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Silencing of lipoxygenase pathways in the diatom genus *Pseudo-nitzschia*

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Abstract

Diatoms are unicellular eukaryotic organisms that are responsible for about 40% of the marine primary production. They also contribute to the biogeochemical cycles in the oceans. Diatoms produce oxylipins, a varied family of lipoxygenase-derived metabolites that share a structural motif arising from the addition of oxygen to polyunsaturated fatty acids (PUFAs). Oxylipins are widely distributed in animals and plants, where they are involved in a broad spectrum of actions including stress and defense responses, regulation of growth and development, signaling, and innate immunity. In diatoms, oxylipins have been proposed as ecological and physiological mediators but their function has not been proven in any species mostly because of the lack of molecular tools. The aim of this doctoral thesis was the functional study of lipoxygenase (LOX) pathways in the ecologically relevant *Pseudo-nitzschia* genus. Transformation of *Pseudo-nitzschia arenysensis* empowered the use of RNAi to silence for the first time *LOX* genes in this species. Experimental evidence showed that the two LOX pathways previously described in *P. arenysensis* were affected by this process with resulting reduction of the levels of corresponding oxylipins. The silenced diatom cells revealed a reduced growth compared to wild type cells and showed a phenotype with a marked photoinhibition, thus confirming the regulatory role of LOX pathways in diatoms and suggesting a direct link between oxylipins and cell resilience. Overall, this study shows the first correlation between a diatom gene and the synthesis of lipid mediators, providing direct evidence that the reduction of lipoxygenase expression levels are correlated to a marked physiological effect.

Abstract

Le diatomee sono organismi eucariotici unicellulari che sono responsabili di circa il 40% della produzione marina primaria. Essi contribuiscono anche ai cicli bio-geochimici negli oceani. Le diatomee producono ossilipine, una ampia famiglia di metaboliti derivati dalle lipossigenasi che condividono un motivo strutturale dato dall'aggiunta di ossigeno nella catena carboniosa degli acidi grassi polinsaturi (PUFA). Le ossilipine si trovano diffusamente negli animali e nelle piante, dove sono coinvolte in un ampio spettro di azioni che includono stress e risposte di difesa, regolazione della crescita e sviluppo, signaling e immunità innata. Nelle diatomee, le ossilipine sono state proposte come mediatori fisiologici ed ecologici, ma la loro funzione non è ancora chiara, molto probabilmente per la mancanza di strumenti molecolari. Lo scopo di questa tesi di dottorato è stato lo studio funzionale del pathway della lipossigenasi (LOX) nel genere *Pseudo-nitzschia*, di notevole rilevanza ecologica. La trasformazione di *Pseudo-nitzschia arenysensis* ha permesso l'uso dell'RNAi con lo scopo silenziare per la prima volta il gene *LOX* in questa specie. I dati sperimentali hanno mostrato che i due pathway LOX, descritti in precedenza in *P. arenysensis,* sono stati entrambi compromessi in questo processo con la risultante riduzione dei livelli delle ossilipine corrispondenti. Le cellule di diatomea silenziate hanno rivelato una ridotta crescita paragonata alle cellule wild type e hanno mostrato un fenotipo con marcata fotoinibizione, questo a conferma del ruolo regolatorio del pathway delle LOX nelle diatomee, suggerendo un diretto legame tra le ossilipine e la capacità di recupero cellulare. Questo lavoro mostra la prima correlazione tra un gene di diatomea e la sintesi delle ossilipine, dando evidenza diretta che la riduzione dei livelli di espressione della lipossigenasi è correlata ad effetti fisiologici marcati.

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1. INTRODUCTION

1.1 Diatoms

Diatoms are unicellular photosynthetic eukaryotic organisms with a size range from 2 µm to >200 µm. They exist as single cells, chains, or colonies, wherever there are sufficient quantities of light and nutrients to sustain growth, from tropical to polar zones. They are one of the main classes of phytoplankton, which regulates the biogeochemical cycles in the oceans and provides the basis of the food chain for both marine and freshwater micro-organisms and animal larvae. Diatoms belong to the class Bacillariophyceae, and more than 200 genera and about 100,000 species can be found on the earth (Mann and Vanormelingen 2013). The name diatom comes from a Greek word, *diatomos*, that means “cut in half” for its typical cell wall structure. The silica wall known as frustule is made up of two parties called theca, one larger (epitheca) fits in another smaller (hypotheca) like a petri dish. The overlapping region is reinforced with silica girdle bands, this region is known as the cingulum (fig. 1). The frustule contains many pores that permit the exchange of nutrients, waste removal and secretion with the external environment.

