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Ultrastructure of the Periplastidial Compartment of the Diatom *Phaeodactylum tricornutum*

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Diatoms contain a secondary plastid that derives from a red algal symbiont. This organelle is limited by four membranes. The two outermost membranes are the chloroplast endoplasmic reticulum membrane (cERM), which is continuous with the host outer nuclear envelope, and the periplastidial membrane (PPM). The two innermost membranes correspond to the outer and inner envelope membranes (oEM and iEM) of the symbiont's chloroplast. Between the PPM and oEM lies a minimized symbiont cytoplasm, the periplastidial compartment (PPC). In *Phaeodactylum tricornutum*, PPC-resident proteins are localized in "blob-like-structures", which remain associated with plastids after cell disruption. We analyzed disrupted *Phaeodactylum* cells by focused ion beam scanning electron microscopy, revealing the presence of a vesicular network (VN) in the PPC, at a location consistent with blob-like structures. Presence of a VN in the PPC was confirmed in intact cells. Additionally, direct membrane contacts were observed between the PPM and nuclear inner envelope membrane at the level of the chloroplast-nucleus isthmus. This study provides insights into the PPC ultrastructure and opens perspectives on the function of this residual cytoplasm of red algal origin.

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Key words: *Phaeodactylum*; diatoms; secondary plastids; secondary endosymbiosis; periplastidial compartment; SELMA.

Introduction

Diatoms constitute a major group of phytoplankton in oceans and freshwater ecosystems, and are so ecologically successful that they are responsible for up to one fourth of global primary productivity (Field et al. 1998). Based on comprehensive surveys of oceanic biodiversity, diatoms are spread globally and are the most diverse photosynthetic eukaryotic lineage (de Vargas et al. 2015; Massana et al. 2015). A striking feature of diatoms is their

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sophisticated ultrastructure, inside highly packed cells, including a chloroplast bounded by four membranes, known as a 'secondary' or 'complex plastid'. Our understanding of the structure of this organelle and its relationship with the rest of the cell is fragmentary. Plastid-mitochondrion metabolic interactions were recently shown to optimize bioenergetic coupling, being one of the reasons for diatoms' performance in ecosystems (Bailleul et al. 2015). The stroma of diatoms' chloroplast is also the site of production of fatty acids, which are precursors for the biosynthesis of all membrane and storage glycerolipids, but it is still unknown how these fatty acids and glycerolipids can traffic across and inside subcellular membranes to reach their final destination (Abida et al. 2015). Important shuttling of proteins, lipids and other metabolites is therefore expected to occur through the four membranes limiting the plastid. Diatom glycerolipids are considered a promising feedstock for biofuels and other lipid-derived chemicals (Levitan et al. 2014). It is therefore essential, but challenging, to advance knowledge on the subcellular organization and connectivity of membranes within diatom cells.

The secondary plastid derives from the engulfment of a red alga by another eukaryotic cell, followed by the reduction of the symbiont subcellular structures (Cavalier-Smith 2003; McFadden 2014; McFadden and van Dooren 2004; Nisbet et al. 2004). Such secondary plastids are found in groups that are distant from diatoms (Heterokonta), like Cryptophyta, Haptophyta, Chromerida or Apicomplexa (Cavalier-Smith 2003; Dorrell and Smith 2011; Gibbs 1962a, b, c, 1979, 1981; Marechal and Cesbron-Delauw 2001; Petroutsos et al. 2014). Secondary plastids are therefore chimeric organelles, combining host and symbiont-derived structures. The outermost membrane, termed the 'chloroplast endoplasmic reticulum membrane' (cERM, Fig. 1) (Gibbs 1979) is supposed to derive from the host phagocytic membrane (Cavalier-Smith 2003; McFadden and van Dooren 2004; Nisbet et al. 2004) and is therefore expected to be phospholipid rich (Abida et al. 2015; Petroutsos et al. 2014). In diatoms, the cERM is directly connected to the host outer nuclear envelope membrane (oNE) and the ER (Bouck 1969; Kroth et al. 2008). In other groups, like Apicomplexa, the cERM and the endomembrane system are not continuous and transfers of material occurs *via* vesicular trafficking (Heiny et al. 2014; van Dooren et al. 2000, 2001). Underneath, the 'periplastidial membrane' (PPM, Fig. 1) is considered to derive from the symbiont plasma membrane (Grosche et al. 2014), although an alternative origin from the host ER has been recently proposed (Gould et al. 2015). The nature of the two innermost membranes of the chloroplast is not debated, being reminiscent of the galactolipid-rich chloroplast envelope of the symbiont, called the 'outer' and 'inner envelope membranes' (oEM and iEM, Fig. 1) (Botte and Marechal 2014; Petroutsos et al. 2014). Between the PPM and oEM lies a minimized symbiont cytoplasm, the 'periplastidial compartment' (PPC, Fig. 1) (Grosche et al. 2014). The cytoplasmic remains of the red algal symbiont show different degrees of reduction. Cryptophytes like *Guillardia theta* contain a minimized version of the nucleus in the PPC, called the nucleomorph (Curtis et al. 2012), whereas other groups including diatoms have completely lost the symbiont nucleus.

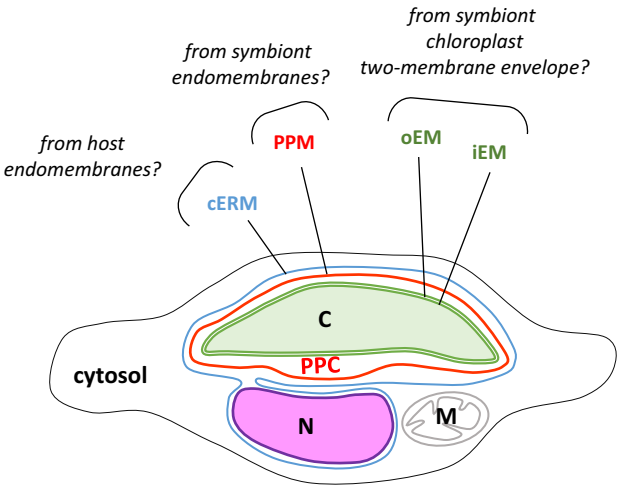

Figure 1. Chimeric organization of the secondary plastid in diatoms. The scheme shows a fusiform cell of *Phaeodactylum*. The plastid is limited by 4 membranes. The chloroplast endoplasmic reticulum membrane (cERM), shown in blue, is continuous with the outer nuclear envelope membrane. The periplastidial membrane (PPM) is shown red. The outer and inner envelope membrane (oEM and iEM), shown in light green, are tightly apposed. The presence of a specific periplastidial compartment (PPC) is based on the detection of blob-like structures observed by confocal microscopy, in which protein precursors fused to GFP and crossing only the cERM and the PPM reside. The presence of a vesicular network (VN) in the PPC is addressed here. C, chloroplast; N, nucleus; M, mitochondrion.

