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Large lipid droplets in leaf epidermis of angiosperms

Anatoly Pautov[a,*], Olga Yakovleva[b], Elena Krylova[a], Galina Gussarova[a,c]

[a] Department of Botany, Faculty of Biology, St. Petersburg State University, Universitetskaya nab. 7-9, St. Petersburg 199034, Russia
[b] Laboratory of Plant Anatomy and Morphology, V.L. Komarov Botanical Institute RAS, Prof. Popov Str., 2, St. Petersburg 197376, Russia
[c] Natural History Museum, University of Oslo, NO-0318 Oslo, Norway

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ABSTRACT

Methods of light, scanning and transmission electron microscopy were used to study the lower epidermis of the leaves in evergreen *Acokanthera oblongifolia* (Apocynaceae), *Exbucklandia populnea* (Hamamelidaceae), *Ternstroemia gymnanthera* (Theaceae), and *Viburnum suspensum* (Caprifoliaceae). All these species show similar leaf anatomy. Our results together with those of earlier studies show that large lipid droplets (LDs) can regularly be found in leaf epidermis of some flowering plants. Their diameter varies in studied species from 3 to 11 μm. Large lipid droplets are typical for cells of stomatal complexes and can have irregular shape. They also occur in some ordinary cells. The formation of large LDs is accompanied by the presence of thick cuticle covering ordinary and subsidiary cells, prominent cuticular outer ledges and rims on stomata, waxy plugs in the outer stomatal cavities (in *A. oblongifolia*), and abundant plastoglobules in plastids. All these indicate that intensive lipid synthesis occurs in leaf epidermis. The stomatal complexes are the place with the highest occurrence of the largest LDs. They are formed in subsidiary cells, which lie beneath the stomata. In these cells plastids and mitochondria are always present. The former contain lipid inclusions. We demonstrated that breakdown of LDs takes place in dying leaves of *V. suspensum*. This might indicate mobilization of lipids from the globules into the LDs. Based on these new data we suggest that the LDs found in cells of stomatal complexes play important role in maintaining normal function of stomata.

1. Introduction

Cytosolic lipid droplets (LDs) occur in all major groups of plants (Murphy, 2012). Their size varies broadly, from 3 to 18 μm. Some authors state that there is no qualitative difference between the larger and the smaller LDs. They use terms lipid droplets, lipid globules, lipid bodies, oil bodies, oleosomes and spherosomes interchangeably (Parker and Murphy, 1981; Chapman et al., 2012). According to others, application of terms for large and small LDs requires additional histochemical, biochemical and ultrastructural analyses of both types (e.g. Lersten et al., 2006). It could be that they are not only different in size, but also in chemical composition, as well as in organization of boundary membrane or other lipid and protein interfaces (Lersten et al., 2006). The most commonly accepted term in the current literature is "lipid droplets" (Murphy, 2012), which we use throughout the text. In addition to cytosolic LDs, plastids can also accumulate LD-like structures known as plastoglobules (Bréhélin et al., 2007; Murphy, 2012; Piller et al., 2012). The tapetal cells of some flowering plants contain organelles called tapetosomes. They are composed of lipid droplets and vesicles (Hsieh and Huang, 2005).

Lipid droplet is a cellular organelle that stores neutral lipids as a source of energy and carbon (Fujimoto and Parton, 2011; Chapman et al., 2012; Liu et al., 2013). New data, complementing this classical point of view, are available (Murphy, 2001; Fujimoto et al., 2008; Murphy et al., 2009; Beller et al., 2010; Murphy, 2012; Kohlwein et al., 2013). Lipid droplets are highly dynamic cellular organelles. They are involved in many processes, such as stress response, pathogen resistance, and hormone metabolism (Chapman et al., 2012; Gidda et al., 2013). For example, there are data that small LDs function in the leaves of *Arabidopsis* as subcellular factors for production of a stable phytoalexin in response to fungal infection and senescence (Shimada et al., 2014). Aubert et al. (2010) showed that *RD20*, a stress-inducible caleosin localized to LDs of the guard cells of leaves, participates in stomatal control of transpiration and drought tolerance in *Arabidopsis*. Lipid droplets take part in the formation of various structures. They provide fatty acids to cellular processes including synthesis of membranes (Thiam et al., 2013; Herms et al., 2015). It has been also established that resources of

* Corresponding author.
E-mail address: a.pautov@spbu.ru (A. Pautov).