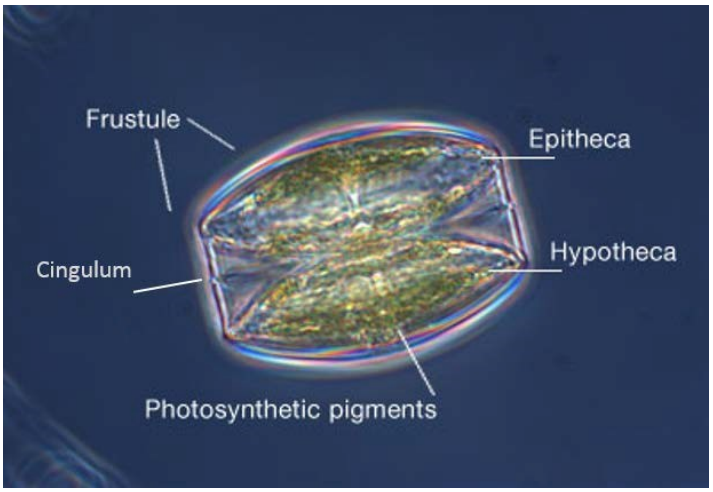

Fig. 1 Schematic representation of a diatom cell.

Diatoms are distinguished in two classes according to their overall shape; the centric diatoms that have a radial symmetry and the pennate diatoms that have a bilateral symmetry (fig. 2). An additional subdivision is due among pennates for the presence or the absence of the raphe, an elongated slit that diatoms can use to move over surfaces (Round et al. 1990).

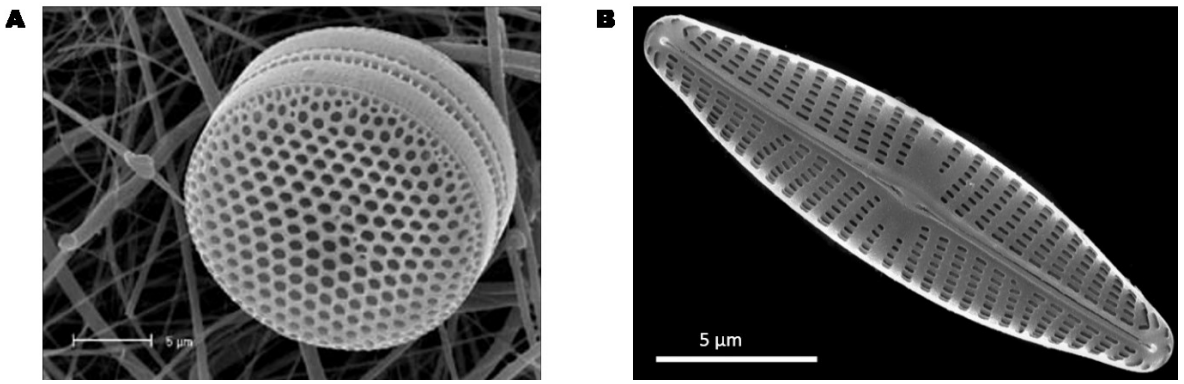

Fig. 2 Pictures at SEM microscopy. **a** Centric and **b** pennate diatom.

Diatoms live in aquatic and semi-aquatic habitats. Depending on their habitats, diatoms are either planktonic (living suspended on the water), benthic (growing associated to a substrate), or both planktonic and benthic. Usually planktonic diatoms have fine frustules or long appendices that allow flotation. Benthic diatoms can be motile when living on sediment and grow closely attached to a substrate, for this reason they are used as indexes of water quality.

Diatoms have a complex evolutionary history that is distinct from algae and plants. The formation of the photosynthetic plastids of land plants and red and green algae occurred about 1.5 billion years ago from a primary endosymbiosis when a eukaryotic heterotroph engulfed a photosynthetic cyanobacterium (Yoon et al. 2004). Instead, diatom plastids might be the result of a second event of endosymbiosis, about 500 million years later, in which a different eukaryotic heterotroph captured a red alga (fig.3). The loss of red alga nucleus and

mitochondrion gave rise to Stramenopiles, the group that includes diatoms, brown macroalgae and plant parasites (Gibbs 1981, Parker et al. 2008). Diatom plastids possess four envelope membranes. The two innermost membranes of the plastids originate from the two envelope membranes of the primary plastid, whereas the third membrane is derived from the plasma membrane of the captured eukaryotic alga. Maybe the outermost membrane represents the phagotrophic vacuole membrane in which the primary alga was engulfed.