Phaeodactylum tricornutum is by far the most studied pennate diatom, following the complete sequencing and annotation of its genome (Bowler et al. 2008), the development of molecular tools for

gene expression and functional characterizations (Apt et al. 1996; De Riso et al. 2009; Falciatore et al. 1999; Siaut et al. 2007) and the production of reference data for membrane lipidomic studies (Abida et al. 2015). *P. tricornutum* is pleiomorphic, with three major morphotypes, *i.e.* fusiform (shown in Fig. 1), triradiate and oval. A series of axenic strains have been collected in various marine environments worldwide, including Pt1, which has been analyzed here (De Martino et al. 2007). Subcellular localization of proteins in the secondary plastid of *P. tricornutum* relies on confocal imaging of cells expressing the green fluorescent protein (GFP) fused to various addressing sequences (Gould et al. 2006; Grosche et al. 2014; Gruber et al. 2007; Hempel et al. 2009; Kilian and Kroth 2005; Moog et al. 2011; Peschke et al. 2013; Sommer et al. 2007).

Most plastid proteins in *P. tricornutum* are nuclear encoded: their sequences contain a bipartite topogenic signal (Bts), comprising an N-terminal signal peptide (Sp), a chloroplast-like transit peptide (Ctp) and an amino acid motif at the cleavage site of the Sp, termed the ASAFAP motif (Gould et al. 2006; Gruber et al. 2007; Kilian and Kroth 2005; Moog et al. 2011). Three major translocating systems are involved to import plastid proteins harboring a Bts (Supplementary Material Fig. S1). Firstly, a sec61 complex operates by co-translational mediation of pre-proteins across the cER (Bolte et al. 2009). Secondly, the symbiont endoplasmic reticulum-associated degradation (ERAD) machinery has evolved to give rise to a translocon called the 'symbiont-specific ERAD-like machinery', or SELMA (Felsner et al. 2011; Hempel et al. 2007, 2009; Lau et al. 2015; Sommer et al. 2007; Stork et al. 2012, 2013). Thirdly, transport across the oEM and iEM involves components related to the classic chloroplast translocon, *i.e.* TOC and a TIC respectively (Bullmann et al. 2010; Heinz and Lithgow 2014; Schleiff and Becker 2011; Schleiff et al. 2011; Sommer et al. 2011; Stork et al. 2013; Wunder et al. 2007).

Following docking of ribosomes at the surface of the secondary plastid, the Sp determines the targeting *via* the cERM and PPM (Supplementary Material Fig. S1). The presence of a phenylalanine (F) or an aromatic residue at position +1 of the Ctp determines the transport across the oEM and the iEM. In the absence of such aromatic amino acid, proteins remain resident in the PPC (Gould et al. 2006; Gruber et al. 2007; Kilian and Kroth 2005; Moog et al. 2011). A recent study has shown that pre-proteins could be N-glycosylated prior their transport through the PPM (Supplementary Material Fig. S1), probably by the action of an oligosaccharide transferase (OST) (Peschke et al. 2013). An important question is then posed by this discovery, regarding the possibility to import some of the plastid proteins, most notably folded glycoproteins, *via* membrane translocators or *via* unknown vesicular trafficking systems.

The evidence for a protein to reside (or be blocked) inside the PPC lies on the detection of GFP fusions inside single spot-like structures at the periphery of the plastid, called "blob-like-structures' (Gould et al. 2006; Gruber et al. 2007; Kilian and Kroth 2005; Moog et al. 2011). The presence of membrane vesicles in blob-like structures was considered as possible based on the arrest of protein import by treatment with Brefeldin A (Kilian and Kroth 2005), however in following studies, this hypothesis was never confirmed. Consistently with the absence of vesicles, no PPC-specific component involved in vesicular lipid trafficking, such as Rabs, SNAREs, COPI, COPII, chlathrin, calveolin, ESCRT, GEFs or GAPs could be predicted (Moog et al. 2011).

In a comprehensive electron microscopy study of the chrysomonad *Ochromonas danica*, a vesicular network (VN) was observed in the PPC (Gibbs 1979). This VN has been initially called a periplastidial reticulum (Gibbs 1979). This network did not extend around the whole chloroplast of *O. danica*, but was found restricted to particular locations, close to the nucleus (Gibbs 1979). Apparent increase of this VN after treatment with cycloheximide and disappearance after treatment with chloramphenicol suggested a relation with protein import (Gibbs 1979). This study is often considered a reference to suggest that the PPC of diatoms may contain a VN, but the detection of membrane translocators and the lack of putative PPC proteins acting in lipid trafficking have been repeatedly used as an argument to consider the presence of vesicles as unlikely, or generated by unknown components (Gould et al. 2006; Moog et al. 2011; Peschke et al. 2013; Sommer et al. 2007). Ultrastructural study of chloroplasts in diatoms other than *Phaeodactylum* has supported the existence of a VN in the PPC (Bedoshvili et al. 2009), but location within the cell and conservation in the diatom phylum have not been assessed. The ultrastructure of the PPC needs therefore to be characterized in *P. tricornutum*. We analyzed by electron microscopy series of ultrathin sections of *P. tricornutum* cells, revealing the presence of a VN in the PPC, at a location corresponding to blob-like structures.