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lipids, represented in cell by LDs, are soon exhausted during the rapid synthesis of cuticle (Sack and Paolillo, 1983).

Similar to cytosolic LDs, plastoglobules play important roles in a wide range of physiological processes (Murphy, 2012). For example, their proteins probably participate in the formation, disassembly and turnover of plastid membrane complex, stress responses and other (Chen et al., 1998; Rottet et al., 2015). Tapetosomes play a predominant role in pollen-coat formation (Hsieh and Huang, 2005, 2007). This cover is a hydrophobic barrier that protects the pollen grains from dehydration and other negative factors.

The mature cytosolic LDs are spherical. They have diameter of 0.5–2 μm in most cell types (Murphy, 2012). Lipid droplets consist of a neutral lipid core that is bounded by a phospholipid monolayer membrane including proteins (Yatsu and Jacks, 1972; De Domenico et al., 2011; Chapman et al., 2012). Lipid droplets found in tissues of vegetative organ tend to be larger compared with those from seeds. For example, large LDs occur in leaf cells of many angiosperms. A study of 302 species and hybrids representing 113 families showed their presence in 24.5% of dicotyledonous and in 7.3% of monocotyledonous plants (Lersten et al., 2006). Diameter of the LDs varies from 3 to 18 μm. The lower boundary of sizes is arbitrary. It has been observed that in grasses a rapid increase in size of the LDs happens immediately after full emergence of the leaves. Smaller LDs develop into larger ones by adhesion and coalescence with each other. The rate of their growth decreases with leaf senescence (Chonan et al., 1980, 1984).

Different opinions exist in regard to the function of the large cytosolic LDs. They can serve as a short-time storage energy reserve (Parker and Murphy, 1981), or act as an intermediate storage products of photosynthesis (Lersten et al., 2006). Some data do not confirm these suppositions. For example, the diameter of LDs, found in seeds is less than the same of the cytosolic LDs, only 0.6–2.0 μm. This range of sizes is considered to provide the most efficient packaging and mobilization during germination (Napier et al., 2001). Large quantities of LDs, which are accumulated in the upper leaves of grasses, do not decrease during grain ripening (Chonan et al., 1984). They remain in dead leaves without any visible destruction (Chonan et al., 1980). In all of the examples discussed above, there is neither indication of uptake of lipids from the globules by developing grains, nor of the outflow of lipids from the dying leaves into other organs. There are however other data. In some cases seasonal changes of the LDs were observed. They were observed in *Diapensia lapponica* L., collected in northern Finland. Each mesophyll cell of this plant contains a single large LD during summer and many smaller LDs during cold months (Pihakaski et al., 1987). It is possible that this kind of changes decreases the freezing point of cells.

Large LDs are localized in the cytoplasm. They occur in mesophyll and bundle-sheath cells (Chonan et al., 1980, 1984; Parker and Murphy, 1981). In mesophyll LDs are formed in cells of chlorenchyma. Hypodermis of growing leaves of poplars contains large quantities of LDs with diameter 0.6–1.4 μm. Their lipids are mobilized during hypodermal cell differentiation. In these plants small vacuoles merging with each other appear in place of LDs (Pautov and Telepova-Texier, 1999; Pautov, 2002). It is noted that large LDs are normally not observed in epidermal cells of leaves (Price, 1912; Linsbauer, 1930; Lersten et al., 2006).

While examining stomatal complexes of plants from various taxonomic groups, with different ecological preferences, we discovered large LDs in epidermis of some of the species. This finding was in conflict with the earlier view accepted in botanical literature and gave impetus to current study. The species in which large LDs were found became our research focus. Our study aim is to demonstrate the obligatory occurrence of large LDs, which are formed in stomatal complexes of leaf epidermis in some flowering plants. The research goals are: (i) examination of structure of the leaves having epidermis with large LDs; (ii) identification of structural type of cells where large LDs are formed; (iii) evaluation of regularity of their formation.