In the entire genome sequence of two different diatoms, *Thalassiosira pseudonana* (Armbrust et al. 2004) and *Phaeodactylum tricornutum* (Bowler et al. 2008) genes involved in mitochondrial fatty acid oxidation pathways and a urea cycle incorporated within the framework of a photosynthetic cell were found (Allen et al. 2006). Only few genes, about 170, were found with a red algal origin (Bowler et al. 2008) and many genes, over 1700, with a green algal origin (Worden et al 2009, Moustafa et al. 2009). It is possible that a green algal endosymbiont preceded the red alga, and that many of its genes were retained while red algal genes were not retained.

In *P. tricornutum* a large number of genes of bacterial origin were also found, about 5% of total gene content, and some of these are shared with *T. pseudonana*. Horizontal gene transfer occurred between diatoms and different groups of bacteria (archaea, proteobacteria and cyanobacteria) independently of endosymbiotic events. Very close associations of diatoms and bacteria in nature could explain these high rates of bacterial gene transfers. There are numerous examples of diatom dependency on bacterial metabolites and of bacterial dependency on released diatom metabolites (Croft et al. 2005; Reimann et al. 2000; Amin et al. 2012).

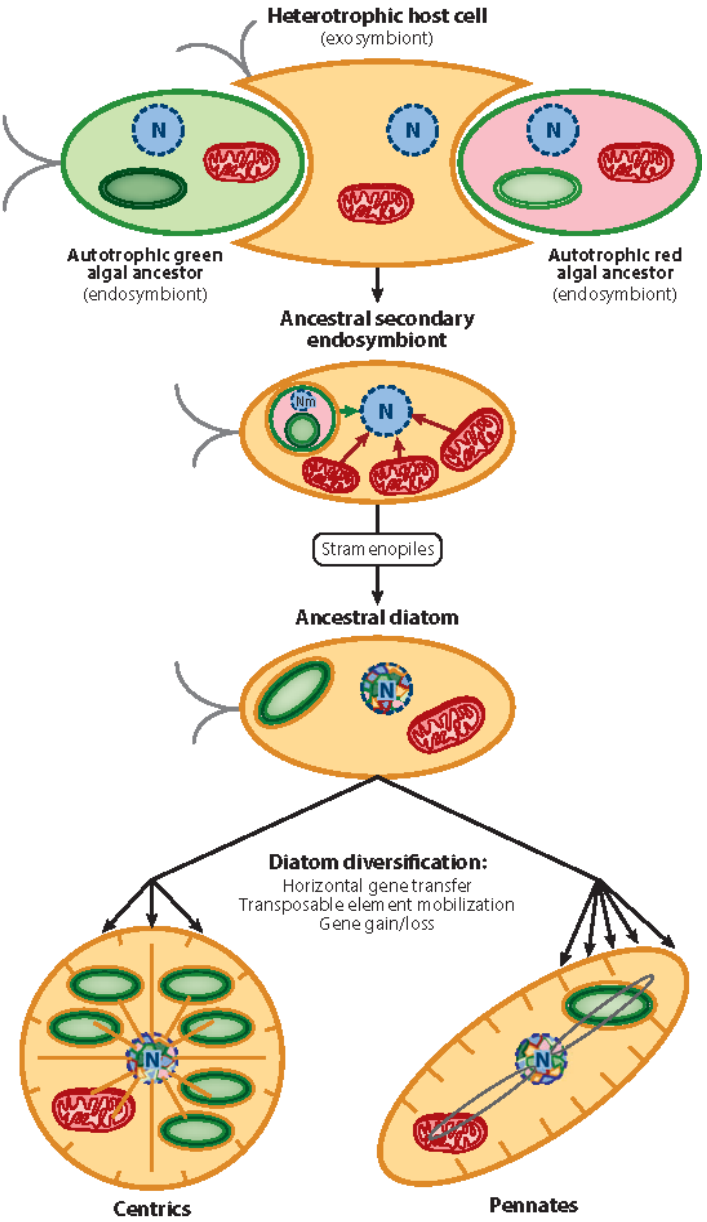

Fig. 3 Schematic representation of the first and second endosymbiotic events (Bowler et al. 2010). N is the nucleus.

1.2 Life cycle

The life cycle of diatoms is composed of two phases: a long phase of vegetative multiplication and a very short period of sexual reproduction. The vegetative multiplication occurs during mitotic division in which the two valves of frustule separate from each other to form two daughter cells. Both daughter cells acquire one of two parental valves that become a new epitheca of daughter cells and will generate a new hypotheca. The nucleus, after the division, moves to the side where the silica deposition vesicle (SDV) forms the new hypotheca (fig. 4). This process leads to the generation of two differently sized daughter cells, one keeps the same size of the parent cell whereas the other is a little bit smaller in length (pennate diatoms) or diameter (centric diatoms). Over successive generations, mitotically dividing populations decrease in mean size until the extinction of the population (MacDonald 1869). These events are very similar in pennate and centric diatoms.

