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NOTE

MONITORING RAPID VALVE FORMATION IN THE PENNATE DIATOM *NAVICULA SALINARUM* (BACILLARIOPHYCEAE)[1]

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After each division of a diatom cell, a new siliceous hypovalve is formed inside the silica deposition vesicle (SDV). We present the sequence of this early formation of the new valve in the pennate marine diatom *Navicula salinarum* (Grunow) Hustedt, visualized by using the fluorescent probe 2-(4-pyridyl)-5-((4-(2-dimethylaminoethylamino-carbamoyl)methoxy)phenyl)oxazole (PDMPO). Our observations confirm that two-dimensional expansion of the growing valve is a rapid process of no more than 15 min; three-dimensional completion of the valve appears to be slower, lasting most of the time valve formation takes. The results are relevant to studies of the timing of molecular processes involved in valve formation (i.e. the bio- and morphogenesis of the SDV) in relation to uptake and transport of silicic acid. Use of this probe helps us to identify specific developmental stages for further detail analysis of diatom basilica formation, which eventually could lead to obtaining enriched SDV fractions.

Key index words: **diatoms; fluorescence; *Navicula salinarum*; PDMPO; silica deposition vesicle; SDV; valve formation**

***Abbreviations:*PDMPO, 2-(4-pyridyl)-5-((4-(2-dimethylaminoethylamino-carbamoyl)methoxy)phenyl) oxazole; SDV, silica deposition vesicle**

The rigid part of the cell wall (frustule) of diatoms is composed of amorphous silica (Pickett-Heaps et al. 1990, Parkinson and Gordon 1999, Vrieling et al. 2000), located in the girdle bands and two valves: the epitheca consisting of an epivalve and epicingulum and the hypotheca consisting of hypovalve and hypocingulum (Round et al. 1990a). The timing of diatom valve formation has not been directly determined in an individual cell; however, some estimates were made based on measurements of whole synchronized cultures. For instance, Reimann (1960) determined that two-dimensional valve formation in *Melosira varians* Agardh takes approximately 8 min. Cell wall morphogenesis in *Ditylum brightwellii* (West) Grunow and in *Navicula pelliculosa* (Brébisson et Kützing) Hilse occurs during, respectively, a 53-min and a 2- to 3-h period of the cell cycle (Sullivan and Volcani 1981).

Commonly, in pennate diatoms the development of the new hypovalve starts from the axial area and grows toward the outer cellular edges (Pickett-Heaps et al. 1990). Subsequently, the frustule thickens in the third dimension and is completed by coverage with a casing followed by exocytosis of the valve and girdle bands and finally cell separation (Darley and Volcani 1971, Darley et al. 1976, Pickett-Heaps et al. 1990, van de Poll et al. 1999). Assessment of the exact time of these latter two events remains elusive; for *N. pelliculosa*, however, it has been estimated that cell separation requires 3 to 4 h (Darley and Volcani 1971). We have occasionally noticed that the initial valve formation in *Navicula salinarum* can proceed rapidly (Vrieling et al. 2005). This prompted us to analyze the development of the early intermediate stages in more detail to get a better insight in the timing of two-dimensional valve growth and three-dimensional thickening in this species.

We batch cultured a pennate diatom, *N. salinarum* (strain L1261), in artificial seawater (Veldhuis and Admiraal 1987) at a salinity of 20 psu. To synchronize cells at cytokinesis arrest, presumably at the phase boundary of S/G2 as has been shown for the closely related *N. pelliculosa* (van de Poll et al. 1999), silicon starvation was applied according to the approach of Coombs and Volcani (1968) with an incubation time of 70 h in Si-free medium. Synchronized growth was initiated by repleting the culture with 100 mM silicic acid to a final concentration of 0.2 mM. Simultaneously, 2-(4-pyridyl)-5-((4-(2-dimethylaminoethylamino-carbamoyl) methoxy)phenyl)oxazole (PDMPO, LysoSensor™ yellow/blue DND-160, Molecular Probes, Europe BV, Leiden, The Netherlands) was added to a final concentration of 1.0 μM. The PDMPO probe is readily

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imported into diatom cells and transported once inside due to membrane potentials. It typically fluoresces green when the intercompartmental silica concentration rises above 3 mM, allowing analysis of valve formation by fluorescence microscopy (Shimizu et al. 2001). Probed cells were harvested by centrifugation (5 min at 1000*g* at 16° C) at the following intervals: $t = 0$ (before addition of silicic acid and PDMPO) and $t = 5$, 10, 15, 20, 25, 35, 45, 55, 85, 115, 235, 355, and 475 min. At every interval the cells were immediately transferred to cups and washed three times with 1.5 mL MilliQ (Millipore BV, Etten-Leur, The Netherlands) using an Eppendorf centrifuge (1 min at 890*g* at 4° C). The harvested cells were fixed in paraformaldehyde according to Hawes (1988) and stored at −20° C in methanol until further analysis. The first centrifugation step (5 min) was incorporated in the harvest times, assuming that valve formation ceases upon removal of complete medium; this means the samples were designated as 10, 15, 20, 25, 30, 40, 50, 60, 90, 120, 240, 360, and 480 min after resupply of silicic acid. Fluorescence microscopy was applied using

