PROGRAMMA DI RICERCA STM: Relazione scientifica dell'attività di ricerca svolta

Characterization of Nitric Oxide-Associated protein 1 (NOA) in the model diatom *Phaeodactylum tricornutum* and his role under stress conditions.

The oceans, the blue lung, cover approximately 70% of the Earth's surface and photosynthetic organisms living in the photic zone are responsible for half of global primary productivity[1]. These organisms drive most of the major oceanic processes; for >3 billion years, they have actively influenced the composition of the Earth's atmosphere, ultimately creating conditions that have allowed multicellular organisms to evolve[2]. These organisms are exposed to environmental perturbations conditions and most, if not all, feed this information into molecular works that allow them to anticipate changes in their environment and to synchronize their main biological processes. In the contemporary oceans, the most successful organisms are photosynthetic prokaryotes and a class of eukaryotic unicellular algae known as diatoms[3]. Diatoms constitute a hugely diverse group of photosynthesizing microorganisms that occur in the plankton and benthos of marine and freshwater habitats[4]. In particular, recent assessments suggest that marine diatoms, responsible for about 20% of global primary productivity.

Despite the extraordinary ecological flexibility and dominance of diatoms, and their enormous importance in influence on geochemical cycles and climate, very little is known about the molecular underpinnings of their success. Resources invested by the research community for the study of marine photosynthetic organisms like diatoms, are not comparable with those of terrestrial plants and consequently their biology is still largely unexplored. We need a better understanding of their physiology at the molecular level in order to understand how they affect planetary level processes.

In order to identify novel regulators of diatoms growth and distributions in adverse enviromental conditions we tried to elucidate the physiological and cellular roles of Nitric Oxide-Associated protein 1, NOA, in the diatom model species *P. tricornutum*, in response to stress conditions.

Given the multi-faceted roles and multi-target effects of the NOA protein, the project aims to characterize the still unknown role of this protein in diatoms, by using a reverse-genetics approach.

The already available and partial characterized overexpressing strains[4] (Vardi et al., 2008) and knockdown mutants, generated in collaboration with the host laboratory and the Laboratory of Computational and Quantitative Biology at the Sorbonne Université, UPMC Université, Paris 06, UMR 7238 in Paris, directed by Dr A. Falciatore, were analyzed during this period of stay at the Weizmann Institute of Science, using a suite of physiological and biochemical tools and approaches developed in the host laboratory in order to study cell signaling and stress responses in these marine alga.

Phenotypic, physiological analyses were performed in order to investigate the role of NOA in modifying different metabolic processes following various environmental stresses such as exposure to diatom derived infochemicals, bioactive compounds that have an important role in regulating cell fate and nutrient limitation. We used a suite of cellular markers for oxidative stress and Programmed Cell Death-PCD in response to environmental cues.

Modulation of NOA expression and function in response to different environmental signals

Analysis of clones with altered NOA activity

Overexpressing transgenic clones and knockdown mutants of PtNOA were confirmed by western blot, proteins were detected with antibodies against NOA and the PSII subunit D2 was used as loading control. Two clones were identified, OE1 and OE2, which showed protein accumulation and three; R7, R17, R27; reduced protein accumulation compared to the wild type (Figure 1).

Evidences suggested that diatoms induce hallmarks of a Programmed Cell Death (PCD)-like mechanism as a result of environmental stress such as nutrient limitation, culture age and exposure to infochemicals. The infochemical aldehyde (2E,4E/Z)-decadienal (DD) can trigger intracellular calcium transients and the generation of nitric oxide (NO) by a calcium-dependent NO synthase-like activity, which results in cell death. We performed a phenotypic characterization of the PtNOA overexpressing (OE) and knockdown mutants by testing the sensitivity to the DD. As shown in Figure 2, overexpression of PtNOA altered the cellular growth in response to sublethal DD concentrations, 7 and 10 µg/ml. Cell survival after DD exposure was monitored after 1 week of growth under normal light conditions. OE cells exhibited hypersensitivity to concentrations of DD that were sublethal to wild-type and knockdown cells. Thus confirming a role of NOA in aldehydes sensing. Treatment with methanol (1%) alone, as control, failed to induce cell death. Experiments were repeated two times with similar results.

Moreover, the infochemical volatile halocarbon (BrCN) that mediate trophic-level interactions in marine diatoms can induce cell death as well. Cell death was measured by flow cytometry, using Sytox Green, a high-affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes, in the clones with altered NOA activity (overexpressing and knockdown) treated either with 50 and 100 µM DD and 0.1 and 0.5 µM BrCN. Samples were incubated in the dark for 30 minutes prior to measurement. We obtained preliminary results that confirm the hypersensitivity to the infochemicals for the PtNOA overexpressing clones. The fraction of sytox-positive cells 24, 48 and 72 hours after exposure to the examined stress conditions, is significantly higher in these clones compared to the wild-type and knockdown cells that on the contrary, show a lesser number of sytox-positive cells. Treatments with methanol (1%) and acetone alone, as control, failed to induce significant cell death.

Moreover, preliminary reported results demonstrated that (DD) can also trigger the generation of nitric oxide (NO). Endogenous NO generation was measured by flow cytometry, using the NO-sensitive dye 4-amino-5-methylamino-2',7'-difluorofluo resceindiacetate (DAF-FM) in overexpressing, wild-type and knockdown cells, 24, 48, 72 and 96 hours hours after exposure to 50 and 100 µM DD. Unfortunately, we could not reach any conclusion, the limited time available and a series of technical problems did not allow us to detect a direct coupling between DD induced stress and NO generation.

