

'Elaioplasts' identified as lipotubuloids in *Althaea rosea*, *Funkia sieboldiana* and *Vanilla planifolia* contain lipid bodies connected with microtubules

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Abstract

"Elaioplasts" observed in *Vanilla planifolia*, *Funkia Sieboldiana* and *Althaea rosea* exhibit all the features characteristic of lipotubuloids earlier described in *Ornithogalum umbellatum*. They are cytoplasmic domains containing aggregates of lipid bodies connected with microtubules. The immunogold technique confirmed the presence of tubulin in this domain. These structures do not have their own membranes but they are surrounded by a tonoplast at the side of a vacuole since they invaginate into it. In cytoplasm of this domain among lipid bodies there are numerous ribosomes, ER cisternae and vesicles as well as few mitochondria, Golgi structures and microbodies while at older developmental stages there are also autolytic vacuoles. The fact that they are so similar to *O. umbellatum* lipotubuloids suggest that "elaioplasts" of *V. planifolia*, *F. Sieboldiana* and *A. rosea* can also be named lipotubuloids.

Keywords: elaioplasts, immunogold technique, lipid bodies, lipotubuloids, microtubules, tubulin

Introduction

In many publications the term "elaioplasts" denotes, in accordance with the meaning, plastids containing lipids [1,2]. However, in the past this name was used according to Wakker [3] who was the first to apply it with reference to lipidic structures, often as big as a cell nucleus, which he identified in five *Vanilla* species. Similar structures were later described in more than 120 species belonging to Monocotyledoneae and Dicotyledoneae (literature data – Tab. 1). The observations in the light microscope showed that some of these plants contain lipidic structures which were definitely not plastids but aggregates of lipid bodies (lipid droplets, oleosomes), e.g. "elaioplasts" in *Malva neglecta* and *Althaea rosea* [4], 12 species of *Gentiana* [5], *Dahlia variabilis* [6], *Ornithogalum umbellatum* [7], *Haemanthus albiflos* [8] ([9] – EM observations). However, it should be noted that some "elaioplasts" in plants listed in Tab. 1 are plastids (e.g. in *Iris*, [10]).

The above literature clearly indicates that the issue is very old, but taking into consideration the papers from the last decade it has become very "hot" once again. Lipid bodies used to be treated as passive structures being only reservoirs of storage substances. However, nowadays they are seen as active

organelles playing an important role in lipid homeostasis, intracellular signal transduction and in temporary protein retention. Disturbances in their functioning cause health problems in animals and humans [11-14]. On the other hand, in plants lipids are an important industrial and food product [15,16]. All this led to the revival of interest in these structures. It has been shown that in COS7 fibroblast [17] the surface of lipid bodies is the site of lipid synthesis because it contains an enzyme, diacylglyceroyltransferase (DGAT) involved in the conversion of diacylglycerols (DAG) into triacylglycerols (TAG). These data were obtained through EM/immunocytochemical studies with the use of colloidal gold. "Elaioplasts" being great aggregates of many lipid bodies seem ideal for this type of research.

Up till now a precise description of light microscopic observations of "elaioplast" development in *O. umbellatum* has been given by Raciborski [18] and Kwiatkowska [7]. They are present in ovary and stipule epidermis. Ultrastructural studies have shown that they are big cytoplasm domains surrounded not by their own membranes but by a tonoplast because they invaginate into vacuoles. They consist of numerous lipid bodies, covered with a phospholipid monolayer, which contain mainly triacylglycerols and free fatty acids. EM observations at huge magnifications demonstrated that lipid bodies were entwined with microtubules running in different directions and joining them together thus the whole domain moved in a cell as one body with a progressive-rotary movement. These structures were called lipotubuloids as they contain lipid bodies and microtubules [19-27]. In the lipotubuloids in addition to lipid bodies there are numerous ribosomes, ER cisternae and vesicles and few mitochondria, microbodies, Golgi structures and at the late developmental stages autolytic vacuoles containing acid phosphatase and lipase [20]. Moreover, electron microscopic observations

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Tab. 1 Examples of plant family of mono- and dicotyledonous class in which have been identified "elaioplasts".

Family	References
Monocotyledonous	
Liliaceae	[7-9,18,28,31-35]
Iridaceae	[10,31]
Discoraceae	[31]
Orchidaceae	[3,10,18,28,31]
Dicotyledonous	
Malvaceae	[4,31,36]
Gentianaceae	[5,37]
Cucurbitaceae	[38]
Campanulaceae	[39]
Compositae	[40]
Pirolaceae	[41]
Orobanchaceae	[41]

revealed actin filaments in them [23]. Lipotubuloids of *O. umbellatum* form aggregations consisting of a growing numbers of lipid bodies during cell development [20,25]. Lipotubuloids appear in intensively growing cells, i.e. in ovary and stipule epidermis, and probably enable intensive growth of cells [25]. However, when the growth of the epidermis is over they gradually disperse into single lipid bodies. The process of disintegration of lipotubuloids is preceded by the disappearance of microtubules joining individual lipid bodies one with another [20,25].