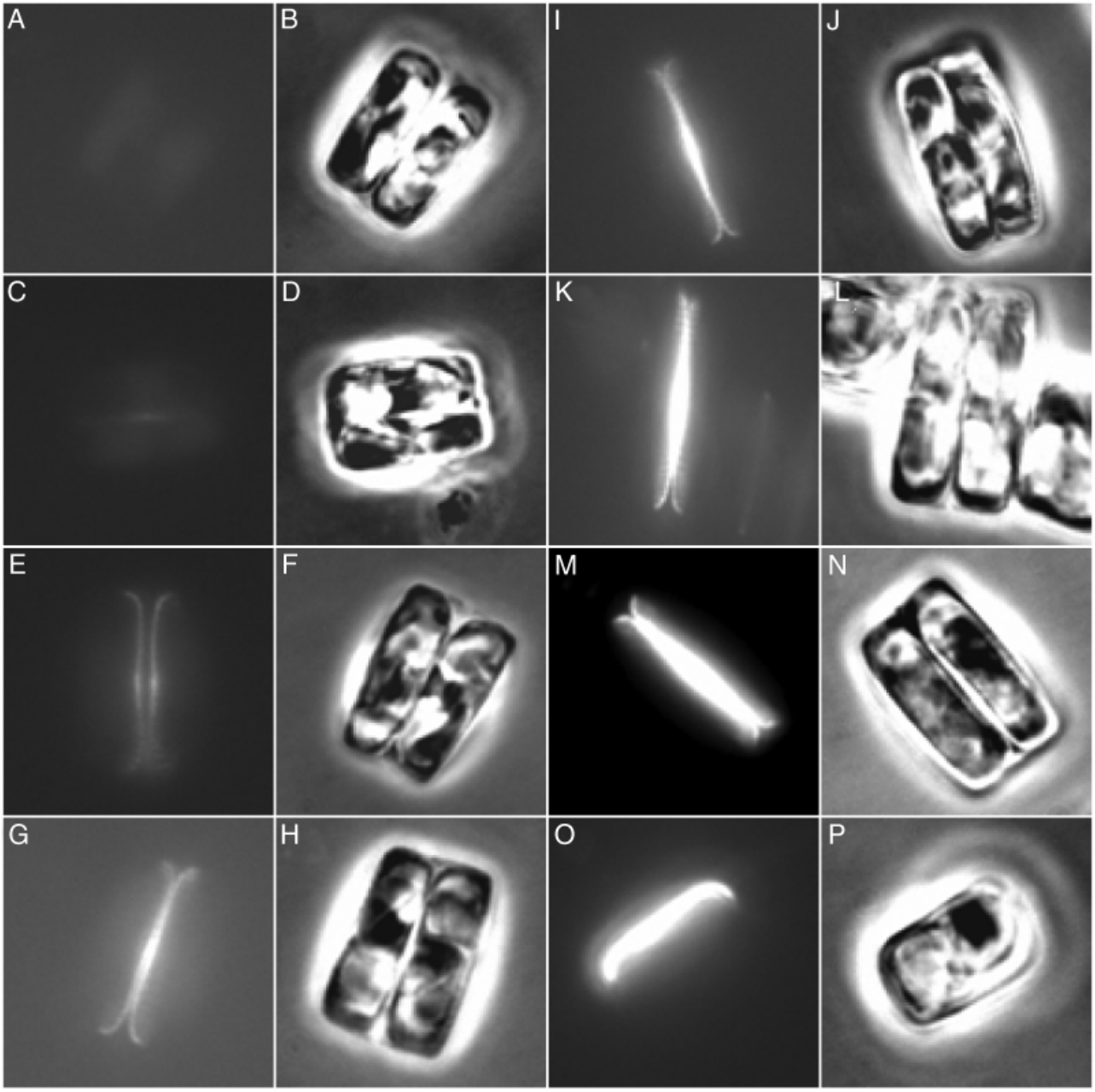

FIG. 1. Valve formation in *Navicula salinarum* visualized by the PDMPO probe and observed in girdle view. Fluorescence images (A, C, E, G, I, K, M, and O) are aligned by their bright field ones (B, D, F, H, J, L, N, and P). The sequence of valve formation is as follows: $t = 0$, before addition of silicic acid (A, B) and subsequently after addition of silicic acid and PDMPO at 10 min (C, D), 15 min (E, F), 25 min (G, H), 40 min (I, J), 60 min (K, L), 90 min (M, N), and 240 min (O, P).

an Axioscope (Zeiss, Oberkochen, Germany) equipped with a bandpass filter for excitation at 365 nm and a longpass filter to record emitted light at wavelengths above 397 nm.