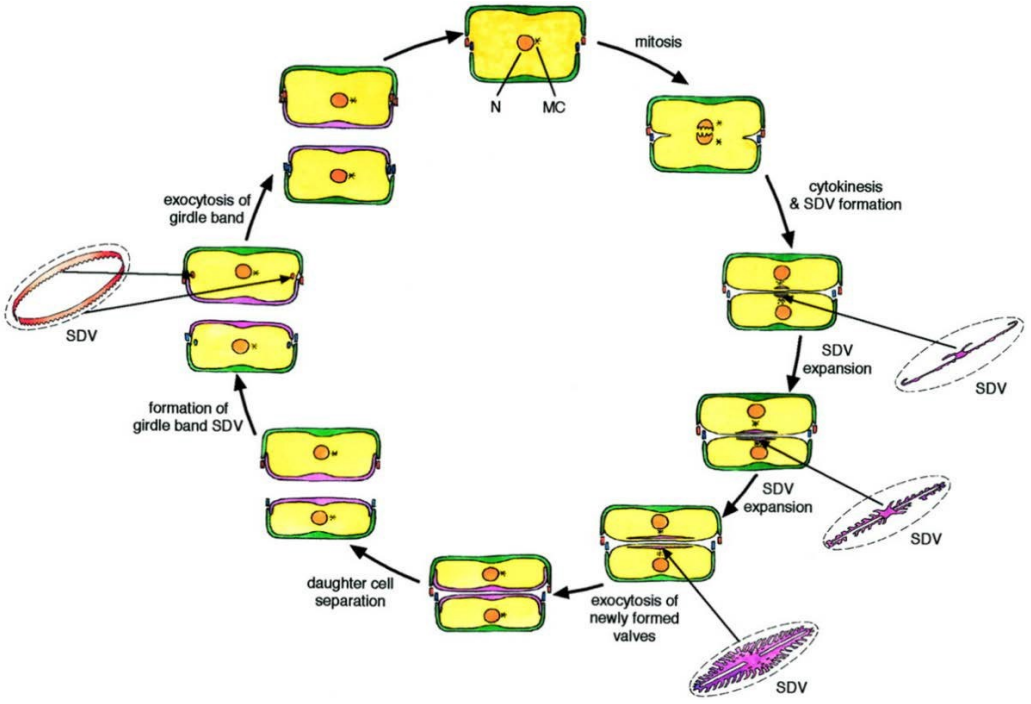

Fig. 4 Vegetative reproduction. Hypovalve and girdle band formation in pennate diatoms. N nucleus, MC microtubule center, SDV silica deposition vesicle (Zurzolo and Bowler 2001).

To regenerate the cell size, diatoms can undergo sexual reproduction. The transition between vegetative and sexual reproduction can occur only when they reach a specific size threshold, typically 30 to 40% of their initial size (Geitler 1932). When favorable conditions exist, such as cell size, correct environmental and physiological factors and the presence of the sexual partner, cells can shift from mitotic to meiotic division. Sexual reproduction is different between pennate and centric diatoms. In pennate diatoms (fig. 5), different strains of the same species with opposite mating type (MT+ and MT-) produce gametes that fuse and form a zygote. The auxospore takes shape from a zygote and when the silica cell wall is deposited it becomes an initial cell. Then the initial cell, the biggest size cell, resumes vegetative multiplication. Instead, centric diatoms are oogamous, they produce eggs (female gametes) which are fertilized by sperm cells (male gametes). Spermatozoa swim actively toward the egg soon after they are set free in the water. However, the attraction mechanisms towards the egg and the subsequent recognition between the two gametes are poorly known. After fertilization of the egg cell, the zygote is wrapped by an organic wall and becomes an auxospore.