2. Materials and methods

We studied leaves of *Acokanthera oblongifolia* (Hochst.) Codd (Apocynaceae), *Exbucklandia populnea* (R.Br. ex Griff.) R.W.Brown (Hamamelidaceae), *Ternstroemia gymnanthera* (Wight & Arn.) Bedd. (Theaceae) and *Viburnum suspensum* Lindl. (Caprifoliaceae). All plant material was collected in the Botanical gardens of the St. Petersburg State University and Komarov Botanical Institute (St. Petersburg, Russia). The cross sections of leaves were made from the middle part of a blade between its margin and midrib.

The fragments of the lower epidermis were analyzed. They were cut from fresh leaves using razor blade. Sudan III dye and Sudan black B dye were added to test for lipids. Presence/absence of the oil bodies in intact cells was recorded using light microscope, Leica DM1000 (Germany). All photographs were taken with a Leica EC3 camera (Germany).

The fragments of leaves were prefixed in the mixture of 3% glutaraldehyde and formaldehyde in 0.1 M phosphate buffer, (pH 7.4). Then they were rinsed in phosphate buffer and fixed in 2% osmium tetroxide. The material was dehydrated in acetone series embedded in epon-araldite mixture. Ultrathin sections were made by Ultracut E ultramicrotome (Reichert-Jung) and stained with lead citrate. The ultrathin sections of leaves were studied and photographed using transmission electron microscopes (TEM): Tesla BS-500 (Czech Republic) and Zeiss Libra 120 (Germany).

For scanning electron microscopy (SEM) samples were dehydrated in ethanol and isoamyl acetate series, critical-point dried in liquid CO_2, mounted on aluminum stubs, sputter coated with a layer of gold (70–100 nm), and observed with SEM JSM-6390LA (USA).

3. Results

All studied plants are evergreen. Their leaves are simple. *A. oblongifolia* has leathery leaves. Blades of *A. oblongifolia*, *T. gymnanthera*, *V. suspensum* have an average area of 20–40 cm^2, in *E. populnea*—150 cm^2. Leaves are thick. The blade width is 300–360 μm in *V. suspensum*, *E. populnea*, *A. oblongifolia*, and circa 500 μm in *T. gymnanthera.* The number of cell layers in mesophyll varies from 9–10 in *E. populnea*, *V. suspensum* and to 13–14 in *A. oblongifolia*, *T. gymnanthera.* Leaf blades in all species are dorsiventral. The ratio of the palisade tissue width to the mesophyll width is 15–20% in *T. gymnanthera* and *A. oblongifolia*, 30–40% in *E. populnea* and *V. suspensum.* Mesophyll of *T. gymnanthera* contains astrosclereids. The leaf blades are covered with thick cuticle. For example, in *V. suspensum* its average width in lower epidermis reaches more than 2 μm. Cuticle also covers the surface of subsidiary and adjacent ordinary cells turned towards the intercellular spaces. In *V. suspensum* the average width of this is 0.2 μm. Leaves are hypostomatic. The large LDs were found in all species in the cells of stomatal complex.

An average stomatal index in studied leaves is from 4% (*A. oblongifolia*) to 8% (*T. gymnanthera*). The number of stomata per 1 mm^2 varies from 90 to 120 (*A. oblongifolia*, *E. populnea*, *T. gymnanthera*), and up to 180 (*V. suspensum*). Their average length is 30 (*V. suspensum*)—38 μm (*A. oblongifolia*, *T. gymnanthera*). Stomata have large outer ledges and stomatal rims (Fig. 1A–C). In *A. oblongifolia*, *E. populnea* and *T. gymnanthera* stomatal rims are located on the outer ledges (Fig. 1B). In *V. suspensum* stomatal rims surround outer ledges and occur directly on the walls of guard cells (Fig. 2B).

Stomata of *A. oblongifolia* and *E. populnea* are cyclocytic. In *V. suspensum* they are laterocytic, or sometimes paracytic. Their guard