This is the first time intermediate stages of valve formation in *N. salinarum* have been visualized sequentially at very short intervals. Within only 10 min after silicic acid and PDMPO addition, fluorescence became visible at the central nodule of the cells (Fig. 1C). At this stage all the cells that contained PDMPO fluorescence revealed the pattern as shown in Figure 1C, although some differences appeared in fluorescence intensities. These differences, however, could not be distinguished at clear-cut stages of valve formation. In side view, these immature fluorescent silica deposition vesicles (SDVs) appeared to be located at the side of the cleavage furrow (Fig. 2C). The clear difference of this fluorescence in the initial stages (Figs. 1, A and B, and 2, A and B) confirms earlier findings of valve formation starting from the axial area and proceeding peripherally toward the outer valve edges (Pickett-Heaps et al. 1990, Round et al. 1990). Only 5–10 min later (t = 15–20 min) the full two-dimensional size of the new hypovalve had been reached, indicating that macromorphogenesis (Crawford et al. 2001) was completed. At t = 15 min the majority of the PDMPO probed cells (75%, with SD = 2.8, coefficient of variation (CV) = 0.038) contained a new valve the same size as the mature one (Fig. 1, E and F). The remainder of the probed cells had a more intense fluorescence and may be considered to have made more progress in valve formation. In apical view, the trans apical ribs were clearly visible within 20 min of the beginning of valve formation (Fig. 2C), suggesting that within the fully expanded SDV further development of more detailed structures had started. At this stage, the fluorescence at the central nodule was more intense compared with the fluorescence of other parts of the valve. In pennate diatoms this region is more heavily silicified; in our *N. salinarum*, thickening in the third dimension apparently had started within 20 min of valve formation. Micromorphogenesis (Crawford et al.