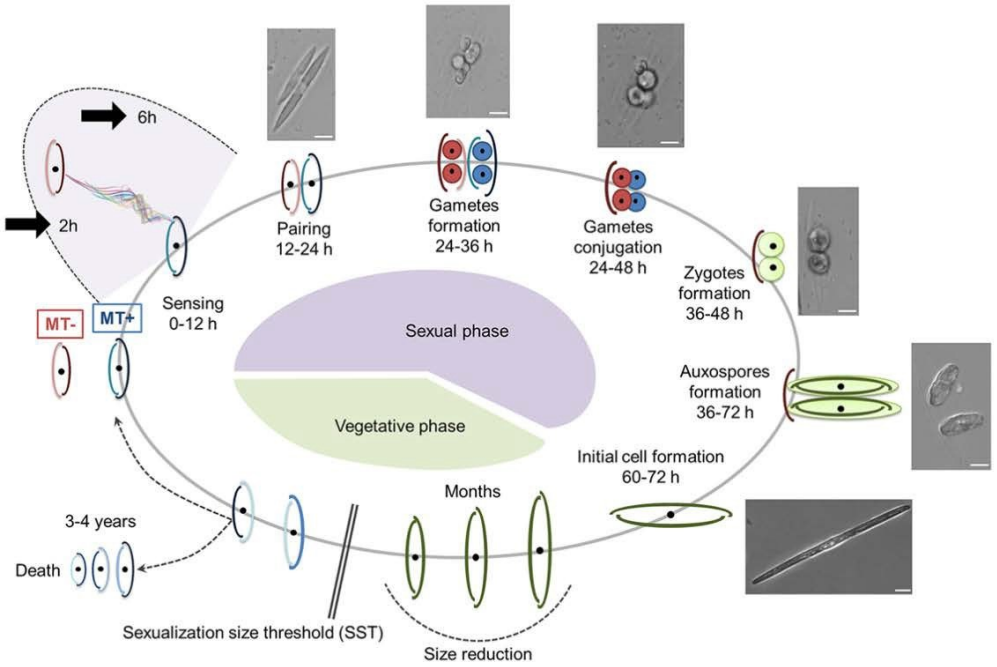

Fig. 5 Sexual reproduction in pennate diatoms. When MT+ and MT- strains (blue and red cells respectively) within appropriate sexual size threshold are mixed together they form gametes which later fuse together to form the zygote, which in turn develops into auxospores. The auxospores eventually develop into large sized initial cells, restoring the original cell size (Basu et al., 2017).

1.3 Ecological and economical role of diatoms

About 20–25% of total terrestrial photosynthesis comes from diatoms. Diatoms are responsible for approximately 40% of annual marine biomass productions making them the most dominant group of organisms sequestering carbon from the atmosphere (Falkowski et al. 1998). Diatoms are important bloom-forming phytoplankton. Diatom blooms have a duration of weeks, they increase quickly in cell number by many orders of magnitude. Their initiation is driven by incident irradiance, nutrient availability and surface mixed-layer shallowing (Platt et al. 2009; Yamada and Ishizaka 2006).

Diatoms are the most crucial organisms in biogeochemistry of silica. They build their glass-like silica shell through the assimilation of silicic acid dissolved in sea water. Immense deposits of silica can be found on the sea floor, derived from cell walls of dead diatoms. These diatom deposits can be used for the production of toothpaste, insulator, and different other purposes. For their capability to form their silica structure in three dimensions, diatoms are also studied for use in nanotechnology (Drum and Gordon 2003; Lang et al 2013; Yang et al. 2011)

Iron is an essential micronutrient for phytoplankton, being involved in cellular processes such as photosynthesis, nitrate reduction, N_2 fixation (Geider and La Roche 1994; Morel and Price 2003). The request of iron in diatoms is often higher than the iron availability in the sea. In vast areas of the world's oceans, classified as High Nutrient Low Chlorophyll (HNLC) regions, there are low concentrations of iron. Diatoms reduce their iron requirements under iron-limiting conditions (Marchetti et al. 2006; Sunda et al. 1991). It seems that centric and pennate diatoms react to deprivation of iron in different ways. For example, *Thalassiosira* (centric) seems to have modified its photosynthetic apparatus permanently (Strzepek and Harrison

2004) while *Phaeodactylum* (pennate) downregulates processes that require a lot of iron avoiding irreversible compromises (Allen et al. 2008). Centric and pennate diatoms also differ in their ability to store iron. Some pennates, like *Pseudo-nitzschia* species, produce ferritin, an iron storage protein that protects against oxidative stress (Marchetti et al. 2009), but in other diatoms the ferritin was not found. This can explain the numerical dominance of *Pseudo-nitzschia* in diatom blooms that result from iron fertilization. In the HNLC regions, where the iron is low, the dominance of both *Pseudo-nitzschia* and *Fragilariopsis* is due in part to high iron storage mediated through ferritin and provides an explanation for the importance of these organisms in regulating oceanic CO_2 (Marchetti et al. 2009).

