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Short communications

Macroautophagy and microautophagy in relation to vacuole formation in mesophyll cells of *Dendrobium* tepals

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ABSTRACT

Prior to flower opening, mesophyll cells at the vascular bundles of *Dendrobium* tepals showed a large increase in vacuolar volume, partially at the expense of the cytoplasm. Electron micrographs indicated that this increase in vacuolar volume was mainly due to vacuole fusion. Macroautophagous structures typical of plant cells were observed. Only a small part of the decrease in cytoplasmic volume seemed due to macroautophagy. The vacuoles contained vesicles of various types, including multilamellar bodies. It was not clear if these vacuolar inclusions were due to macroautophagy or microautophagy. Only a single structure was observed of a protruding vacuole, indicating microautophagy. It is concluded that macroautophagy occurs in these cells but its role in vacuole formation seems small, while a possible role of microautophagy in vacuole formation might be hypothesized. Careful labeling of organelle membranes seems required to advance our insight in plant macro- and microautophagy and their roles in vacuole formation.

Introduction

The early development of many plant cells is characterised by an increase of vacuolar volume. In many mature plant cells the cytoplasm is eventually present as a thin layer between the vacuole and the cell wall (Smith et al., 1992; Inada et al., 1998; van Doorn et al., 2003). It has been suggested that the increase in vacuolar volume, at the expense of the cytoplasm, involves autophagy, possibly both macroautophagy and microautophagy (Matile, 1997; van Doorn and Woltering, 2005; Bassham, 2009). However, the experimental evidence for this idea is still quite scant.

In plants, macroautophagy is initiated either by the formation of small tubes, similar to the endoplasmatic reticulum, or of small sheets consisting of two membranes. Hydrolases are apparently present from the outset, inside the membranes of these tubes or sheets. The tubes form a cage-like network around a portion of the cytoplasm. The tubules of this network merge laterally, forming a continuous membrane-bound space in the cytoplasm. A portion of the cytoplasm becomes sequestered inside. The same sequestration occurs when sheets formed by a double membrane fold around a portion of the cytoplasm (van Doorn and Papini, 2013).

The resulting organelle shows close morphological similarity to the autophagosome in animals and yeasts. However, autophagosomes in animals and yeasts do not contain hydrolases from the outset of their formation, but merge later on with a compartment containing such hydrolases. In the plant-type autophagosome the hydrolases degrade the cytoplasmic contents and the inner of the two membranes (van Doorn and Papini, 2013). It has been noted that the sequestered portion of the cytoplasm is often broken down before the degradation of the inner membrane, suggesting that the hydrolases are actively transported over the inner membrane (Coulomb et al., 1982).

Microautophagy in plants is the uptake of cellular constituents by an invagination or evagination at the vacuolar membrane. The invaginated or evaginated space contains a portion of the cytoplasm, usually excluding large organelles. The invagination/evagination becomes restricted at the outer membrane of the vacuole, resulting in a vesicle that moves into the vacuole. The vesicle membrane and the vesicle contents then degrade (Thompson and Vierstra, 2005; Bassham et al., 2006). Although the

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presence of vesicles in the vacuoles has been suggested to be due to microautophagy (Smith et al., 1992; Matile and Winkenbach, 1971; Hiratsuka and Terasaka, 2011), it has been concluded that adequate proof of microautophagy in plants has not yet been given (van Doorn and Papini, 2013).

This paper examines the presence of macroautophagy and microautophagy in the mesophyll of *Dendrobium* tepals, in relation to the increase in vacuolar volume. Using TEM we evaluated what kind of autophagous structures were present, and studied the formation of large vacuoles and the structures in the vacuolar lumen. We investigated the hypothesis that autophagy plays an important role in the increase in vacuolar volume.

Materials and methods

Dendrobium cv. Lucky Duan inflorescences were harvested at a commercial farm near the laboratory at Kamphaeng Saen, Nakhom Pathom, Thailand. The inflorescences had 4–6 fully open flowers and 4–6 closed floral buds. Inflorescences were transported dry to the laboratory, packed in cardboard boxes, and arrived within an hour after cutting. Upon arrival, the stem ends were immediately placed in distilled water. The second and third fully open flowers on the inflorescences were excised at the distal end of their pedicels, using a sharp razor blade. The flower pedicels were immediately individually placed in 10 mL vials containing distilled water. All isolated flowers were held in an air-conditioned room at 25 °C and 80% relative humidity. The photosynthetically active photon flux was about 15 $\mu mol\ m^{-2}\ s^{-1}$, for 12 h per day.

The leafy parts of orchid flowers are all non-green, called tepals. The tepals of the inner whorl are homologous to petals in other flowers. In orchids one of the leafy organs in the inner whorl is called lip. We used the two tepals of the inner whorl that are not the lip. The present work refers to cells in or close to the vascular bundles.