Results and Discussion

Since blob-like structures remain associated to chloroplasts after cell disruption (Kilian and Kroth 2005), we took advantage of this property and analyzed by electron microscopy series of ultrathin sections of disrupted *P. tricornutum* cells (Fig. 2A, B). Series of thin sections allow the detection of membrane connectivity in the three dimensions. Here, the thickness of section slices was 4 nm, and 200 to 600 sections were collected per sample, allowing the ultrastructure scanning of single organelles (chloroplast, mitochondrion or nucleus) from tangential sections (edges) to cross sections (Fig. 2C).

Focusing on a disrupted unpacked cell, the scanning of the region between a nucleus and a secondary plastid is shown in Figures 3, 4 and 5. In these series, the identity of the membranes is assessed by the connectivity in the two dimensions of the sections and the conservation from one section to the following, *i.e.* the third dimension. To help trace membrane identity and connectivity between sections, schematic representations are also shown: sections 1', 48', 140', 145', 152', 178', 184' and 208'.

Firstly, Figure 3 shows the most tangential region of the nucleus. Section 1 corresponds to the edge of this organelle, *i.e.* a tangential view of the oNE. Section 16 shows the tangential view of the iNE. The following sections, *e.g.* 30, 44 or 48, are transverse views of the nucleus, containing the chromatin, and allowing the visualization of nuclear pores (Fig. 3, section 48, NP). The uncondensed chromatin indicates that the cell is in interphase. In section 120, the cross section of the nuclear envelope is slightly irregular and shows a constricted area (Fig. 3, section 120, dashed circle). In this constricted

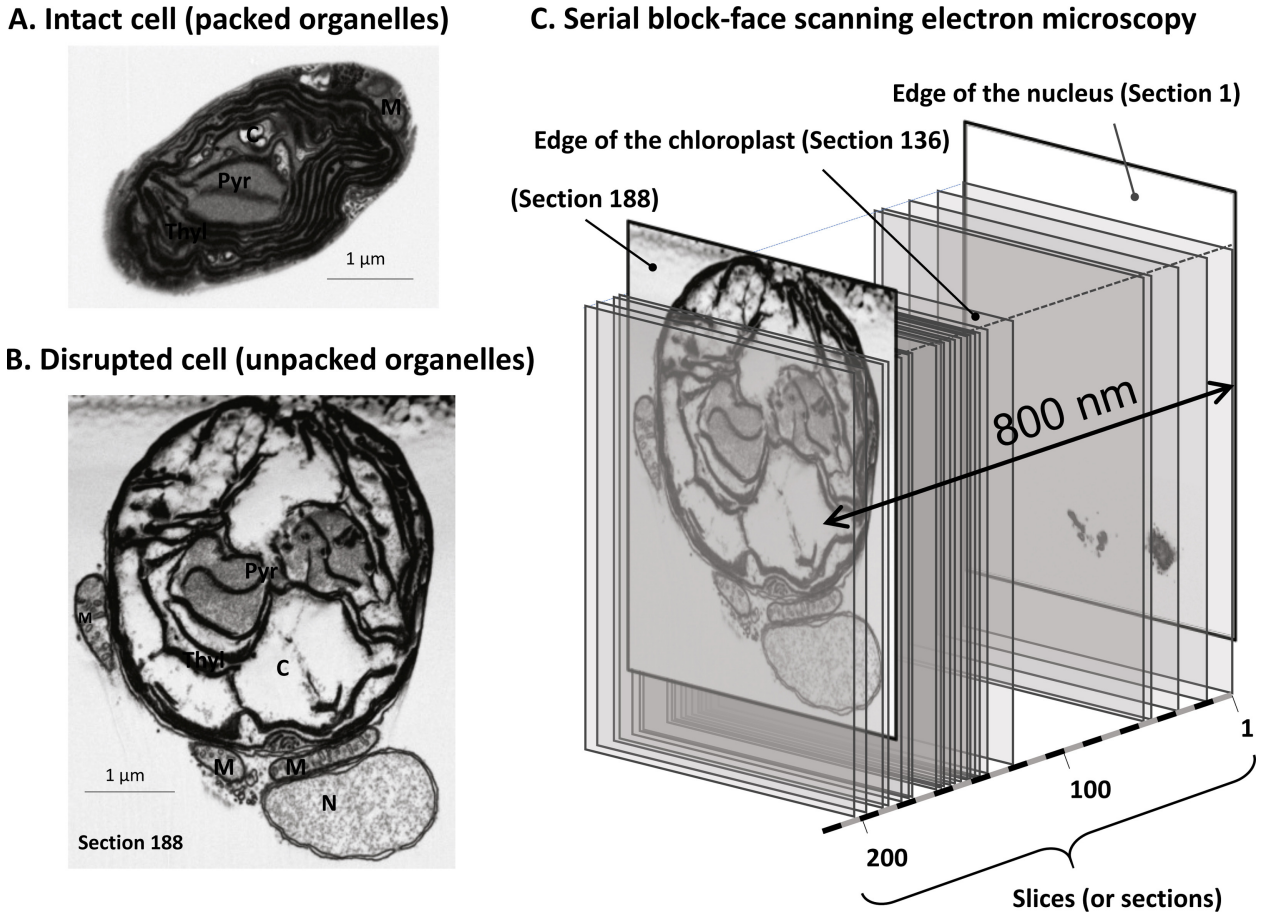

Figure 2. **A**. Electron micrograph of an intact *Phaeodactylum* cell. **B**. Disrupted cell. **C**. Lateral view of the serial scanning method. Slices or section are 4 nm-thin, and allow the detection of membrane continuity between successive cross sections. For 200 sections, the depth of the scanning is 0.8 µm. In the disrupted cell shown in Figures 3, 4 and 5, the tangential view of the nucleus is in section 1, and that of the chloroplast is in section 136. C, chloroplast; M, mitochondrion; N, nucleus; Pyr, pyrenoid; Thyl, thylakoids.