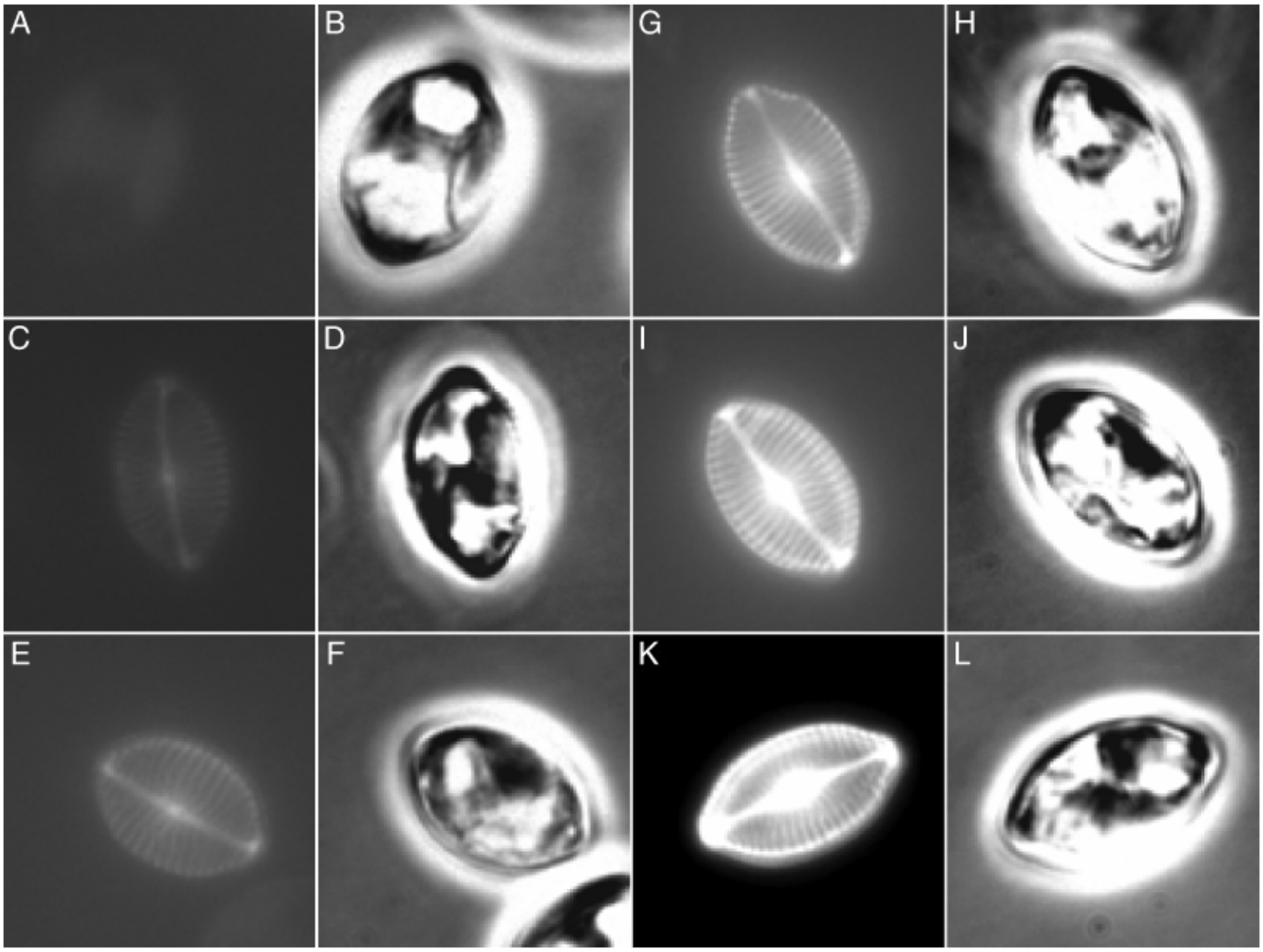

FIG. 2. Valve formation in *Navicula salinarum* visualized by the PDMPO probe and observed in transapical view. Fluorescence images (A, C, E, G, I, and K) are aligned by their bright field ones (B, D, F, H, J, and L). The sequence of valve formation is as follows: t = 0, before addition of silicic acid (A, B) and subsequently after addition of silicic acid and PDM PO at 20 min (C, D), 30 min (E, F), 60 min (G, H), 90 min (I, J), and 120 min (K, L).

2001) may also have been initiated at this stage, but we were not able to prove this; only very powerful noninvasive tools may provide evidence. Valve thickening was clearly visible by the increase of the fluorescence intensity in the newly formed valve when seen in girdle view (Fig. 1, E–N). Also in apical view we observed this increased fluorescence intensity (Fig. 2, C–L). After 240 min the first cells of *N. salinarum* had completed valve formation and started to separate (Fig. 1, O and P). In fact, 55% of the PDMPO probed cells (SD = 0.7, CV = 0.013) were separated. Preliminary trials to visualize valve formation *in vivo* confirmed that valve formation is fast: We detected silica-induced PDMPO fluorescence from stages after 5 min of resupply of silicic acid to Si-synchronized cells (unpublished results).

As Shimizu et al. (2001) stated, PDMPO is a fluorescent compound that can accumulate in acidic cell compartments; the SDV is one of these (Vrieling et al. 1999b). The PDMPO probe is useful for silica biomineralization research because it is co-deposited with diatom biosilica. Incorporation of a fluorescent probe in diatom biosilica has been shown to be proportional to the amount of silica deposited (Brzezinski and Conley 1994, Shimizu et al. 2001, Vrieling et al., unpublished results). This explains our observation of increased fluorescence intensity at the stages at which the largest amounts of silica are deposited; in fact, such stages appear when the valve has been completed in the second dimension and thickening in the third dimension follows (Figs. 1, E–N, and 2, D–L).

The cells of *N. salinarum* were synchronized by silicon limitation, arresting the cells at the stage after cytokinesis (Coombs and Volcani 1968, van de Poll et al. 1999, Vrieling et al. 1999b). This stage of arrest may well be favorable for diatoms to start cell wall formation as soon as silicic acid is available again in the medium (Sullivan 1977). The fast incorporation of PDMPO (and at that time also deposition of silica) relies on the rate of uptake of probe (and silicic acid) from the environment. In diatoms, such a fast uptake mechanism apparently is in operation and different silicon transporters are involved (Hildebrand et al. 1998, Hildebrand 2003, Hildebrand and Wetherbee 2003). The membrane potential of the transport mechanisms may even be functionally supported by sodium-dependent symport mechanisms (Bhattacharyya and Volcani 1980), whereas the high membrane permeability of PDMPO allows a concerted uptake of both silicic acid and probe.

Molecular and physicochemical processes play a central role in the formation of diatom biosilica, the expansion of the SDV, and the morphogenesis of the various siliceous cell wall components (Simpson and Volcani 1981, Pickett-Heaps et al. 1990, Round et al. 1990, Vrieling et al. 1999a). For a better understanding of diatom valve formation and for unraveling of the (bio)chemical and molecular processes involved, a detailed analysis of the time course of processes in the SDV is required. For this purpose, sufficient immature and intact SDVs should become available, making enrichment of SDVs essential. With respect to the initial sequences of valve formation in *N. salinarum*, we may obtain enriched SDV fractions using a tracer method based on silica-embedded PDMPO fluorescence.

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