During the first part of our *in vivo* and ultrastructural studies with the use of the immunogold technique we concentrated on two questions (*i*) whether "elaioplasts" observed in light microscope in *Vanilla planifolia* [3], *Funkia Sieboldiana* [28] and *Althaea rosea* [4] which are not plastids exhibit the structure characteristic of *O. umbellatum* lipotubuloids, (*ii*) whether lipid bodies of these "elaioplasts" are also connected with microtubules. Hence, the aim of this work was to check whether the presence of lipotubuloids is limited only to one species, *O. umbellatum*, or they are more widespread which would prove that the correlation between lipid bodies and microtubules is a more general feature.

Material and methods

Plant material

Seeds of *Althaea rosea* were germinated for four days in darkness at 25°C on moistened filter paper in Petri dishes. Plants of *Vanilla planifolia* and *Funkia Sieboldiana* were delivered from Botanic Garden in Łódź. Epidermis of *Vanilla planifolia* young leaves, *Funkia Sieboldiana* ovaries as well as epidermis of *Althaea rosea* roots and hypocotyls were used.

Observations in living cells

Before fixation the epidermis specimens were thoroughly observed in phase light microscope with immersion in order to affirm the presence of "elaioplasts" and their motions.

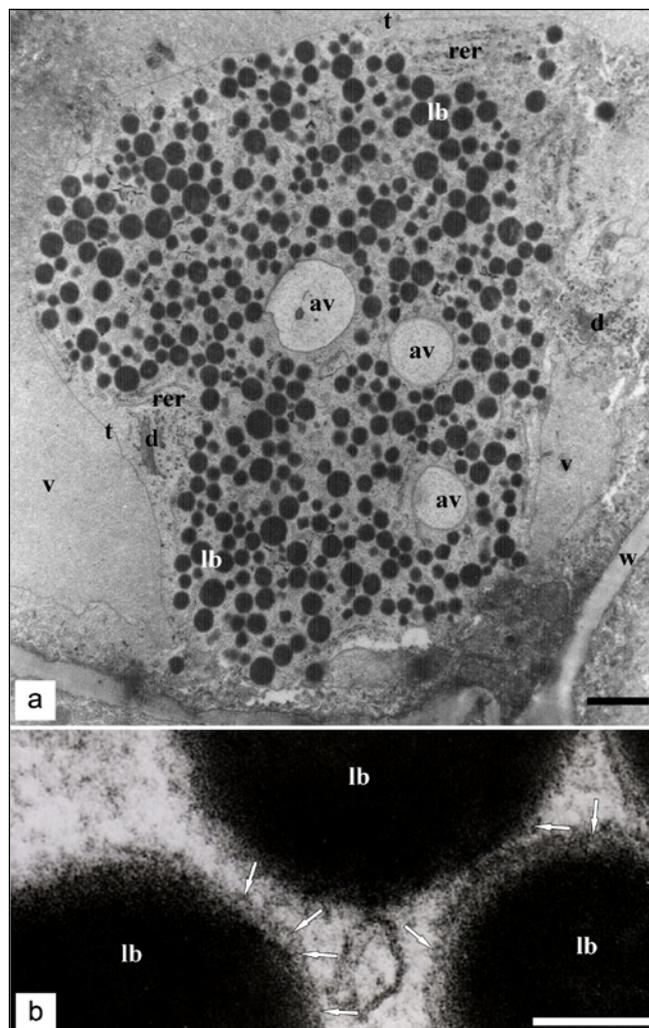


Fig. 1 Fragment of the cell with lipotubuloid in *Funkia Sieboldiana* ovary epidermis. **a** Ultrastructure of lipotubuloid; scale bar: 2 μ m. **b** Lipid bodies entwined by microtubules in cross sections (arrows); scale bar: 200 nm. av – centrally located autolytic vacuole; d – dictyosome; lb – lipid bodies; r – ribosomes; rer – rough endoplasmic reticulum; t – tonoplast; v – vacuole; w – cell wall.

Electron microscopy

Epidermis of plants belonging to the above species was fixed in the mixture of 1% OsO₄ and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h and postfixed in 1% OsO₄ in the same buffer at 4°C for 3 h. After dehydration in the ethanol series, the material was embedded in the medium consisting of Epon 812 and Spurr's resin. Ultrathin sections (70 nm) cut using Reichert Jounq Ultracut ultra microtome with the glass knife were double stained with uranyl acetate and lead citrate according to Reynolds [29]. The sections were examined and photographed in a JEOL JEM 1010 transmission electron microscope at 80 kV acceleration voltage.