We further examined the different responses to iron and nitrogen limitation, as well. Both, are major nutrients which control diatom growth and distribution in the ocean. Nutrient limitation experiments were done by washing the cells in nutrient free media and resuspending them in f/2 without the appropriate nutrient (nitrogen or iron). For iron limitation, 1 µM of the efficient iron

chelator desferrioxamine B (DFB) was added.

Iron limitation led to high induction of cell death. We analyzed again the content of the "live" and "dead" cells by flow cytometry using Sytox Green dye after 1, 3, 5 and 7 days post starvation. In contrast to what we observed in the different clones treated either with DD and BrCN, we detected only minor differences between the different clones (OE and RNAi) and the wild type control cells, in response to iron limitation. While an increase of dead in cells overexpressing PtNOA, was detected in response to nitrogen limitation at 5 and 7 days post starvation.

One of the early responses of plants and algae to allelochemicals and nutrient starvation is also the generation of reactive oxygen species (ROS). In order to test if the protein NOA, in diatoms, is also involved in the control of oxidative damage, we measured the oxidation values by flow cytometry, using the reduced glutathione, also known as GSH, as day, after exposure to the NOA OE and RNAi clones to 100 and 150 µM H2O2 for 24 and 48 hours. As a result of ROS production we observed an alteration in GSH content in OE PtNOA clones, after 48 hours of exposure to 150 µM H2O2.

Thaken together these preliminary results strongly suggest a pivotal role of NOA in cell fate determination and suggest a strong correlation between the NOA expression and the ability to adapt at various stress conditions such as oxidative stress, nutrient limitation and exposure to diatom derived infochemicals. We will continue to study the NOA gene, by characterizing, his signalling pathways and his function by using genetic and biochemical approaches. I hope that I will have the oppuntunity to join the labiratory of Dr. Vardi again in the future to continue our collaboration.

Materials and Methods

Culture growth: *P. tricornutum*, accession Pt1 8.6 (CCMP2561 in the Provasoli-Guillard National Center for Culture of Marine 115 Phytoplankton) was purchased from the National Center of Marine Algae and Microbiota (NCMA, formerly known as CCMP).
Cultures were grown in f/2 media (882.4 µM NaNO3, 35.21
µM Na2HPO412H2O, 1.83 µM FeCl36H2O) (Guillard & Ryther 1962) at 18 ºC with 16:8 hours light:dark cycles and light intensity of 80 µmol photons·m-2·sec-1 supplied by 120 cool-white LED lights (Edison). All experiments were performed with exponentially growing cultures at ~5·105 cells·ml-1.

Spot test: We performed the spot test on NOA OE and RNAi clones and Pt1 as control, using two different concentrations of DD 7µg/ml and 10µg/ml and also two different cells concentrations 0.5 and 1 x106 (concentration 1 day before DD add).

Infochemical preparation: The infochemical (E,E)-2,4-decadienal (DD) (Pohnert 2002; Miralto et al. 1999), (Acros Organics) solutions were prepared by diluting the 95% stock in absolute methanol on ice. DD was added to the cells by 1:200 dilution. Control cultures were treated by addition of

1:200 methanol to the culture.
The halocarbon BrCN (Vanelslander et al. 2012), (Sigma) solutions were prepared by diluting the stock of 5 M in acetone. BrCN was added to the cells by 1:1000 dilutions. Control cultures were treated by addition of 1:1000 acetone to the culture.

Cell death analysis: Membrane permeability was determined by Sytox Green (Invitrogen) at a final concentration of 1 µM. Samples were incubated in the dark for 30 minutes prior to measurement.

Chemicals: Sytox Green nucleic acid stain (5 mM stock in DMSO); DAF-FM (5 mM stock in DMSO); Reduced Glutathione, GSH (5 mM stock in DMSO).

BIBLIOGRAPHY

1. Falkowski, P. G. a. R. J. A. Aquatic photosynthesis. (Blakwell Science UK, 1997).
2. Falkowski P., K. A. Evolution of primary producers in the sea. (The Netherlands: Elsevier Academic Press, 2007).
3. Falkowski, P. G., Barber, R. T. & Smetacek, V. V. Biogeochemical Controls and Feedbacks on Ocean Primary Production. Science 281, 200-207 (1998).
4. Vardi A, Bidle KD, Kwityn C, Hirsh DJ, Thompson SM, Callow JA, Falkowski P, Bowler C. A diatom gene regulating nitric-oxide signaling and susceptibility to diatom-derived aldehydes. Curr Biol. 2008 Jun 24;18(12):895-9

Fig.1

Western blot with OE and RNAi lines

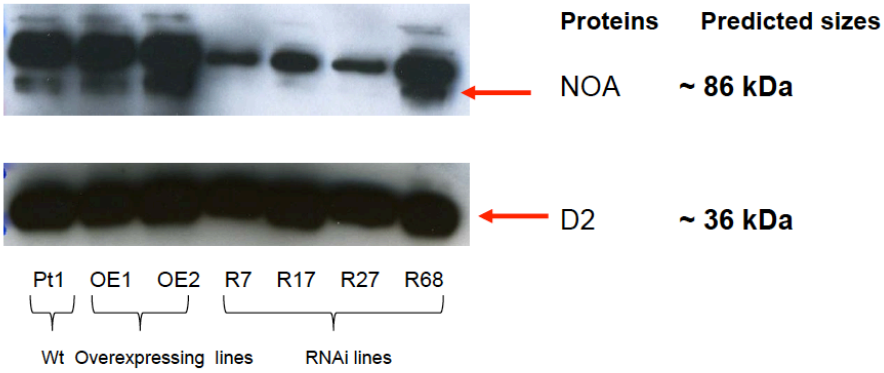

Fig.2