Diatoms are excellent lipid accumulators. Lipid production by diatoms is an important process in the aquatic ecosystem (Obata et al. 2013). A primary target for biofuels production is the accumulation of lipids, either in the form of total lipids or triacylglycerol (TAG), which provide carbon for conversion to a variety of types of fuel (Pienkos and Darzins 2009). Global climate change, caused by burning petroleum and other fossil fuels, has motivated an urgent need to develop renewable alternatives. Diatom lipids have been suggested as a potential diesel fuel substitute with an emphasis on the neutral lipids due to the possibility to modulate their accumulation in algal cells (d'Ippolito et al. 2015; Levitan et al. 2014; Tanaka et al. 2015; Valenzuela et al. 2013). In 1998, the Aquatic Species Program recommended a list of 50 microalgal strains, selected from a pool of over 3000 candidates, with promising potential as biofuel-production organisms (Sheehan et al.1998). Sixty percent of the selected strains were diatoms, which were chosen based on criteria such as high growth rates and lipid yields, tolerance of harsh environmental conditions, and performance in large-scale cultures (Hildebrand et al. 2012). More recently, d'Ippolito an coworkers (2015)

screened seventeen diatom species for their capacity to produce biomass and lipids, in relation to their growth rate. Two strains of *Thalassiosira weissflogii* and *Cyclotella cryptica* were selected as best candidates for biofuel production. In these diatoms, nitrogen limitation induced a remarkably high level of TAG, above 80% of glycerolipids as a result of remodeling of the lipid pools and *de novo* synthesis of neutral lipids. The study suggested that appropriate changes in the culture conditions can be effective tools to modulate lipid metabolism and positively affect oil production in these photoautotrophic organisms.

1.4 Gene function studies with molecular tools

Interest in the ecological and economic roles of diatoms led to the need to develop techniques that can be used to monitor and manipulate diatom genes. Available resources for diatom studies include sequenced genomes of only few species (Armbrust et al. 2004; Bowler et al. 2008; Lommer et al. 2012; Tanaka et al. 2015; Mock et al. 2017, Basu et al. 2017) and a collection of about 170 transcriptomes (Keeling et al. 2014). Gene function can be determined by mutating the gene and studying the mutant phenotype for any possible alterations. It is possible to use two different approaches to mutate genes, reverse and forward genetics. Forward genetics seeks to identify genes involved in a biological pathway or process through the screening of cells (or organisms) that contain random modifications throughout the genome that can alter gene function. The advantage of forward genetics is that it does not require any prior knowledge of the gene sequence. The first step in any forward genetic approach is the creation of heritable mutagenic lesions that can be screened for phenotypic effects. Reverse genetics is an opposite approach. Analyzing the phenotypic effects of specific mutated gene sequences it is possible to deduce the function of a specific gene.

The manipulation of the expression of single genes by genetic transformation technologies offers powerful ways of studying genes by reverse genetics. In diatoms, different methods as electroporation (Niu et al. 2012; Zhang and Hu 2014) and bacterial conjugation (Karas et al. 2015; Diner et al. 2016) have been used for generating transgenic cells but they have been performed only for the model diatom *P. tricornutum*. The most commonly used technique is based on helium-accelerated bombardment of gold or tungsten microparticles coated with the DNA that is to be introduced into host cells by a gene gun machine (fig. 6). The

bombardment methodology was initially reported for *Cyclotella cryptica* and *Navicula Saprophila* (Dunahay et al. 1995) and subsequently for *P. tricornutum* (Apt et al. 1996).