Immunogold technique

The above material was prepared as it is described in EM section. Freshly cut ultrathin sections were mounted on nickel Formvar coated grids for EM investigations. Prior to immunogold reaction the sections were treated with 10% hydrogen peroxide for 15 min to remove osmium which changes antigen structure [30] and washed in distilled water and then in PBS (0.01 M, pH 7.4, Sigma). Air dried grids with the sections were blocked with 0.5% BSA and 0.05% Tween 20 in PBS for

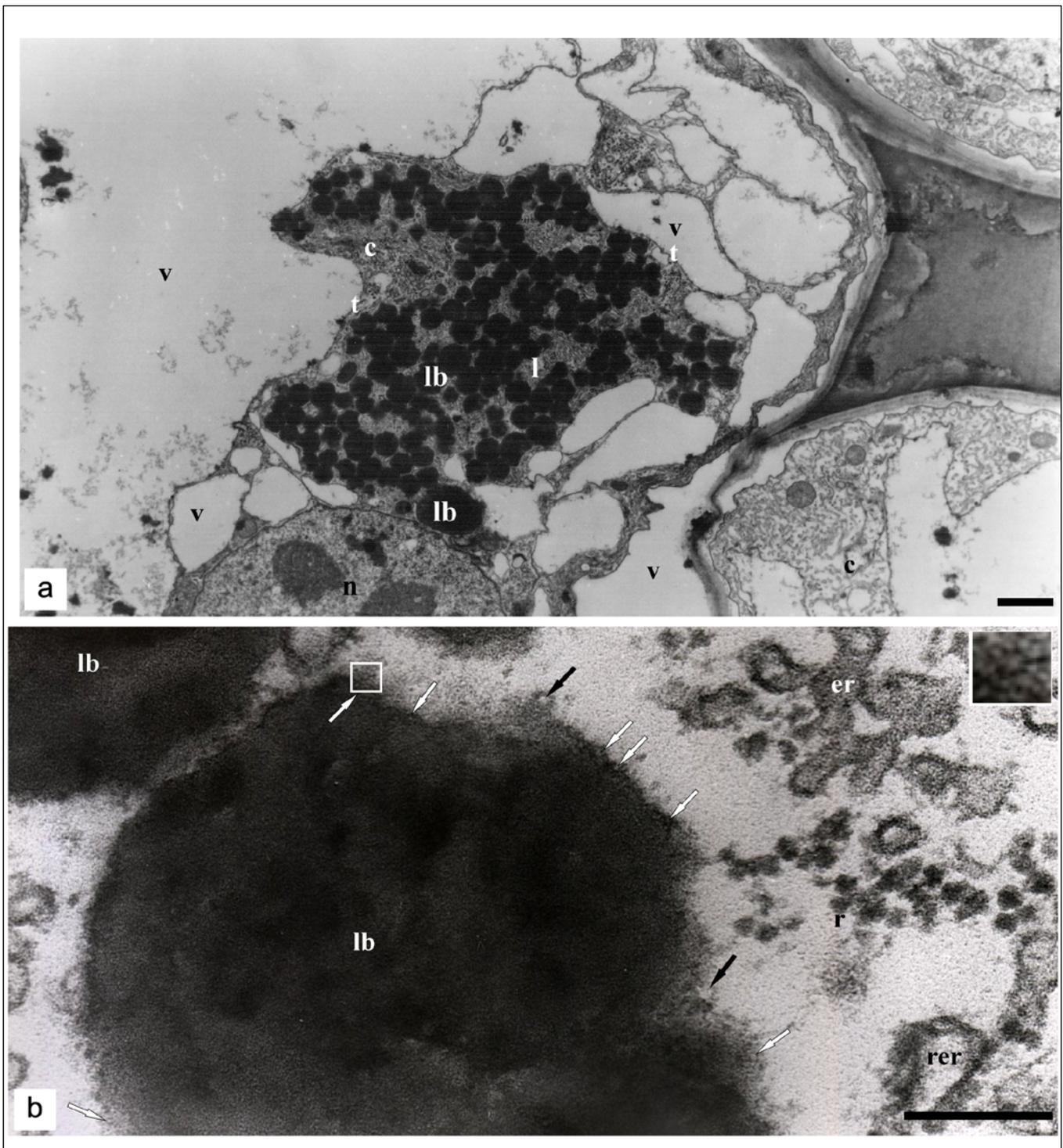


Fig. 2 Fragment of the cell with lipotubuloid in epidermis of young *Vanilla planifolia* leaves. **a** Ultrastructure of lipotubuloid; scale bar: 2 μm . **b** Lipid bodies with microtubules (white arrows). Black arrows indicate depolymerizing microtubules seen as single subunits. Cross section of the microtubule is viewed at higher magnification of the outlined area; scale bar: 200 nm. c – cytoplasm; er – endoplasmic reticulum; l – lipotubuloid; lb – lipid bodies; n – nucleus; r – ribosomes; rer – rough endoplasmic reticulum; t – tonoplast; v – vacuoles.

20 min and then dried with tissue-paper, and incubated overnight at 20°C with the primary anti- α -tubulin mouse monoclonal antibody (T-5168, Sigma) diluted 1:300 in an antibody diluent (pH 8.0, S 0809, DAKO). The material not treated with the primary antibodies was a negative control. After washing 10 times for 5 min each in PBS the grids were incubated with the secondary antibody [anti-mouse IgG conjugated with 10 nm gold (No. EM.GMHL10, Polysciences)] diluted 1:70 in the antibody diluent for 1.5 h at the same temperature, next the grids were rinsed again in PBS and distilled water (10 times for

5 min each). Ultrathin sections were double stained, examined and photographed as above.

