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3-D analysis of dictyosomes and multivesicular bodies in the green alga *Micrasterias denticulata* by FIB/SEM tomography

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ABSTRACT

In the present study we employ FIB/SEM tomography for analyzing 3-D architecture of dictyosomes and formation of multivesicular bodies (MVB) in high pressure frozen and cryo-substituted interphase cells of the green algal model system *Micrasterias denticulata*. The ability of FIB/SEM of milling very thin 'slices' (5–10 nm), viewing the block face and of capturing cytoplasmic volumes of several hundred μm^3 provides new insight into the close spatial connection of the ER–Golgi machinery in an algal cell particularly in *z*-direction, complementary to informations obtained by TEM serial sectioning or electron tomography.

Our FIB/SEM series and 3-D reconstructions show that interphase dictyosomes of *Micrasterias* are not only closely associated to an ER system at their cis-side which is common in various plant cells, but are surrounded by a huge "trans-ER" sheath leading to an almost complete enwrapping of dictyosomes by the ER. This is particularly interesting as the presence of a trans-dictyosomal ER system is well known from mammalian secretory cells but not from cells of higher plants to which the alga *Micrasterias* is closely related. In contrast to findings in plant storage tissue indicating that MVBs originate from the trans-Golgi network or its derivatives our investigations show that MVBs in *Micrasterias* are in direct spatial contact with both, trans-Golgi cisternae and the trans-ER sheath which provides evidence that both endomembrane compartments are involved in their formation.

1. Introduction

The plant Golgi apparatus positioned at the cross-road between the secretory-biosynthetic and the endocytotic-vacuolar pathway fulfills a great number of different functions ranging from polysaccharide synthesis, protein glycosylations, and transfer, sorting of products, regulation of vesicle trafficking up to vacuole formation and participation in degradation processes (among numerous others, see reviews by Faso et al. (2009), Hawes and Satiat-Jeunemaitre (2005)). As synthesis site for most constituents of the cell wall, it is central in plant development and plays an important role in response to environmental impact. The diverse functions of the Golgi apparatus are reflected in the unique morphology and structural integrity of the numerous motile dictyosomes of a plant cell (Hawes, 2005). While spatially and functionally tightly associated to the ER from where they are supplied with proteins and lipids, the flattened closely attached cisternae of a dictyosome usually display clear cis–trans-polarity. Cis-, median- and trans-cisternae of a Golgi stack are involved in different steps of product processing and the tubular-reticular trans-Golgi network (TGN) acts as a sorting station that marks outgoing cargo for its destination and also holds the role of an early endosome (Hwang and Robinson, 2009; Staehelin and Moore, 1995; Viotti et al., 2010). The early endosome is the first compartment that receives cargo endocytosed from the plasma membrane (Otegui and Spitzer, 2008). Via different intermediate stages it may then mature to the late endosome which is frequently named multivesicular body (MVB). MVBs are essential for membrane recycling back to the TGN, may act as constituents of the degradation pathway ending up in the vacuole (Lam et al., 2007; Otegui and Spitzer, 2008; Robinson et al., 2008; Tanchak and Fowke, 1987) or may even be involved in the formation of autophagosomes (for references see below).

By means of three-dimensional high-voltage electron microscopy early studies by Marty (1978, 1999) provided evidence that the TGN acts as starting point for generation of vesicle like provacuoles which develop to tubular prevacuoles by microinvagination of their membranes (see also Bassham et al. (2006)). Provacuoles, prevacuoles or prevacuolar compartments are regarded as

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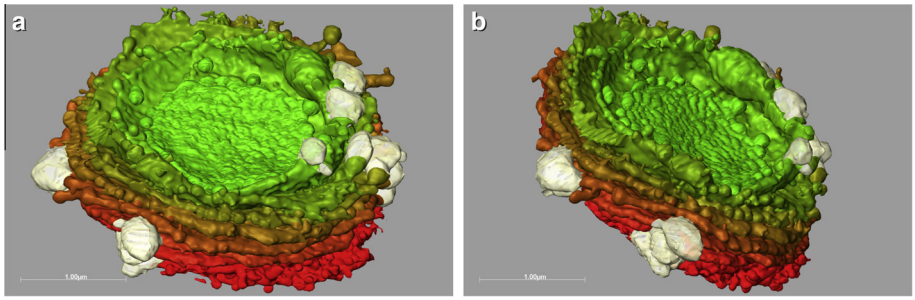

Fig.1. (a), and (b) Different 3-D views of the trans-side of a dictyosome of *M. denticulata* reconstructed from FIB/SEM series. Cisternal rims are lacerated, outermost-trans-cisterna are shorter than others and undulated. Secretory vesicles (grey) are connected to edges of trans-cisternae. From red to green: cis- to trans-cisternae.

