Three-dimensional analysis of chloroplast protrusion formed under osmotic stress by polyethylene glycol in rice leaves

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ABSTRACT

Chloroplast protrusions (CPs), large stromal areas lacking thylakoid membranes, are present before the formation of Rubisco-containing bodies (RCBs). RCBs are round bodies found within the cytoplasm and transported to the vacuole via the autophagy pathway. In the present study, we observed CPs and RCBs in rice mesophyll cells under polyethylene glycol-induced osmotic stress following 3D reconstruction of transmission electron microscopy images of serial sections. Osmotic stress induced the formation of CPs and RCBs whose features were similar to those observed under salt stress, suggesting that osmotic effect contributes to the formation of CPs and RCBs. The 3D image of CPs revealed that some CPs were formed far from the main chloroplast body, and one of the CP was in physical contact with a mitochondrion and a peroxisome, which were located far from the main chloroplast body. Additionally, a CP with a connection to the main chloroplast body by a narrow structure was observed three-dimensionally. Since the volume of CPs was similar to that of RCBs, the CP with the narrow structure may be a precursor structure just before the release of an RCB into the cytoplasm. In the present study, the volumes of two CPs among 24 CPs were markedly larger than the volumes of other CPs and RCBs. Thus, the large body of the CP could be an unviable structure that fails to release RCBs.

ARTICLE HISTORY

Received 29 July 2019
Revised 15 November 2019
Accepted 3 December 2019

KEYWORDS

Chloroplast protrusion; Polyethylene glycol; Rice; Rubisco containing body; Three dimensional reconstruction; Ultrastructure

Introduction

Chloroplast protrusions (CPs) are structures containing a large stromal area and lack a thylakoid membrane (Holzinger et al., 2007b). Although the physiological significance of this arrangement has not been fully elucidated, the structure might be necessary for the association with...
other organelles such as mitochondria and peroxisomes by increasing the surface area of chloroplasts (Holzinger et al., 2007a). In addition, it has been suggested that CPs with thin connections to the main chloroplast body could contribute to the degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Michaeli et al., 2014; Yamane et al., 2012). Rubisco is degraded by pathways both dependent on and independent of autophagy (Otegui, 2018). As degradation proceeds via autophagy, the structure of Rubisco-containing bodies (RCBs), which are small round bodies observed in the cytoplasm by transmission electron microscope (TEM), are first released from chloroplasts. RCBs can be derived from stromules and CPs. Stromules and CPs are tubular projections including stroma without thylakoid membranes, and a distinction between stromules and CPs is described using a ‘shape index’, which is a ratio between length and radius of these structures (Holzinger et al., 2007b). Generally, stromules are the structure with long and thin tubules. On the other hand, CPs are the structure with short and large stromal area or tubular projections which could be the structure just before the separation from the main chloroplast body (Yamane et al., 2012). In addition, CPs with large stromal area occasionally include membrane structures (Yamane et al., 2012). In our previous study, we observed that CPs including Rubisco were almost separated from the main chloroplast body, and the isolated structures similar to the CPs were observed in the cytoplasm and vacuoles under salt stress (Yamane et al., 2012). A CP that has separated from the main chloroplast body can also be observed under carbon starvation or salt stress using living cells expressing the fused protein of ATG8 interacting protein 1-green fluorescence protein (ATI1-GFP; Michaeli et al., 2014). Gunning (2005) observed the round body at a stromule tip, which maintains its connection to the main chloroplast body through a narrow structure. This research did not directly observed the release of the round body into the cytoplasm. Thus, it is expected that when the CP is connected to the chloroplast by the narrow structure it will be imminently released as an RCB. However, the formation process of CPs before the release has not yet been well observed.

The purpose of this study was to elucidate the 3D ultrastructure of CPs and RCBs through the reconstruction of 3D architecture using serial sections of leaf tissues under osmotic stress observed by TEM, and to discuss the change in the ultrastructure of CPs before the release of RCBs and the factors related to their formation.

### Materials and methods

#### Plant materials and stress treatment

After seeds of rice (Oryza sativa L. cv. Nipponbare) were surface sterilized, they were imbibed according to the methods of Yamane et al. (2012). The growing culture, condition, and period were matched to the methods of the relevant literature (Yamane et al., 2012). After the rice plants were grown for 3 weeks, they were affected by osmotic stress by adding polyethylene glycol (PEG) 4000 to the water culture medium for 4 days. The water potential was measured with an osmometer (OSMOTRON-5, Orionriken Co., Ltd.) and the PEG containing medium was −0.5 MPa, which was equivalent to 100 mM NaCl.