Statistics of α -tubulin immunolabeling

The percentage of gold grains in two lipotubuloid domains (*i*) around and within lipid bodies and (*ii*) rest of the lipotubuloid cytoplasm, in three plant species, was calculated. Thirty lipotubuloid micrographs randomly chosen from 5 roots were analysed. Significance of difference between these two domains was assessed by Student *t*-test taking $p = 0.05$ with the use of

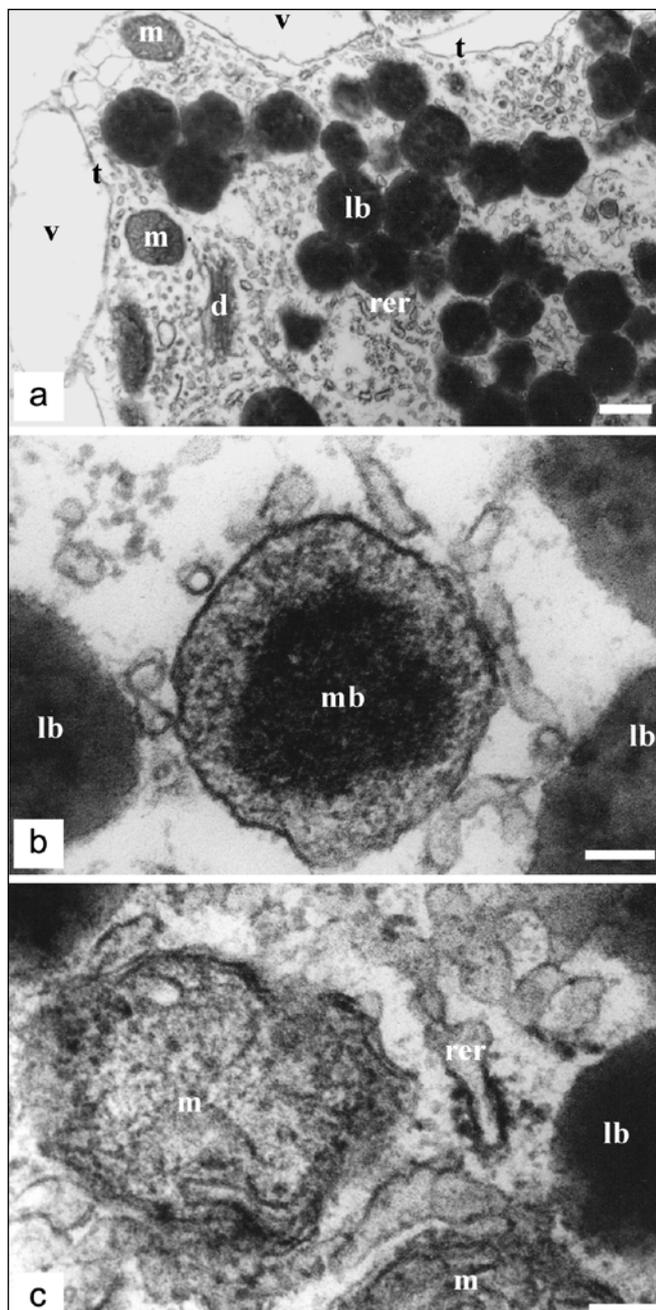


Fig. 3 a-c Fragments of lipotubuloid ultrastructure of *Vanilla planifolia* leaves with different cell structures. **a** Scale bar: 500 nm. **b,c** Scale bar: 100 nm. d – dictyosome; lb – lipid bodies; m – mitochondrion; mb – microbodies; rer – rough endoplasmic reticulum; t – tonoplast; v – vacuole seen among lipid bodies.

Microsoft Excel 2000; means \pm SE were also estimated.

Results

Funkia Sieboldiana 'elaioplasts'

F. Sieboldiana "elaioplasts" are located in ovary epidermis and are the cytoplasm domains containing a great number of lipid bodies. They are spherical structures often localized near a nucleus and mostly surrounded with a tonoplast (Fig. 1a). They look very much like *O. umbellatum* lipotubuloids, however mitochondria, Golgi structures and microbodies are more often visible at the "elaioplasts" peripheries while ribosomes,

ER cisternae and vesicles are inside the domain among lipid bodies. In the centre of "elaioplast" autolytic vacuoles are visible (Fig. 1a). The lipid bodies of "elaioplasts" are surrounded by microtubules in cross (Fig. 1b) or longitudinal sections. The above observations indicate that *F. Sieboldiana* "elaioplasts" are lipotubuloids.

Vanilla planifolia 'elaioplasts'

Under small EM magnification epidermal cells of young leaves contain, like the previous ones, irregular aggregates of lipid bodies surrounded by a tonoplast and connected with parietal cytoplasm and nucleus with a strand of cytoplasm (Fig. 2a). Among lipid bodies there are numerous ribosomes, ER cisternae and vesicles (Fig. 2, Fig. 3) and some Golgi structures (Fig. 3a), microbodies (Fig. 3b), mitochondria (Fig. 3c), and autolytic vacuoles (not demonstrated). Under great magnification microtubules in cross (Fig. 2b) or (less often) longitudinal sections are visible near lipid bodies. These microtubules are not so distinct and numerous as those in *O. umbellatum* which may result from their weaker stability. Nevertheless, since *V. planifolia* "elaioplasts" exhibit features characteristic of *O. umbellatum* lipotubuloids they can be treated as lipotubuloids.

