-1 were significantly fewer and larger than those in the wild type (Fig. 5B, C, Supplementary Fig. S2). The phenotypes of the two double mutants were quite similar to each other and to the single mutant leb-1 (Fig. 5).

Discussion

The traditional method of data presentation in cell biology, namely showing one or a few microscopic images, provides limited and sometimes biased information. A better way to
present such information is to quantify the characteristics in the images and treat the data statistically. However, manual measurement of many images is not only laborious, but also at risk for subjective bias by the measurer. Here we developed an automated image quantification system for ER bodies (Fig. 1) that allowed us easily to obtain quantitative data about the number and morphology of ER bodies. These quantitative data enabled us to perform statistical studies on the differences between the wild type and mutants (Figs. 2B, C, 5B, C).

An amino acid substitution of Cys29 with tyrosine (C29Y) on PYK10 was found in an ER body morphology mutant leb-1 (Fig. 3B). PYK10 protein number was decreased in the mutant (Fig. 4A), although the accumulation of PYK10 mRNA did not decrease (Fig. 4B). These results suggest that the C29Y substitution reduces the stability of the PYK10 protein in the ER. However, we could not eliminate the possibility that these substitutions reduced the translational efficiency of PYK10.

Non-reducing SDS–PAGE revealed that the electrophoretic mobility of PYK10 in the leb-1 mutant was clearly different from that in the wild type (Fig. 4C). This difference suggests that the C29Y amino acid substitution caused a tertiary structural change of the PYK10 protein. The PYK10 protein forms a homodimer joined by a disulfide bound (Nagano et al. 2005). The cysteine in the 29th position probably contributes to the intermolecular disulfide bond in the PYK10 dimer.

The bglu21-1 and pyk10-1 single mutations only very slightly (if at all) affected the number and morphology of the ER bodies (Fig. 5). In contrast, the number of ER bodies decreased and the bodies were larger in the bglu21-1 pyk10-1 double mutant compared with the wild type or the single mutants (Fig. 5). These results indicate that bglu21-1 and pyk10-1 have synergistic effects on at least the size of ER bodies (on the number of ER bodies, this may have been additive rather than synergistic). The phenotypes of the ER bodies of the leb-1 mutant were quite similar to those of the bglu21-1 pyk10-1 double mutant (Figs. 2, 5). In addition, the leb-1 mutation allele acted as a semi-dominant allele to...
the PYK10 wild-type allele (data not shown), and BGLU21 is a close homolog of PYK10 (Xu et al. 2004).

Based on the above results, we hypothesized that the leb-1 mutation allele acted dominantly to the BGLU21 wild-type allele with respect to the number and morphology of the ER bodies. If this hypothesis is true, then the phenotypes of bglu21-1 leb-1 would be similar to those of bglu21-1 pyk10-1 and leb-1. If the hypothesis is not true, then bglu21-1 leb-1 would exhibit an additive or synergistic phenotype compared with leb-1. Quantification revealed that the phenotypes of the bglu21-1 leb-1 mutant were similar to those of the bglu21-1 pyk10-1 and leb-1 mutants (Fig. 5), making our hypothesis consistent with the quantification results.

![Fluorescent images of epidermal cells in Col-0, bglu21-1, pyk10-1, bglu21-1 pyk10-1, bglu21-1 leb-1 and leb-1. Bars = 5 µm. (B) Average areas and numbers of ER bodies per image. The bglu21-1 pyk10-1, bglu21-1 leb-1 and leb-1 mutants are significantly different from Col-0 (*P < 0.001, pairwise comparison by Tukey–Kramer’s method, see also Supplementary Fig. S2). Error bars indicate the SD (n > 5). (C) Distribution of area of each ER body. ER bodies in bglu21-1 pyk10-1, bglu21-1 leb-1 and leb-1 are significantly larger than those in Col-0 (*P < 0.001, Kruskal–Wallis test and pairwise comparison, see also Supplementary Fig. S2).](image-url)
The molecular basis of the dominant manner of the leb-1 allele to the PYK10 and BGLU21 wild-type alleles remains unsolved. One possible mechanism is that the leb-1-type PYK10 protein competitively inhibits interactions between the wild-type BGLU21 or PYK10 protein and an unknown partner, possibly an NAI2 protein. NAI2 is a key component of ER body formation (Yamada et al. 2008). Although it is not confirmed that PYK10 interacts with NAI2, the phenotypes of leb-1 (i.e. fewer and larger ER bodies) are similar to those of NAI2 knock-down plants (Yamada et al. 2008). This may mean that in the leb-1 mutant, the NAI2 protein is trapped by leb-1-type PYK10 proteins and cannot interact with the wild-type BGLU21 and PYK10 proteins. To reveal the molecular mechanisms, further biochemical and structural analyses of leb-1-type PYK10, wild-type PYK10 and wild-type BGLU21 proteins are needed.

Our results showed not only the effectiveness of our quantitative approach to study organelle morphology, but also the possibility of controlling organelle morphology by modifying the component protein. It is expected that quantification and modifications will be essential techniques in the study of organelle morphology.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) and Landsberg erecta (Ler) were used as wild-type plants. All plant lines used in this study except Ler carried a GFP-h locus coding for constitutively expressed ER-targeted GFP. The GFP-h locus was incorporated by crossing various mutants and the GFP-h plant (Hayashi et al. 2001). The leb-1 mutant was isolated from an EMS-mutagenized M2 population (Alonso et al. 2003). Although it is not confirmed that PYK10 interacts with NAI2, the phenotypes of leb-1 (i.e. fewer and larger ER bodies) are similar to those of NAI2 knock-down plants (Yamada et al. 2008). This may mean that in the leb-1 mutant, the NAI2 protein is trapped by leb-1-type PYK10 proteins and cannot interact with the wild-type BGLU21 and PYK10 proteins. To reveal the molecular mechanisms, further biochemical and structural analyses of leb-1-type PYK10, wild-type PYK10 and wild-type BGLU21 proteins are needed.

Immunoblot analysis

Roots of 20-day-old plants were homogenized in SDS−PAGE sample buffer with or without 2-mercaptoethanol (Nagano et al. 2005). The homogenates were centrifuged at 500×g and 22°C for 1 min to remove debris. The supernatant was collected as a root extract, and was subjected to SDS−PAGE followed by either Coomassie Brilliant Blue staining or immunoblot analysis as previously described (Nagano et al. 2005).

Confocal laser scanning microscopy and automated image quantification

Cotyledons of seedlings (6 d after sowing) were used for confocal laser scanning microscopic analysis. The adaxial epidermis cells in the mid-cotyledon were observed. We took images of the adaxial surface of cells, because most ER bodies were observed by their longitudinal side. We use a confocal laser scanning microscope (LSMS10, Carl Zeiss, Jena, Germany) to observe fluorescent proteins. To observe GFP, we used an argon laser (488 nm) and a 505/530 nm bandpass filter. All images were taken under the same parameters: laser output strength, 22%; magnification, 40×; resolution, 512×512 pixels/112.5×112.5 μm; pinhole, 100 μm; and 12-bit coloring.

All image analysis procedures were performed using ImageJ software (Abramoff et al. 2004). For detailed information, including feature description and our original ImageJ programs, see also our website, http://hasezawa.ib.k.u-tokyo.ac.jp/zp/Kbi/ERbody. Statistical analysis of the quantification results was performed using the program R.

Supplementary data

Supplementary data are available at PCP online.

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