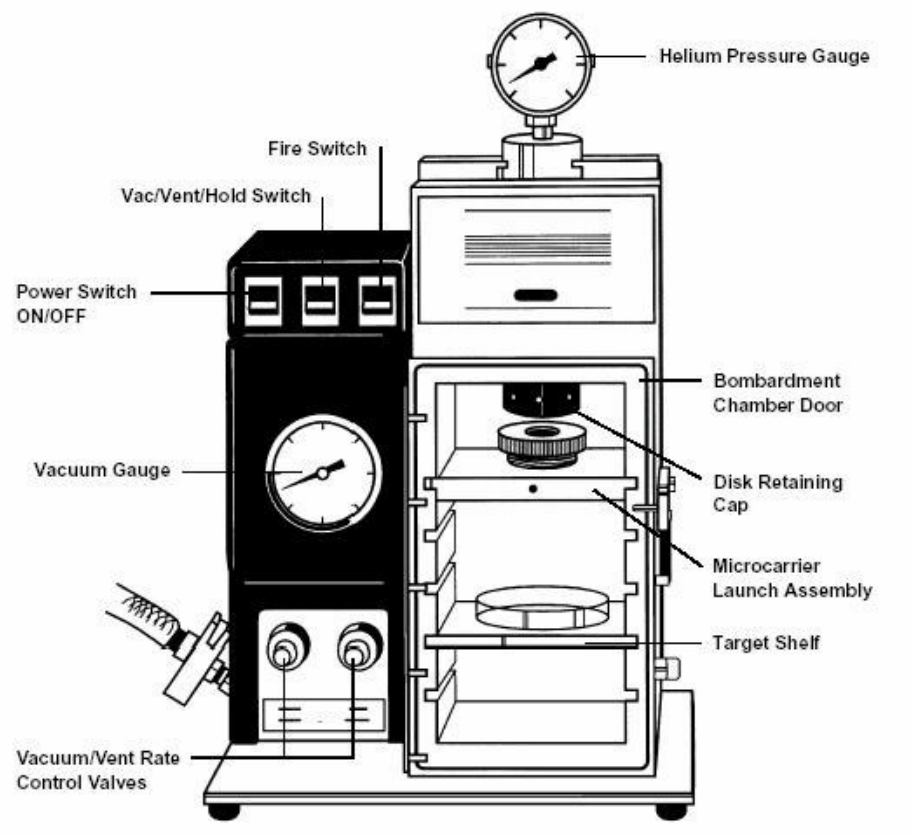

Fig. 6 Gene gun machine used for biolistic transformation.

The transformation methods can be used for different applications, such as for the inactivation and overexpression of genes, and for the localization of a gene product inside the cell by fusing it to fluorescent reporters. Using green fluorescence protein (GFP) as a reporter for protein localization, is possible to follow a protein of interest in live cells, for instance it was possible to study the mechanisms of protein translocation and import into diatom plastids (Kroth 2007). Reporter genes can be used to follow the activity of a promoter, DNA sequences upstream of protein

coding sequence that drive and regulate gene expression, and to determine the expression efficiency. One of the most used in plants and in diatoms also is GUS, a gene encoding β-glucuronidase (Falciatore et al. 1999; Apt et al. 2002). Another important and crucial application for elucidating gene function is the gene silencing, a process that generates small RNAs molecules that inhibit the expression of the gene of interest. De Riso (2009) reported for the first time this technique in diatoms. A complementary approach to the gene silencing is the overexpression of a gene of interest that can cause the phenotype alteration and can be helpful when the inactivation of a gene is lethal. Overexpression of specific genes has already been reported in diatoms (Zaslavskaia et al. 2000). In recent years, the research of new methods for genome editing gave rise to TALEN (Transcription Activator-Like Effector Nucleases) and CRISPR (Clustered Regulatory Interspaced Short Palindromic Repeats)/Cas9 systems development. These new tools are able to cut specific sequences of DNA double-strand that is repaired by one of two major mechanisms: non-homologous end-joining or homologous recombination. In *T. pesudonana* and in *P. tricornutum,* TALEN and CRISPR have been used successfully (Daboussi et al. 2014; Hopes et al. 2016; Nymark et al 2016).

These molecular techniques provide an excellent starting point for exploring novel aspects of diatom biology as inter- and intracellular signaling mechanisms that regulate population proliferation and programmed cell death in response to environmental signals.

1.5 The genus *Pseudo-nitzschia*

Pseudo-nitzschia is a marine planktonic pennate diatom that has a worldwide distribution. The genus *Pseudo-nitzschia* was separated from *Nitzschia* by Peragallo in 1908, but only in 1994 some species were transferred from *Nitzschia* to *Pseudo-nitzschia* (Hasle 1994). This latter genus can form intense, visible blooms, such as those reported in the Monterey Bay (Walz et al. 1994), and more often can form less dense blooms that are not visible to the naked eye. More than 40 species are known. Fourteen species of *Pseudo-nitzschia* are reported to produce the toxin domoic acid (DA), although some of the strains tested were not always toxigenic under test conditions (Trainer et al. 2012). It has been also reported a significant variability in DA production not only among different species but also among different strains of the same species (Bates, 1998). During a large *Pseudo-nitzschia* bloom off Prince Edward Island in 1987, 100 people were sickened and three died after eating cultured mussels (Bates et al. 1989). This was the first incident that brought recognition of *Pseudo-nitzschia* as a producer of DA. Symptoms from exposure to DA are gastrointestinal and neurological, and can include short-term memory loss, giving the syndrome the name of Amnesic Shellfish Poisoning (ASP).