#### TEM observation

The middle part of the fully expanded uppermost leaves (fifth leaves) of control and PEG-treated plants was used for TEM observation. Small pieces of leaves (2 mm square) were cut and fixed in Karnovsky’s fixative (mixture of 4% paraformaldehyde and 5% glutaraldehyde in 50 mM phosphate buffer [pH 7.2]) for 5 h. The pieces were incubated in 50 mM phosphate buffer for 2 h and then fixed with 2%
osmium tetroxide in the same buffer for 14 h. The samples were dehydrated in a series of graded acetone and propylene oxide solutions and embedded in Spurr’s resin.

Serial ultrathin sections (100 nm thickness) were cut using a diamond knife on an ultramicrotome (Ultracut E; Leica, Germany) and placed on one-slot (1 × 2 mm) copper grids coated with formvar. Around 50 serial sections were placed on a copper grid. The grids were stained with 2% uranyl acetate for 5 min, followed by staining with lead citrate for 2 min. The serial sections were observed using TEM (H7500; Hitachi, Japan) at 100 kV and photographed using a CCD camera (Advanced Microscopy Technique, USA) connected to the TEM. The serial images of eight chloroplasts from mesophyll cells in control and PEG-treated leaves were obtained, and 24 CPs were observed in the chloroplasts in total. In addition, the serial images of 50 RCBs within the cytoplasm were obtained.

Reconstruction of 3D image

The images of serial sections obtained by the observation of TEM were treated with drift correction using the open source software, Fiji (http://fiji.sc/Fiji). Contours of chloroplasts were traced manually by using Paint Tool SAI software (SYSTEMAX Inc., Japan). The traced images were used for 3D reconstruction using Image-Pro Premier 3D software (Ver. 9.3, Media Cybernetics, USA).

Statistical analysis

The statistical difference of the mean (the symbol of cross marks in Figure 9) between the CP and RCB was assessed with two-sided test of Student’s t-test with the level at P < 0.05 using Excel 2012 for Windows (SSRI Japan, Co. Ltd). The mean of the CP for the statistical analysis was calculated using the data except or the two large CPs.

Results

CPs and RCBs observed using TEM under PEG stress

Figure 1 shows the ultrastructure of chloroplasts in the mesophyll cells of rice under control and PEG stress. The chloroplast in mesophyll cells of control plants showed no ultrastructural distortion and possessed well-developed grana and stromal thylakoids (Figure 1(a)). By contrast, the formation of CPs was observed in chloroplasts in the mesophyll cells of rice subjected to PEG stress for four days (Figure 1(b), arrows).

Figure 2 shows the ultrastructure of CPs in the mesophyll cells of rice subjected to PEG stress. Some CPs were almost separated from the main chloroplast body, while the envelope of the CPs was still continuous with the envelope of the main chloroplast body (Figure 2(a–c)). Three patterns of CPs: without inner structure (Figure 2(a)), with the crystalline inclusion (Figure 2(b), asterisk) and the inner membrane structure (Figure 2(c), arrowheads) were observed under PEG stress. Some inner membrane structures found in CPs were connected to the chloroplast envelope (Figure 2(d), dashed arrows).

Figure 3 shows the ultrastructure of RCBs found in cytoplasm under PEG stress. A similar ultrastructure to that of the CPs was observed, specifically lacking an
inner structure (Figure 3(a)), with the crystalline inclusion (Figure 3(b), asterisk), and with the inner membrane structures (Figure 3(c), arrowheads).

3D reconstruction of CPs and RCBs by using TEM serial images

Reconstructing the 3D architecture for part (Figure 4) or whole structures (Figures 5 and 6) of chloroplasts with CPs was conducted using the traced serial images. Figures 4–6 show parts of traced serial images and the 3D architecture of the chloroplasts with one CP (Figures 4 and 5) and five CPs (Figure 6) under PEG stress. In Figure 4, the CP was almost separated from the main chloroplast body. The CP was observed from sections 18 to 26 (Figure 4), and the connection of the CP to the main chloroplast body was observed only in sections 21, 22 and 23 (Supplemental Figure 1). We also observed the enlarged CP (Figure 5). The CP appeared in sections 10 to 40 (Figure 5 and Supplemental Figure 2). The CP connected to the main chloroplast body from sections 10 to 23 and separated from section 24 onwards (Supplemental Figure 2). In Figure 6, the two CPs (circled number 1 and 5) were partially connected with the main chloroplast body. The connections of CPs circled number 1 and 5 to the main chloroplast body were observed between sections 5 to 17 and from 7 to 21, respectively. However, the CPs circled 1 and 5 were separated from the main chloroplast body at sections 8 and 22, respectively. The CP circled number
1 was associated with the mitochondria (pink in Figure 6(b)) and peroxisome (blue in Figure 6(b)), which were located far from the main chloroplast body. The CPs (circled number 2 and 3) in Figure 6(b) were fully connected with the main chloroplast body. Although the protruded body circled number 4 in Figure 6(a)-19 looked like a CP under TEM observation, the stroma-including structure surrounding the mitochondrion was observed between sections 20 to 33 (Supplemental Figure 3). The 3D reconstruction image indicated that the whole structure of the CP was the chloroplast pocket including a mitochondrion (Figure 6(b)).