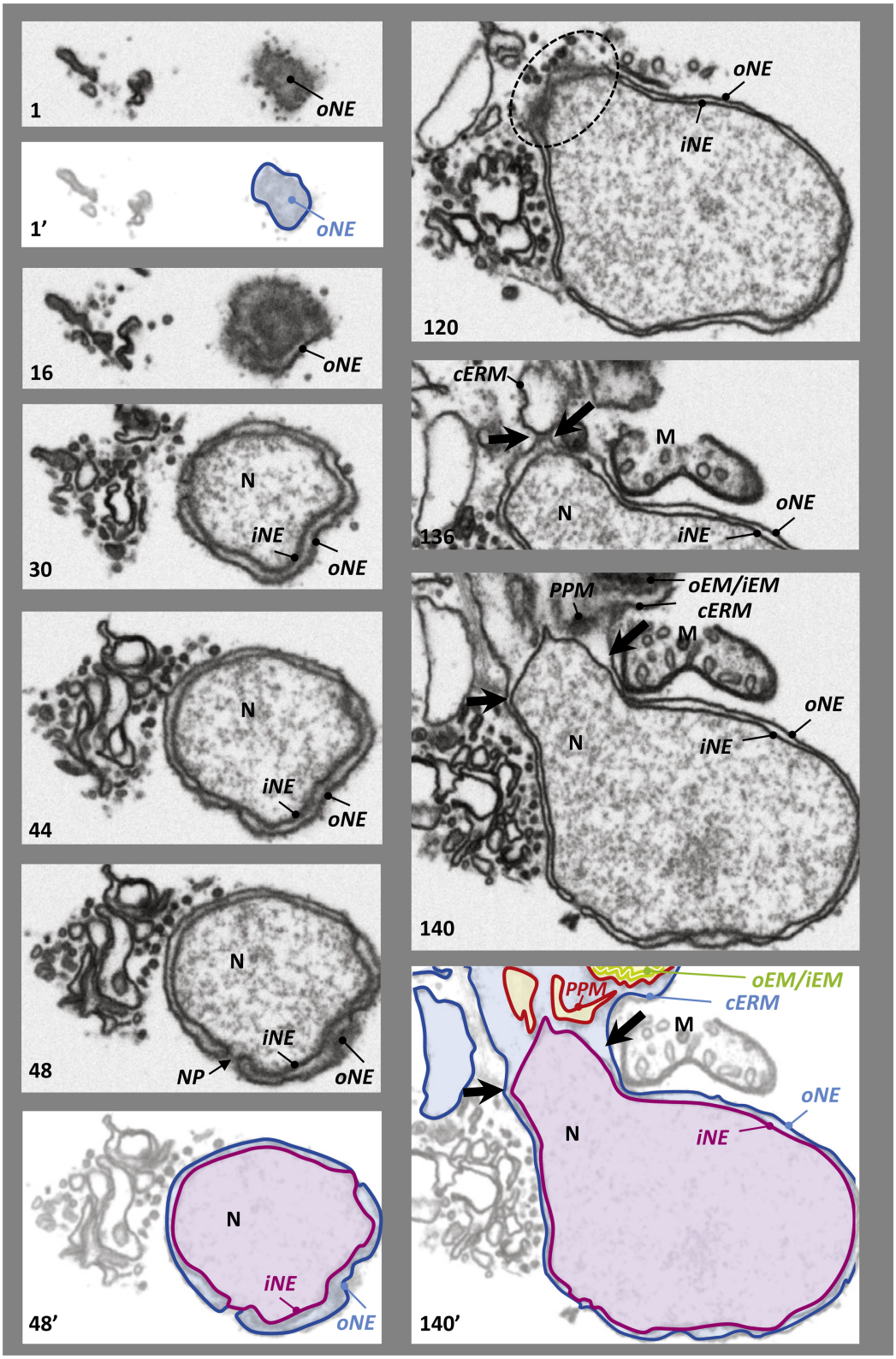

Figure 3. Serial electron micrograph scanning of a *Phaeodactylum* disrupted cell at the level of the cERM–oNE isthmus. The outer nuclear envelope is shown in blue from a tangential section (1) to the level of sub-spherical nucleus (48 and 48'), where it is lined with the inner nuclear envelope (iNE) shown in purple. The nucleus