convergence point for either the endocytotic pathway or autophagy. Provacuoles may sequester portions of cytoplasm by forming digitate extensions thus developing to early double-membrane autophagosomes lateron (Marty, 1978, 1999). Although the observations of Marty were based on chemically fixed tissue in which artificial membrane alterations cannot be excluded, the results are intriguing and deserve verification by means of a more reliable structure preservation technique. More recent publications on high pressure frozen *Nicotiana* BY-2 cells (Tse et al., 2004) and *Arabidopsis* root tips (Scheuring et al., 2011) identified MVBs as prevacuolar compartments or provacuoles arising from one particular domain of the TGN and able to fuse with the vacuole in a non-vesicular way. This process requires both TGN integrity and V-ATPase activity (Scheuring et al., 2011). In protein storage tissue of *Arabidopsis* embryos it has been found that two different populations of TGN derived vesicles containing either the storage protein or the processing enzymes, fuse into MVBs functioning as pre-vacuolar compartments (Otegui et al., 2006). Whereas numerous participants in these different degradation pathways have been identified, the structural transformation from the TGN into the MVB is still obscure. This process however is crucial for both understanding the endocytotic and the degenerative pathway.

High pressure freeze fixation (HPF) for best structural preservation (Staehelin et al., 1990) combined with 3-D analysis such as electron tomography has been proved to be an excellent tool for getting insight into the development of dictyosomal or ER derived structures and their functions at high resolution (Donohoe et al., 2006; Hayashi-Nishino et al., 2009; Kang and Staehelin, 2008; Kang et al., 2011; Knott et al., 2008; Mogelsvang et al., 2004; Ylä-Anttila et al., 2009). Although the benefit of this technique for detailed structural analysis is undoubted, its limitations arise from the maximum thickness of the sections (400 nm; see Donohoe et al. (2006)), from the maximum tilt angle of about 70° causing a "missing wedge" and from the relatively small volume (max. ~25 μm^3) that can be calculated for the 3-D reconstructions. The new technique of focused ion beam milling and viewing by field emission scanning electron microscopy (FIB/SEM) overcomes these problems and provides additional structural information by its ability of sectioning very thin slices (5–10 nm) parallel to the block face and by covering volumes of several hundreds of μm^3 (Knott et al., 2008; Schroeder-Reiter and Wanner, 2009; Schroeder-Reiter et al., 2009, 2012). The resolution of FIB/SEM tomography does not yet reach the resolution of TEM in *x*/*y* but is close to it as clearly demonstrated in a recent publication (Villinger et al., 2012). In respect to analysis of dictyosomal derived membranes an additional advantage of this method is provided by the fact that several dictyosomes of a cell can be captured at the same time.

In the present study we employed this technique for analyzing the 3-D architecture of interphase dictyosomes of the algal model system *Micrasterias denticulata* (Meindl, 1993) and to obtain insight into structural connections between MVBs and endomembrane systems. This alga is very well suited for such investigations as it possesses large dictyosomes with a constant average number of 11 cisternae throughout the cell cycle and their vesicular products during different developmental stages are well defined (Eder and Lütz-Meindl, 2008; Eder et al., 2008; Lütz-Meindl and Brosch-Salomon, 2000; Oertel et al., 2004). Moreover, numerous studies influencing the secretion pathway have provided information on the regulation of the secretory machinery (Lehner et al., 2009; Salomon and Meindl, 1996). Recently evidence has been obtained that *Micrasterias* is capable of performing autophagy and programmed cell death upon induction by abiotic stressors such as oxidative stress, high salinity or cadmium (Affenzeller et al., 2009a,b; Andosch et al., 2012). Coincidently with the occurrence of autophagy, environmental stress evokes severe structural alterations at dictyosomes (Affenzeller et al., 2009a,b; Darehshouri et al., 2008; Volland et al., 2011). Particularly cadmium induces a dose and time dependent complete disintegration of dictyosomes combined with an increase in the number of MVBs (Andosch et al., 2012). Although, like in other plant and animal systems, the ER contributes to autophagosome formation in *Micrasterias* there are several indications that MVBs may functions as sources for stress induced degeneration or even autophagosome formation as well (see above). Information on the structural origin of MVBs as provided by FIB/SEM tomography will therefore be important as a basis to understand stress induced degradation processes. The present study shows that MVBs are frequently found in structural contact with both trans-Golgi cisternae and a "trans-ER". As *Micrasterias* is a member of Streptophyta belonging to the closest relatives of higher plants (Wodniok et al., 2011) we expect that the results obtained may be of general interest for plant cells and may also provide insight into evolutionary aspects of the first steps of degradation.

2. Materials and methods

All chemicals were purchased from Roth (Karlsruhe, Germany) or Sigma–Aldrich (Vienna, Austria) unless stated differently.

2.1. Cell cultures

The unicellular freshwater green alga *M. denticulata* was cultivated in a liquid Desmidiacean medium (Schlösser, 1982) in Erlenmeyer flaks at constant temperature of 20 °C and a light/dark regime of 14/10 h. Cells were subcultured every 4–5 weeks. To obtain defined interphase stages for FIB/SEM analyses, developmental stages were collected and were allowed to grow in nutrient solution for 48 h.

2.2. High pressure freeze fixation for TEM and FIB/SEM tomography

Interphase cells of *M. denticulata* were high-pressure frozen and cryo-substituted following the protocol of Aichinger and