Figure 7 shows all traced serial images and the 3D architecture of an RCB observed in the cytoplasm under PEG stress. The diameter of the RCB was around 0.5 µm, and the images of the RCBs by TEM and 3D reconstruction indicated round and spherical conformation, respectively. Figure 8 shows parts of traced serial images and the 3D architecture of the RCB which was completely separated from the main chloroplast body. The 3D reconstruction image without the treatment with transparent produced an image similar to that of RCBs (Figure 8(b), left upper). Since a mitochondrion was included, the RCB may be produced by a chloroplast pocket.

**Volume of CPs and RCBs**

Figure 9 shows the quantification of the volume of CPs and RCBs observed under PEG stress. In the present study, 24 CPs were observed in eight chloroplasts and 50 RCBs in the cytoplasm were randomly obtained from the mesophyll cells of rice. The number of CPs and RCBs per cell was not studied in the present study because of the difficulty in obtaining a whole cell with the serial sectioning TEM method. The volume of most CPs fell below 2.0 µm³ save for two outlying CPs, which were markedly larger and indicated in Figure 5(b). The chloroplasts with large CPs in the mesophyll cells of rice treated with PEG for four days were relatively rare. By contrast, all RCBs had a volume of less than 2.0 µm³. The distribution pattern of CPs except for the two large CPs was similar to that of RCBs. The mean volumes of CPs and RCBs, except for the two large CPs, were 0.343 and 0.466 µm³, respectively. The median values of CPs and RCBs, except for the two large CPs, were 0.215 and 0.392, respectively. The mean and median value of RCBs were close to those of CPs, and the mean value with and without the large two CPs were not statistically different compared with that of RCBs by Student’s t-test.

**Figure 3.** The ultrastructure of RCBs in cytoplasm under PEG stress. (a) RCB without inner structure. (b) RCB with crystalline inclusion (asterisk). (c) RCB with inner membrane structure (arrowheads). C, chloroplasts; M, mitochondria; N, nucleus. Bars = 0.5 µm.
Discussion

Formation of CPs is induced by osmotic effect

In our previous study, salt stress induced the formation of CPs and the CPs were separated from the main chloroplast bodies resulting in the formation of RCBs (Yamane et al., 2012). Salt stress is the combination of ionic and osmotic effects (Munns & Tester, 2008), and it remains unclear which factors induce the formation of CPs and RCBs. In the present study, osmotic stress by PEG induced the formation of CPs and RCBs (Figure 1–3). In addition, the ultrastructural features of crystalline inclusions and inner membrane structures formed in CPs and RCBs were similar to those observed under salt stress (Figures 2 and 3). The protruding stromule body was also formed in tobacco epidermal cells under osmotic stress by PEG (Gray et al., 2012). Thus, the osmotic effect is one of the factors capable of promoting the formation of a protruded body such as a CP resulting in the formation of an RCB.

Stromules are observed in rice mesophyll cells under normal growth conditions, and could function to trap photorespiratory CO₂ released from the mitochondria (Sage & Sage, 2009). In the present study, however, the protruding body of CPs was not observed in rice mesophyll cells under control. In the previous study, CPs have been mainly observed under stress conditions such as salinity (Yamane et al., 2012), high (Buchner et al., 2007) and low temperature (Stefanowska et al., 2002), and herbicides (Pechová et al., 2003). Thus, CPs could be preferentially formed under stress conditions, though stromules are formed even under normal growth conditions. Further studies exploring the factors related to the formation of the tubular projections such as stromules and CPs should be conducted.
3D structure of CPs and RCBs

Since CPs are the tubular projections or short and large stromal areas formed by chloroplasts, CPs are connected to the main chloroplast body even if the connection part is narrow. On the other hand, RCBs are the separated structure found in cytoplasm, and thus there is no connection to the main chloroplast body. In the present study, the 3D reconstruction images indicated that a CP was constructed by the portions with and without connections to the main chloroplast body (Figures 4(b) and 5(b), arrows). The