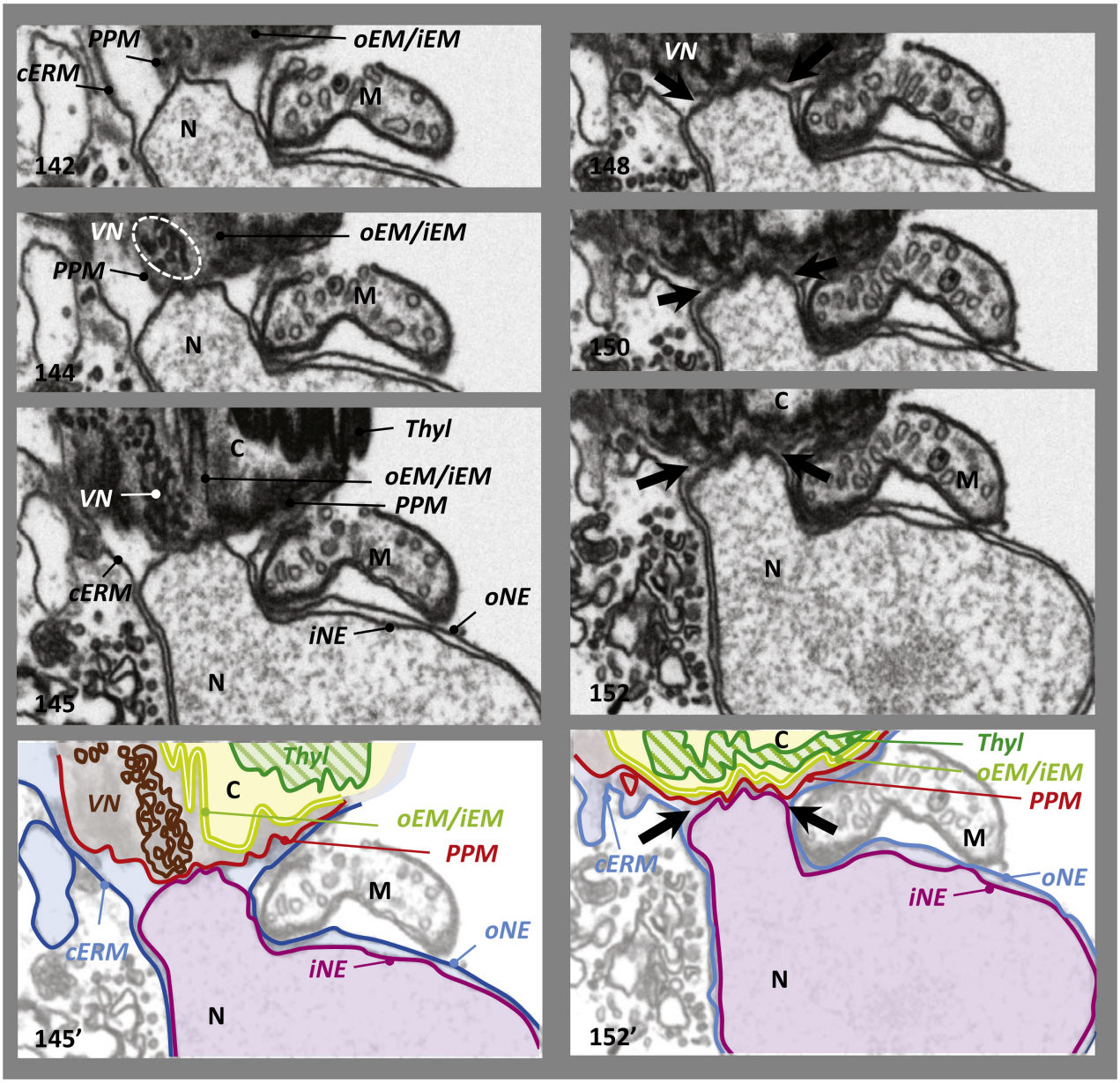

Figure 4. Serial electron micrograph scanning of a *Phaeodactylum* disrupted cell at the level of the iNE-PPM membrane contact. The outer nuclear envelope (oNE) is shown in blue in continuity with the cERM. The inner envelope membrane (iNE) is shown in purple and gets in very tight contact with the irregular periplastidial membrane (PPM) (from 142 and further). A vesicular network (VN) fills the space between the PPM and the two innermost membranes of the chloroplast, the outer and inner envelope membranes (oEM and iEM, respectively), shown in light green. C, chloroplast; M, mitochondrion; N, nucleus; Thyl, thylakoids.

then forms a constricted area shown in dashed lines (120). In the vicinity of the chloroplast, the oNE becomes continuous with the chloroplast endoplasmic reticulum membrane (cERM). The edges of the isthmus are shown with arrows. M, mitochondrion; N, nucleus; NP, nuclear pore.

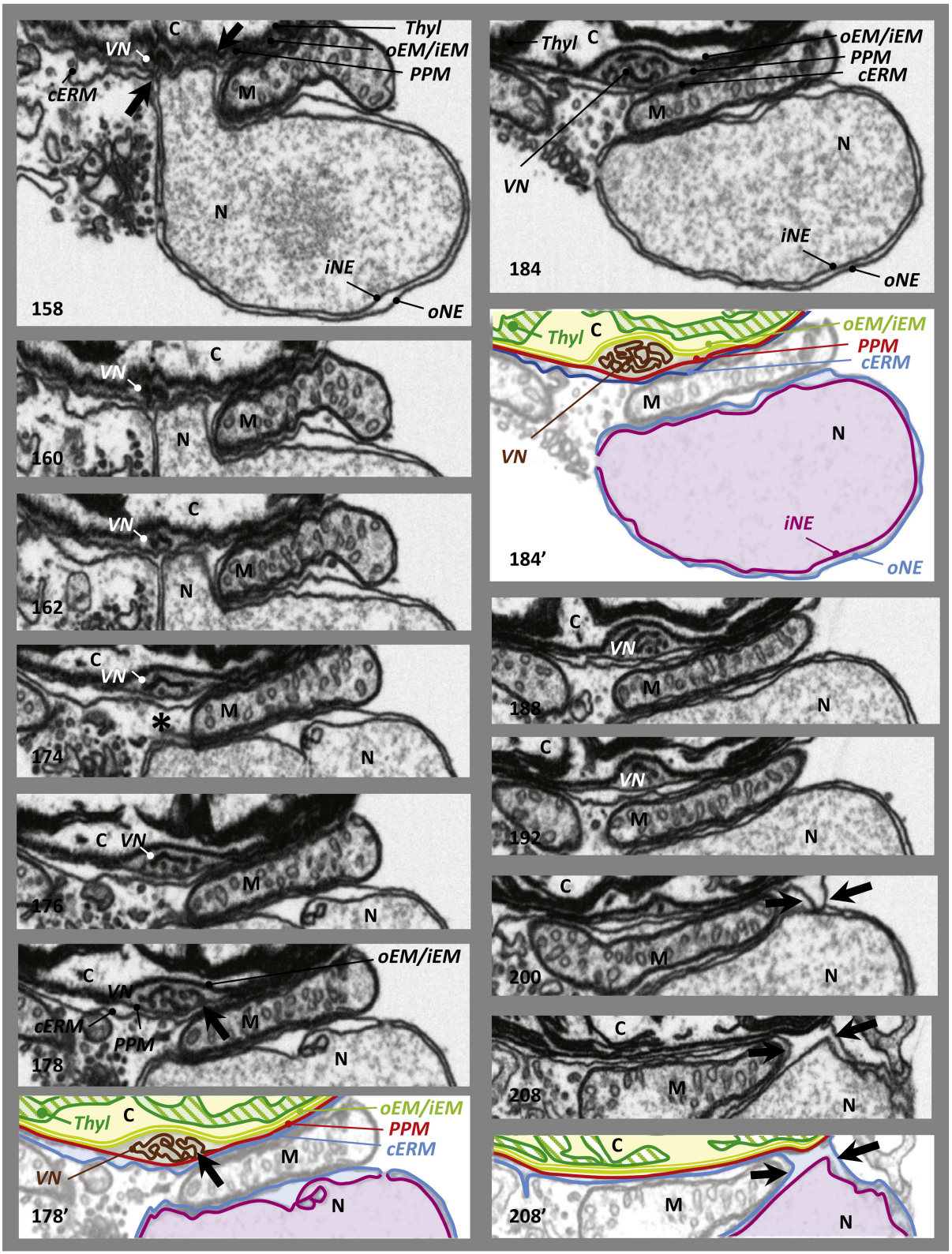

Figure 5. Serial electron micrograph scanning of a Phaeodactylum disrupted cell at the level of the periplastidial compartment. The outer nuclear envelope (oNE) is shown in blue in continuity with the cERM. The inner envelope membrane (iNE) is shown in purple in tight contact with the periplastidial membrane (PPM) at the

region of the nucleus the oNE is connected to the cERM (Fig. 3, sections 136, bold dark arrows). In section 140, this oNE-cERM isthmus gets larger (Fig. 3, sections 140, bold dark arrows) and irregular tangential sections of the PPM are visualized facing directly the iNE. Here, and in following sections, the oEM and the iEM appear as closely apposed membranes.