Althaea rosea 'elaioplasts'

They are localized in seedling hypocotyl and root epidermis. They consist of numerous lipid bodies located in the cytoplasmic domain connected with peripheral cytoplasm and in the vicinity of a cell nucleus, at the vacuole side they are surrounded with a tonoplast (Fig. 4a). They contain numerous ribosomes, ER cisternae and vesicles as well as few mitochondria, Golgi structures and microbodies in the domain (Fig. 5). Near "elaioplast" also some plastids containing starch grains are visible (Fig. 4a). At later developmental stages autolytic vacuoles appear (not demonstrated). At great EM magnification cross sections of microtubules (Fig. 4b) touching lipid bodies are visible among them. All this indicates that *A. rosea* "elaioplasts" are lipotubuloids.

Observations with the use of EM immunogold technique

Immunogold technique with the use of anti- α -tubulin antibodies revealed gold grains near lipid bodies, in the cytoplasm between them and adjacent to microtubules in *V. planifolia* (Fig. 6a), *A. rosea* (Fig. 6b) and *F. Sieboldiana* (Fig. 6c,d). These images confirm the opinion that "elaioplasts" of these plants

Tab. 2 The presence of α -tubulin immunogold labeling in two lipotubuloid domains of three plant species.

Species	Frequency [%]	
	Around and within lipid bodies	Rest of the lipotubuloid cytoplasm
<i>Funkia Sieboldiana</i> *	82 \pm 7.9	18 \pm 1.5
<i>Vanilla planifolia</i> *	59 \pm 4.5	41 \pm 2.8
<i>Althaea rosea</i>	47 \pm 3.8	53 \pm 5.9

The number of gold grains in particular domains is presented as the percentage of the whole pool of grains in the lipotubuloids regarded as 100%. Values represent the mean \pm SE. Significant differences occurred in species marked with *.