Transmission electron microscopy (TEM) was carried out using fresh tepal segments (*ca.* 0.2 × 1.5 cm) which were removed from the tepals using a razor blade. The tissue was fixed in 5% glutaraldehyde in 25 mM sodium phosphate buffer, pH 6.8, for 0.5 h at room temperature. Segments were transferred to 3% glutaraldehyde in buffer, for 2 h, in ice. Samples were rinsed with buffer every hour for 12 h, post-fixed overnight with 1% osmium tetroxide in water, then rinsed with distilled water before dehydration (ethanol series) and gradual substitution of ethanol by propylene oxide. Samples were embedded in Spurr's resin at 60 °C for 24 h. Ultrathin sections were mounted onto copper grids and air-dried before staining in 2% uranyl acetate and lead citrate. Sections were examined using a JEOL TEM-1230 (Tokyo).

Results

Ultrastructure of cells at an early phase of vacuole formation

Cells close to the vascular bundles, or in such bundles, when at an early phase of vacuolation often exhibited an electron-dense cytoplasm, containing a nucleus, endoplasmatic reticulum with attached ribosomes, free ribosomes, mitochondria, plastids, Golgi bodies, and multilamellar bodies (Fig. 1A). Small, spherical parts in the cytoplasm were observed that were less electron-dense than the remainder of the cytoplasm (Fig. 1B, arrows).

Macro-autophagy

Fig. 1C–G show various stages of the formation of plant-type macroautophagous structures. These structures are formed by tubules; no sheet-like macroautophagy was observed in the cells studied. Fig. 1C exhibits a cell at an early stage of vacuolation. Many tubules are found in the cytoplasm. The large arrows point at a series of tubules that form an almost complete ovoid-like configuration. Another group of tubules, indicated by small arrows, is found inside this ovoid-like configuration. These tubules also form a circular-like configuration (Fig. 1C). Fig. 1D shows a structure in which several tubules have merged. The same is found in Fig. 1E, although here only very small gaps are left between a few tubules (black arrows). The white arrow points to tubules that have laterally merged. Fig. 1F and G show the same late autophagosome-like structure (arrows). The portion of the cytoplasm is not yet fully surrounded, but the membranes of the autophagous structure have already dilated, giving rise to a larger vacuolar-like space. Some amorphous, electron-dense material (here called 'debris') is found in this space.

These macroautophagous structures were found in cells at an early stage of vacuolation. The structures were found only in cells in or close to the vascular bundles, not in the mesophyll away from these bundles, which at the time of sectioning exhibited a thin layer of cytoplasm and a large central vacuole (data not shown).

Possible microautophagy, structures in vacuoles

Fig. 2A exhibits a vacuole in which the vacuolar membrane is protruding outward (arrow), which is typical for microautophagy. In Fig. 2B two vesicles, each with a single membrane, are probably still attached to the cytoplasm but are at least partially inside the vacuole. The one at the right side of the micrograph is only barely attached to the cytoplasm (arrow), while the other has a wider base attached to the cytoplasm.

Vacuoles contained several types of vesicular structures (Fig. 2C–E, arrows) as well as material not bound by a membrane (Fig. 2C–E, arrowheads). In Fig. 2E debris is found in the vacuole (arrowhead). Vesicular structures in the vacuolar lumen either have a single outer membrane (Fig. 2C and E, arrows) or a double one (Fig. 2D, arrow). Some inclusions shown in Fig. 2F (arrows) are apparently round but seem to lack an outer membrane.

Fig. 3 exhibits multilamellar bodies, which are found in the cytoplasm (Fig. 3A) or protrude at various depths into a vacuole (Fig. 3B–E). Note what seems an invaginating vacuolar membrane around the multilamellar body in Fig. 3D. The multilamellar body in Fig. 3E is almost entirely in the large vacuole but still connected to the thin strip of cytoplasm (arrow) of the cell. In Fig. 3F, a multilamellar body is located inside a vacuole, without a connection to the cytoplasm at the plane of section.

Possible vacuole fusion

Fig. 4 shows physiological young cells, indicated by the presence of many small vacuoles. The arrows in Fig. 4A and B indicate areas where vacuoles might have fused, but it cannot be excluded that the vacuole is just showing a bulge rather than fuse with another one. When a relatively large vacuole had already become formed, many smaller vacuoles could still be present (Fig. 4C). Some membranes between vacuoles were apparently weak as they had broken (arrows in Fig. 4C) either during preparation for TEM or already *in vivo* prior to preparation. In a few cells no structures that might indicate vacuole fusion or vacuole bulging were apparent at the plane of section (Fig. 4D).

Discussion

The present work was carried out to assess whether macro- or microautophagy would precede and accompany vacuole formation. The work was restricted to mesophyll cells close to the vascular bundles and inside these bundles.