Secondly, Figure 4 shows a focus in the region where the iNE faces directly the PPM. In sections 142, 144 and 145 the PPM is less irregular and shows an increased apposition with the iNE. A vesicular network (VN) appears between the PPM and the oEM/iEM. The PPM/iNE membrane contact site expands from sections 148 to 152, becoming as large as the oNE-cERM isthmus.

Thirdly, Figure 5 allows visualizing the expansion of the VN in regions where the oEM and cERM are not connected. From sections 158 to 160 and 162, the oNE-cERM isthmus and the PPM/iNE membrane contact site are clearly visible, whereas section 174 shows disconnected cross sections of the chloroplast and the nucleus (Fig. 5, sections 174, star). The VN is still visible, indicating that the VN is close, but not strictly dependent on the nucleus-chloroplast isthmus. In sections 176 and 178, direct connections between the VN and the PPM are visible (Fig. 5, section 178/178', bold arrow), whereas no link between the VN and the oEM could be detected. The VN is visible in sections 188, 192 and is tangentially observed in section 200. Section 208 shows a second connection between the nucleus and the chloroplast (Fig. 5, section 208/208', bold arrows), but this time without any VN.

Thus this series illustrates that at the level of a large cERM-oNE isthmus a PPM/iNE membrane contact site is established and a VN appears between the PPM and the oEM, connected to the PPM but not to the oEM. The VN in the PPC is therefore at a location corresponding to that of blob-like structures observed by confocal microscopy in *Phaeodactylum* cells broken by osmotic shock, and initially reported to possibly contain membrane vesicles (Kilian and Kroth 2005).

Based on this analysis of disrupted and unpacked cells, we sought to establish whether the VN could be detected in intact cells. Figure 6 shows three examples. In cell 1, the VN lies within a groove between the PPM and the oEM, and appears therefore at two opposite locations of the chloroplast periphery in the vicinity of the nucleus (Fig. 6A, sections 203, 320 and 332, black arrows), in particular at the level of the nucleus-choroplast isthmus sections 332, star). In cell 2, the VN appears more distant from the nucleus (Fig. 6B, sections 235, 330 and 378, black arrow). In cell 3, the VN appear close to the nucleus, but not in the area where the chloroplast-isthmus occurs. Overall, the VN is therefore most often present close to the chloroplast-nucleus isthmus, where it might play a functional and structural role, although other locations are possible. Using the complete set of sections of cell 1 (Fig. 6A) we used the volume viewer of Fiji image analysis tools (Schindelin et al. 2012) to reconstitute cross sections perpendicular to the main axis of the cell (Fig. 6D). The three-dimensional organization of the VN can thus be viewed in Supplementary Data. The ultrastructure of the VN in *Phaeodactylum* is therefore similar to that of the VN observed in the *Ochromonas*, although in this chrysomonad, the VN was only located close to the nucleus and appeared to have some direct connection with the oEM in addition to the PPM (Gibbs 1979).

We also examined previously published *Phaedoctylum* electron micrographs. In most cases, it is barely possible to identify the limiting membranes of the chloroplast. In the in-depth study of dividing cells published recently (Tanaka et al. 2015), one can see in some of sections, membrane vesicular or reticulated structures in the periphery of the chloroplast, the precise nature of which could not be assessed at that time, and which might be a VN within the PPC. In other diatoms like *Thalassiosira proshkinae*, *Attheya ussurensis*, *Chaetoceros muelleri*, *Aullacoseira baicalensis*, *Synedra acus*, a similar VN structure could be observed at the periphery of chloroplasts (Bedoshvili et al. 2009). A PPM/iNE direct contact could also be observed in *Thalassiosira proshkinae* and *Chaetoceros muelleri* (Bedoshvili and Likhoshvai 2012). Thus, the organization of the PPC characterized here in *Phaeodactylum* is likely conserved in both pennate and centric diatoms.

level of the nucleus-chloroplast contact zone. The vesicular network (VN) fills the space between the PPM and outer envelope membranes (oEM), shown in light green. The VN is also present in regions where the chloroplast and the nucleus are not connected (star in 174, and further). The VN shows continuity with the PPM (178) but not with the oEM. Additional direct connections between the oNE and cERM are visible in regions where no VN can be observed (208). C, chloroplast; M, mitochondrion; N, nucleus; Thyl, thylakoids.