Figure 5. Traced and 3D images of a whole chloroplast with the large body of a CP. (a) Parts of the traced serial TEM images. The number in each photograph indicates the order of serial sections. Green and yellow indicate a chloroplast and CP, respectively. C, chloroplasts; M, mitochondria; P, peroxisomes; N, nucleus. Same number of the labels indicates same organelles through the serial sections. Bars = 1.0 µm. (b) The 3D reconstruction image of the whole chloroplast with the large body of a CP. The number in Figure 5 (b) corresponds to the number of the cross sections shown in Figure 5(a). The reconstruction image is viewed from the direction indicated by white block arrows in the cross-sectional images of each figure. The arrow in Figure 5(b) indicates the area without the connection to the main chloroplast body.
two-dimensional ultrastructure of CPs in the area without the connection was round under TEM observation (for example Figure 6(a)-13 and 19, circled number 1), which was similar to that of RCBs (Figure 3(a)). Thus, 3D analysis is useful in preventing misidentifications of CPs and RCBs when their structures are observed at the ultrastructural level.

The CP which fully connects with the main chloroplast body has been observed by TEM (Holzinger et al., 2007b, 2007a). In the present study, however, the 3D reconstruction image of CPs indicated that some CPs partially connect to the main chloroplast body. The structure has not been reported, which might be due to the confusion of RCBs in the cytoplasm as mentioned.
above. Holzinger et al. (2007a) suggest that chloroplasts enhance the association with other organelles using stromules and CPs, though there have been fewer direct observations of this process. In the present study, the CP circled number 1 in Figure 6 physically contacted the mitochondrion and peroxisome which were located far from the main chloroplast body. Thus, the CP formed far from the main chloroplast body may be the structure capable of enhancing the physical association with other organelles such as mitochondria and peroxisomes.

It has been considered that RCBs appear from stromules and CPs. Gunning (2005) observed plastid stromules in tomato trichomes with video microscopy. The stromules showed small round bodies at stromule-tips, which are still continuous with the main plastid body with the narrow structure. Spitzer et al. (2015) speculated that RCBs are produced by tip-shedding of stromules from the observation of an Arabidopsis thaliana mutant lacking the endosomal protein known as CHARGED MULTIVESICULAR BODY PROTEIN1 (CHMP1).
Thus, the small round body connected to the main plastid body by the narrow structure may be formed before the release of RCBs. Although it has been suggested that the separation of CPs is one of the pathways of RCB formation, the change in the ultrastructure of CPs before the formation of RCBs has not been observed. In the present study, the CP connected to the main chloroplast body by the narrow structure was observed two-dimensionally using TEM (arrows in Figure 2) as observed under salt stress (Yamane et al., 2012). In addition, the 3D reconstruction image clearly showed that the small spherical structure appeared from the main chloroplast body, and the structure was still partially continuous with the thin connection part, while a large portion of the CP was almost separated from the main body (Figure 4). The mean and median values of the volume of CPs were close to those of RCBs (Figure 9). These results suggest that the CP with the narrow
structure was captured just before its release of RCBs into the cytoplasm. In the future, the mechanism underlying the release of the narrow structure leading to the formation of RCBs should be elucidated.

The volumes of the two CPs were apparently larger than that of other CPs and RCBs (Figure 9). Using the mutants lacking autophagy related genes (ATGs) such as atg5-1 and atg7-2, Spitzer et al. (2015) observed that rosyary-like stromules were formed by the impaired release of RCBs, which causes the accumulation of unnecessary stromal proteins in plastids. Thus, the large body of the CP observed in the present study under PEG stress could be caused by the disordered release of RCBs.

**All protruded bodies may be separated from chloroplasts**

In our previous study, a new protruded body of sheet structure was found under salt stress (Yamane et al., 2018). The sheet structure might be related to the formation of chloroplast pockets, which surround other organelles such as the cytoplasm, mitochondria, and peroxisomes. Since the protruded bodies of stromules and CPs are separated from the main chloroplast bodies leading up to the formation of RCBs, it is hypothesized that chloroplast pockets formed by the sheet structure are also released into the cytoplasm and form RCB-like structures including organelles. In the present study, the chloroplast pocket including a mitochondrion, which was partially connected with the main chloroplast body, was observed (Figure 6). In addition, the RCB-like structure including a mitochondrion was observed (Figure 8). These results suggest that chloroplast pockets are separated from the main chloroplast body and released into the cytoplasm as well as stromules and CPs. In this study, however, the fate of the separated pocket is still unclear and requires further study.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This work was partially supported by Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Numbers JP18K05603 (to K.Y.), JP19K15823 (to T.O.), and JP17H03757 (to M.T).

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