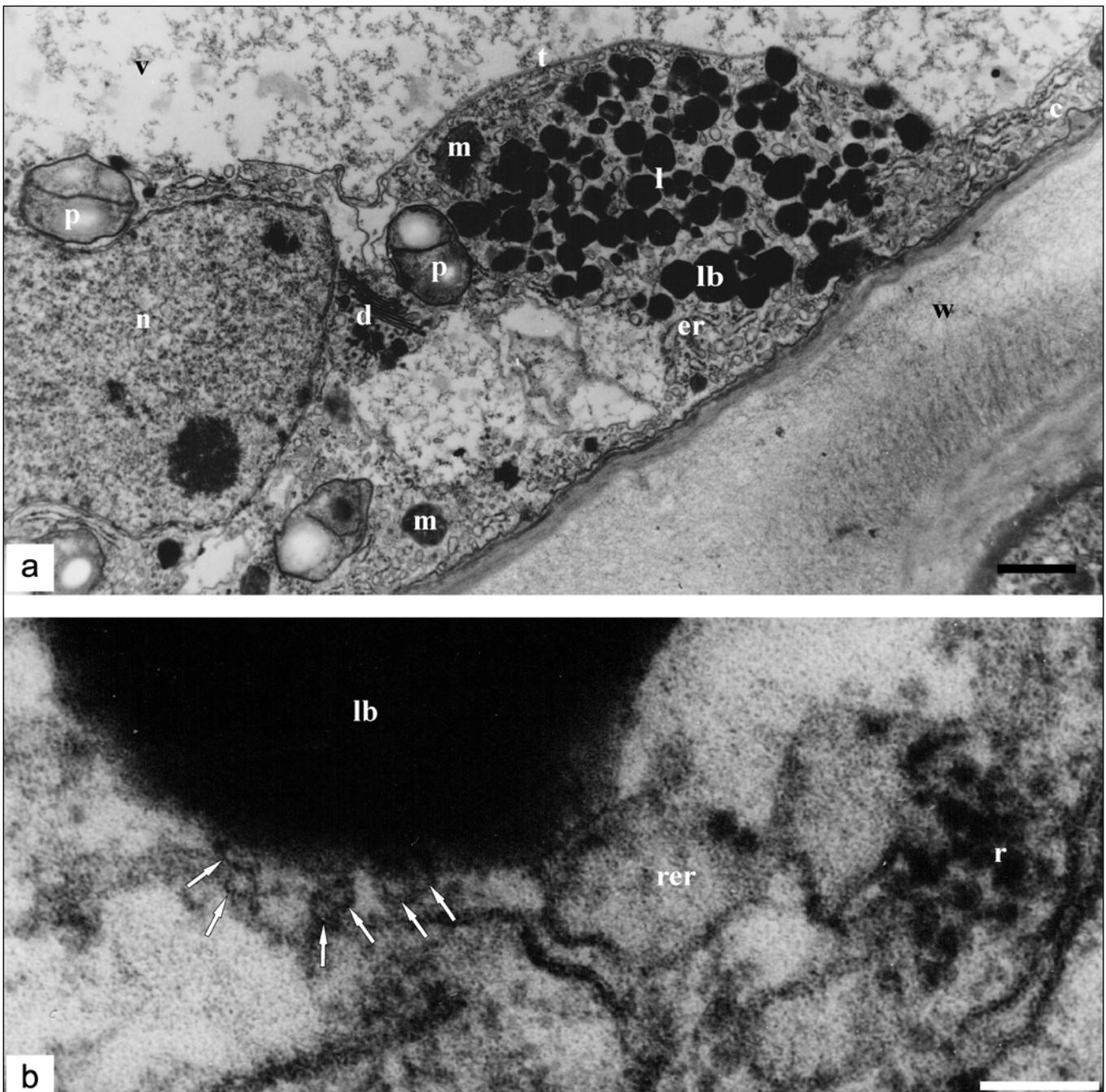


Fig. 4 Fragment of the cell with lipotubuloid in epidermis of *Althaea rosea* root. **a** Ultrastructure of lipotubuloid; scale bar: 2 μm . **b** Lipid body entwined by microtubules in cross sections (arrows); scale bar: 100 nm. c – cytoplasm; d – dictyosom; er – endoplasmic reticulum; l – lipotubuloid; lb – lipid body; n – nucleus; m – mitochondria; p – plastids; t – tonoplast; v – vacuole; w – cell wall.

are lipotubuloids.

Statistical analysis revealed that in *F. Sieboldiana* and *V. planifolia* there are statistically significant differences between the gold grain number present within lipid bodies and the rest of the lipotubuloid cytoplasm. This means that the greatest number of α -tubulin subunits occurs around and within lipid bodies, which proves that the highest density of microtubules is located in this cell compartment (Tab. 2).

Observations in living cells

These observations showed that in all studied species the lipotubuloids of granular structure, strongly refracting light performed a rotary and progressive motions with variable dynamic, similarly to lipotubuloids of *O. umbellatum*.

Discussion

The specificity of the immunogold technique with the use of anti- α -tubulin antibody was first determined by analyzing labeling of *O. umbellatum* lipotubuloids which are characterized with the presence of many stable microtubules [42]. Microtubules of *O. umbellatum* lipotubuloids are precisely marked with gold grains (ca. 61%) both on longitudinal and cross sections of microtubule walls. The other gold grains (ca. 39%) are outside of microtubules around lipid bodies and most probably mark non-polymerised tubulin (unpublished data). It is commonly known that tubulin occurs in a cell in the polymerised form – microtubules, or non-polymerised one as free tubulin subunits [43]. As gold grains marked microtubules, it allows for the conclusion that the immunogold technique is tubulin-specific.

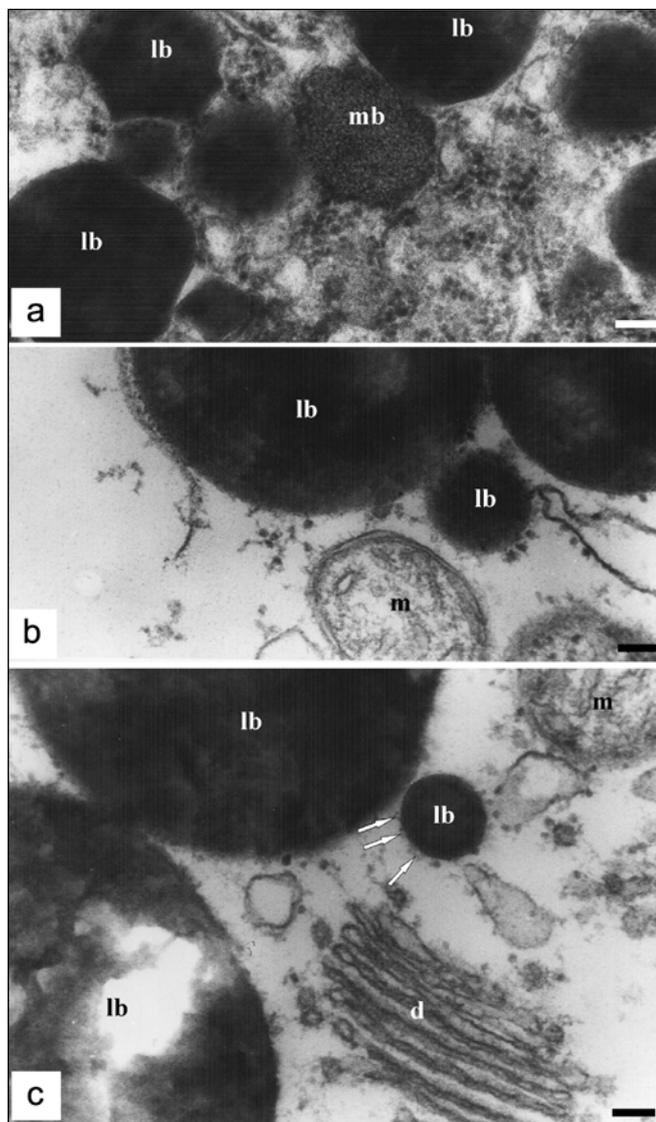


Fig. 5 a-c Fragments of lipotubuloid ultrastructure of *Althaea rosea* root with different cell structures. lb – lipid bodies; d – dictyosom; m – mitochondria; mb – microbodies, seen among lipid bodies. Scale bars: 100 nm.