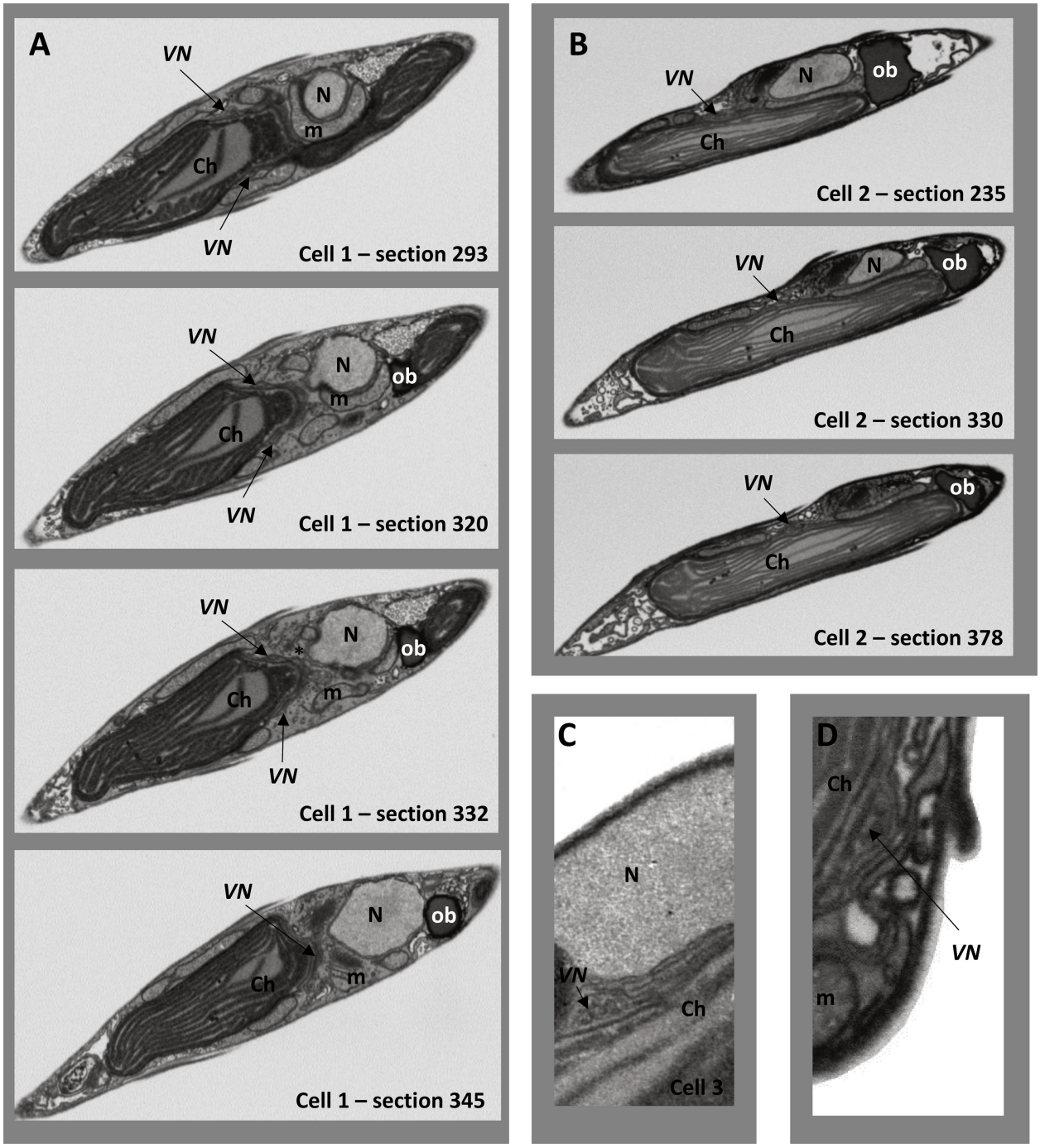

Figure 6. Serial electron micrograph scanning of *Phaeodactylum* intact cells at the level of the periplastidial compartment. Three cells are shown (cell 1, 2 and 3) in **A**, **B** and **C**. A magnified cross-section of cell 1 is shown in **D**, corresponding to the video provided in supplementary data. The vesicular network (VN) within the periplastidial compartment is shown with arrows. Ch, chloroplast; m, mitochondrion; N, nucleus; ob, oil body.

Probably the most important result of this study is that the PPC of diatoms is not empty. A residual cytoplasm exists, containing vesicles. None of the proteins that were reported to reside, or possibly reside, in the PPC could be predicted to act in vesicle formation, like SNAREs, Rabs, COPI, COPII, chlathrin, calveolin, ESCRT, GEFs or GAPS (Moog et al. 2011). The VN must therefore be generated by an unknown process. The identification of the proteins generating the VN in the PPC represents therefore an important challenge for future works.

The glycerolipid composition of each of the four membranes that surround the plastid is unknown, but the present study will be also crucial in future investigation related to membrane lipid biogenesis. It is difficult to speculate on the location of the classical lipids found in the envelope of primary chloroplasts in secondary plastids *i.e.* galactoglycerolipids (monogalactosyldiacylglycerol, MGDG and digalactosyldiacylglycerol, DGDG), sulfoquinovosyldiacylglycerol (SQDG) and phosphatidyldiacylgycerol (PG) (Abida et al. 2015; Boudiere et al. 2014; Petroutsos et al. 2014) or if phospholipids of the ER or nuclear envelope are also present and in the same proportions in the cERM. Lipid composition of the PPM could be related to that of the cERM, by importing phosphoglycerolipids, or to that of the oEM, by importing chloroplast lipids. In photosynthetic organisms, it is usually considered that in standard conditions phospholipids are mostly present in the endomembrane system, whereas non-phosphorus glycolipids are in the plastid. The analysis of isolated secondary plastids has been possible in Apicomplexa, which is non-photosynthetic and disconnected from the nucleus (Botte et al. 2013) and in that peculiar case, presence of galactoglycerolipids could not be shown (Botte et al. 2008, 2013; Botte and Marechal 2014), whereas plastid membranes were clearly enriched in phosphoglycerolipids and even sterols (Botte et al. 2013). Galactolipids could be detected in the photosynthetically active secondary plastid of a Chromerida by immunofluorescence confocal imaging (Botte et al. 2011). The comprehensive analysis of the lipidome of *Phaeodactylum* has shown the presence of MGDG, DGDG, SQDG and even a form of SQDG acylated on its polar head (Abida et al. 2015). Based on our observations, we speculate that galactoglycerolipids are present in the iEM and oEM and that phosphoglycerolipids and betaine lipids are likely present in the cERM and the PPM. Membrane lipid transfers might occur at the level of membrane contact sites, such as that observed here between the PPM and the IEM, or *via* dedicated platforms, such as the VN, or by other non-vesicular systems between adjacent membranes.