Ultrastructural and immunocytochemical analyses with the use of the immunogold technique and anti- α -tubulin antibodies show that “elaioplasts” in *F. Sieboldiana*, *V. planifolia* and *A. rosea* exhibit all the features characteristic of *O. umbellatum* lipotubuloids and are cytoplasmic domains without their own membranes. Great part of their surface is surrounded by a tonoplast because they invaginate into a vacuole. The main components of this domain are great quantity of lipid bodies connected with microtubules. Ribosomes, cisternae and vesicles of ER are located among lipid bodies or at the periphery of the domain. Moreover, few mitochondria, Golgi structures and microbodies being counterparts of peroxisomes and glyoxysomes can also be seen. At later developmental stages autolytic vacuoles appear inside this domain (demonstrated in *F. Sieboldiana* Fig. 1a). Thus the above described structures do not resemble plastids so the term “elaioplasts” is not adequate. These structures should be called lipotubuloids as they are analogous to *O. umbellatum* lipotubuloids, although they differ from the latter ones due to a small number of microtubules which are poorly contrasted and mainly in cross sections adjacent to half unit membrane of lipid bodies. This is surely

the effect of their lesser stability since apart from whole microtubules there are also broken ones or just single subunits representing tubulin whose presence is also evidenced by immunogold signals in the form of gold grains.

Differences in microtubule stability are well known. They concern different microtubules within a cell as well as the same type of microtubules occurring in different organisms. This is connected with posttranslational modifications of microtubules (PTMs) which change their stability [44-46]. Moreover microtubules can form complexes with microtubule-associated proteins (MAPs) which either increase or decrease their stability [47]. For example cortical microtubules characteristic of dynamically growing cells are more stable than inner ones, however, there are also instable cortical microtubules revealed only after taxol application [48]. In the light of these results the fact that the microtubules from the plants examined in this research are less stable than those of *O. umbellatum* lipotubuloids should not prevent calling their “elaioplasts” – lipotubuloids.

When in 1888 Wakker called “elaioplasts” the lipidic structures, which he observed in *Vanilla*, he thought about structures producing lipids (in those days this term was not correlated with plastids, only later it became commonly used with regard to them). It turned out that Wakker’s intuition was right: *O. umbellatum* lipotubuloids indeed synthesize lipids since they intensively incorporate tritium labeled palmitic acid ([22]; see documentation also in minireview [26]). EM autoradiography showed that first, after 40 min of incubation, microtubule strands between lipotubuloids became labeled and after 2 h of incubation this precursor was incorporated into lipid body surface which was covered with a phospholipid monolayer with adjacent microtubules. These observations led to the hypothesis that microtubules might be involved in lipid synthesis which took place at the surface of lipid bodies [22,26]. Also Pacheco et al. [49] believe that microtubules are associated with lipid synthesis on the basis of mouse monocyte studies. Whether lipotubuloids of three examined species are the site of lipid synthesis will be investigated in future research. Moreover, the results of Czabany et al. [50] concerning yeast and of Kuerschner et al. [17] about COS7 fibroblast also suggest that the surface of lipid bodies with different anchored proteins is active during lipid synthesis.

The findings of the current research proving that the presence of microtubules around lipid bodies in lipotubuloids is characteristic of other plant species gives the research on functional correlation between lipid bodies and microtubules more general character. It should be added that microtubules play an important role in the generation of progressive-rotary motion of *O. umbellatum* lipotubuloids [26,27]. Similar motions of lipotubuloids were also observed in the plant species examined in the current work. It is clear that dynamic motion of lipotubuloids in the cell greatly facilitates both incorporation of lipid precursors and enzymatic complexes and spreading of lipid degradation products contained in lipotubuloids. Disappearance of microtubules followed by disintegration of lipotubuloids into singular lipid bodies suggests that microtubules play a role in stabilizing lipotubuloids.

Information can be found in literature that biochemical analyses revealed tubulin in a fraction of isolated lipid bodies [51-53]. It is the more important since microtubules accompany lipid bodies not only in lipotubuloids. The connection between lipid bodies and microtubules has been found in *Marchantia paleacea* [54], in *Lactuca sativa* seeds [55] and in *Gelidium robustum* [56]. In animals there are other elements

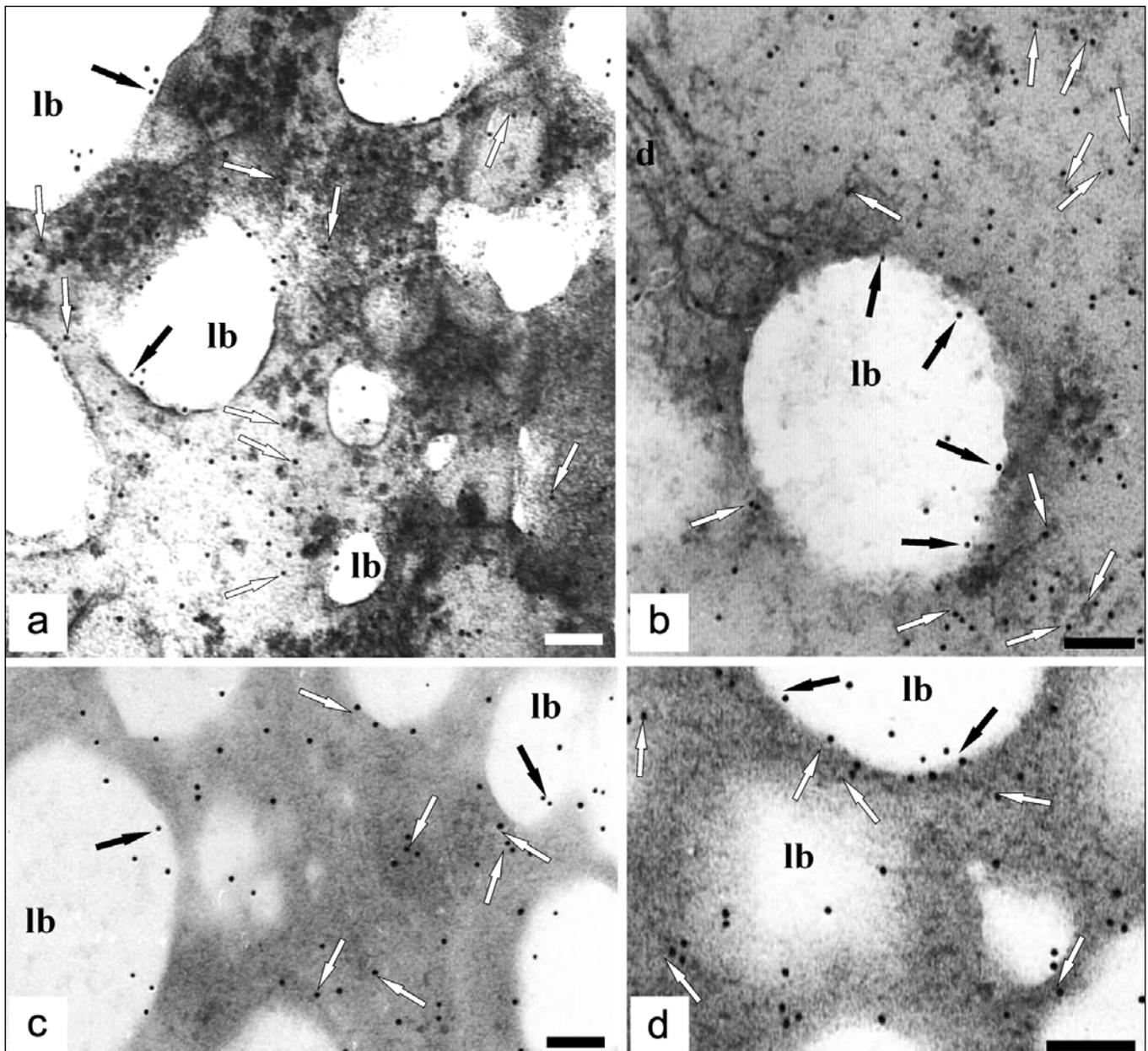


Fig. 6 Detection of α -tubulin with the immunogold technique in: **a** Epidermis of young *Vanilla planifolia* leaves; **b** Epidermis of *Althaea rosea* root; **c,d** Epidermis of *Funkia Sieboldiana* ovary. Black arrows indicate gold grains around lipid bodies, white arrows indicate gold grains at cross and longitudinal sections of microtubules. lb – lipid bodies. Scale bars: 200 nm.

of cytoskeleton around lipid globules, more stable than microtubules, these are 10 nm vimentin filaments, identified with immunogold technique during morphogenesis of adipocytes [57-59]. However, in the cumulus-oocyte complexes of mice there are microtubules visible close to lipid droplets [60]. Microtubules are also present at the surface of lipid bodies in human leukocytes [53]. Moreover, observations of LaPointe and Rodríguez [61] indicate that the synthesis or organization of a microtubule system connected with lipid bodies is involved in fat mobilization from the peritoneal fat bodies.

It is worth noting that in the species examined in these studies, similarly as in the case of *O. umbellatum* lipotubuloids, lipid bodies are accompanied by ribosomes, ER and some mitochondria, Golgi structures and microbodies and autolytic vacuoles containing lipase as well as acid and alkaline phosphatases [25,26]. Functional correlations between these organelles and lipid bodies were also observed during lipid transformations and cell functioning in animals [11].

Acknowledgements

This work is dedicated to memory of Prof. Anna Wałek-Czarnecka (1890-1978), who was pioneer of "elaioplast" research in our laboratory, in 120 anniversary of birth. This study was supported by the National Committee of Scientific Research, grant No. N N303 359035.

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