The continuity between the PPM and the VN suggests that the biogenesis of the VN depends on the PPC and not on the oEM. A summary of a possible scenario for the origin of the PPM and VN is thus given in Figure 7. Important questions raised by the present study lie in the molecular organization and the function of the membrane compartments we have unraveled. Are there structural proteins involved in the opening of a circular isthmus connecting the oNE and the cERM? What are the components maintaining the iME and the PPM closely apposed inside the opened oNE-cERM isthmus? Is the iME/PPM membrane contact site involved in the exchange of metabolites, ions, proteins or nucleic acids? The plastid of diatoms seem to encode its required set of tRNAs and rRNAs (Oudot-Le Secq et al. 2007), but other transfers of RNAs might occur. Retrograde signaling from the chloroplast to the nucleus (Lepetit et al. 2013) might also occur at the level of this chloroplast-nucleus isthmus. What are the proteins involved in the elaboration of the VN? How are the PPM and VN physically connected? What is the function of the VN? The VN could be an important platform for the import of some protein precursors, most importantly those that are folded and glycosylated after crossing the cERM and PPM (Peschke et al. 2013). Protein transport from the VN to the oEM would therefore need a non-vesicular process. This study provides therefore novel insights into the PPC ultrastructure, opening fascinating perspectives to comprehend the origin of the secondary plastid in diatoms, its protein and membrane lipid biogenesis and its sophisticated relationship with other cell compartments.

Methods

***Phaeodactylum tricornutum* cultivation:** The Pt1 *Phaeodactylum tricornutum* strain (CCAP 1055/3) was obtained from the Culture Collection of Algae and Protozoa, Scottish Marine institute, UK. The culture was grown in exponential phase in ESAW (Enriched Seawater, Artificial Water) medium, using 50 mL single-use flasks with 100 rpm shaking (Certomat BS-1 incubator; Sartorius stedim biotech), a low light intensity of 20 μmol photon $m^{-2}.s^{-1}$ and a 12/12 hour light/dark photoperiod at 19 °C.

Sample preparation for electron microscopy: Cells of *P. tricornutum* were harvested at late logarithmic phase (2.10^6 cells/mL) before the offset of the light period at 5000 x g, 10 min, 4 °C. Cells were then fixed in 0.1 M cacodylate buffer (Sigma-Aldrich), pH 7.4, containing 2.5% glutaraldehyde (TAAB), 2% formaldehyde (Polysciences) for 1 h at room temperature and

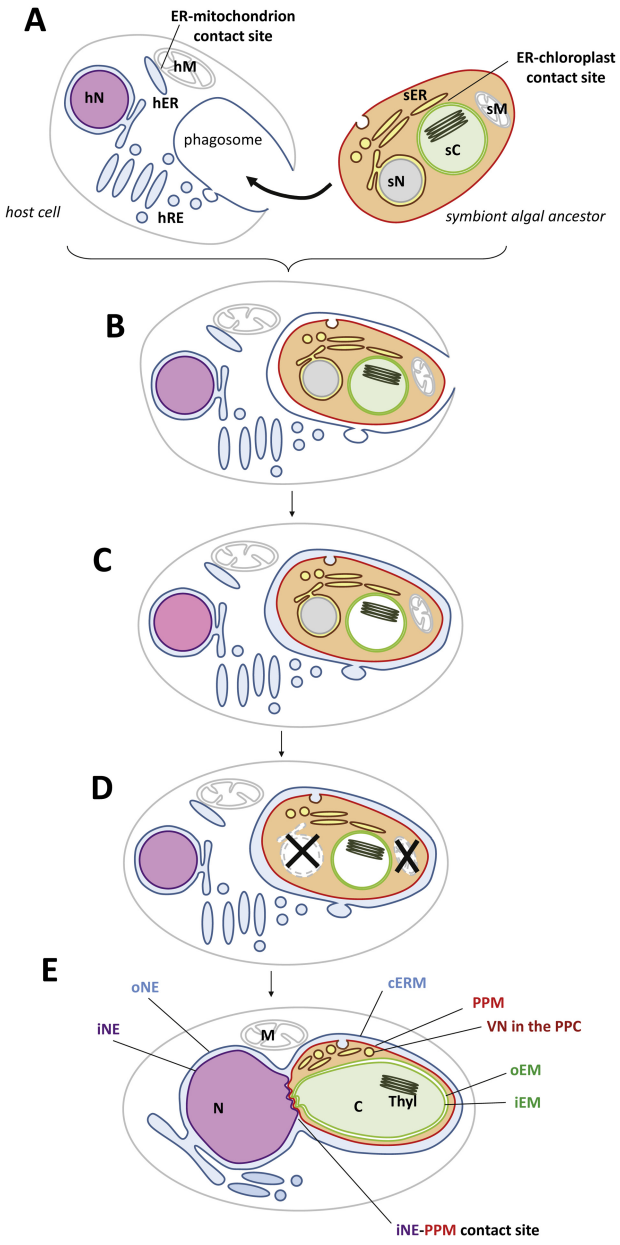

Figure 7. Stepwise reduction of the symbiont cytosol following secondary endosymbiosis in the diatom lineage. **A**. The host cell and red algal symbiont. **B**. Engulfment of the red alga. **C**. Residence and transmission of the red alga within the phagotrophic membrane. **D**. disappearance of symbiont organelles, including the nucleus, and cytosolic structures. **E**. Present status. C, chloroplast; cERM, chloroplast endoplasmic reticulum membrane; iEM, inner envelope membrane; iNE, inner nuclear envelope; M, mitochondrion; N, nucleus; oEM, outer envelope membrane; oNE, outer nuclear envelope; PPC, periplastidial compartment; PPM, periplastidial membrane; Thyl, thylakoid, VN, vesicular network.

prepared according to a modified protocol from T. J. Deerinck (http://ncmir.ucsd.edu/sbem-protocol).

Focused ion beam – scanning electron microscopy (FIB-SEM): Focused ion beam (FIB) tomography has been realized in a Zeiss NVision 40 dual-beam microscope. In this technique, the Durcupan embedded cells of *P. tricornutum* were cut in cross-section, slice by slice, with a Ga+ ion beam (of 700 nA at 30 kV), and each slice was imaged in scanning electron microscopy (SEM) at 5 kV using the in-column EsB back-scatter detector. For each slice, a thickness of 4 nm has been removed, and the SEM images are recorded with a pixel size of 4 nm. The image stack is then registered by cross-correlation using the StackReg plugin in the Fiji software. This procedure gives us directly an image the 3D structure of the sample with an isometric voxel size of 4x4x4 nm^3.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2016.04.001.

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