Almost all the *Pseudo-nitzschia* species hitherto studied have a heterothallic life cycle. Sexual reproduction is obtained only when strains with opposite mating type get in contact (Geitler, 1935). The genus has elongate cells (apical axis considerably greater than transapical and pervalvar axes); two plate-like chloroplasts symmetrically arranged in the median transapical plane; colonies of cells serially joined in valvar contact with overlapping valves ("stepped" colonies); the valves may have both perforated and unperforated open bands; and the raphe is strongly eccentric. Among *Pseudo-nitzschia* species, *Pseudo-nitzschia*

multistriata and *Pseudo-nitzschia arenysensis* (fig.7) are two species commonly found in the Mediterranean Sea that form chains and can reproduce sexually.

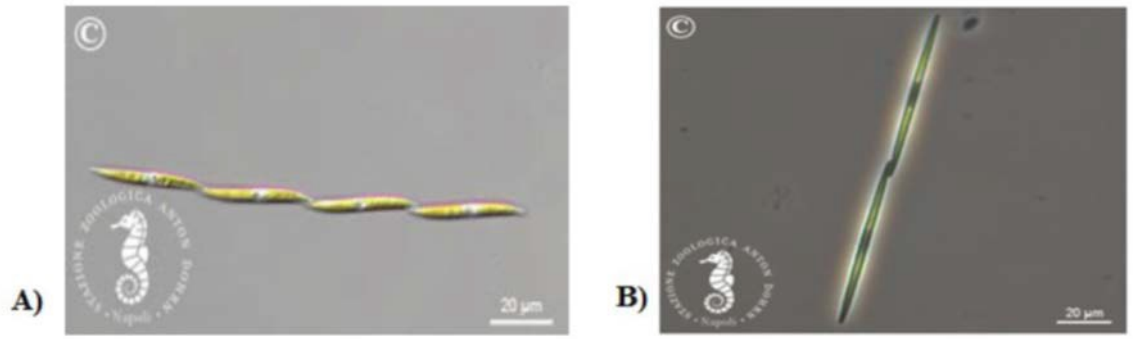

Fig. 7 a *P. multistriata* and **b** *P. arenysensis* cells.

P. multistriata was described for the first time by Takano in 1993 in Japanese waters. It has been reported from different locations including the Mediterranean Sea where it has been shown to produce DA (Orsini et al. 2002). It can be distinguished from other *Pseudo-nitzschia* by its prominent sigmoid shape in girdle view. Toxin content of *P. multistriata* cultures isolated from the Gulf of Naples was within the lowest values reported in the literature (Bates, 1998).

The genome sequence for this species has been generated and will become publicly available shortly (Basu et al. 2017).

P. arenysensis is a species that does not produce DA. In Lundholm et al. (2006), the authors described two new species and two clades based on morphology, molecular probes and phylogenetic analyses in the *P. delicatissima* group. The authors found large genetic variation among the strains of *P. delicatissima* and concluded that this species should be divided into two major clades, representing cryptic species. Then Amato et al. (2007) confirmed the observations, using morphological features, sequence data and mating compatibility. Finally, Quijano-Scheggia et al. in 2009 renamed one of the two clades as *P. arenysensis*.

P. arenysensis has been reported as one of the diatom species capable to produce oxygenated fatty acid derivatives (d'Ippolito et al. 2009; Lamari et al. 2013; Miralto et al. 1999; Zheng & Shimizu 1997). The transcriptome is available within Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) (Keeling et al. 2014). In Di Dato et al. (2015) the transcriptomes of *P. delicatissima*, *P. arenysensis* and *P. multistriata* were compared and the main metabolic pathways involved in the biosynthesis of isoprenoids and in domoic acid synthesis were analyzed.

1.6 Oxylipins

Oxylipins is a collective term for oxygenated compounds derived from fatty acids. They are an important class of signaling molecules in higher eukaryotes (Andreou et al. 2009; Blée 1998; d'Ippolito et al. 2005; Dar et al. 2015; Farmer et al. 2003; Gerwick et al. 1993; Lee et al. 2008; Pohnert & Boland 2002; Savchenko et al. 2013; Serhan et al. 2004; Turner et al. 2002). In animal systems, eicosanoids, derive from C_{20} PUFAs, regulate cell differentiation, immune responses, and homeostasis (Funk 2001). In contrast, terrestrial plants use derivatives of C_{18} (octadecanoids) and C_{16} (hexadecanoids) fatty acids as developmental or defense hormones (Weber 2002).

Marine diatoms produce a number of oxylipins (Cutignano et al. 2011; Fontana et al. 2007) including hydroxy acids, epoxyalcohols, -oxoacids and polyunsaturated aldehydes, mainly derived from eicosapentaenoic acid (EPA) and chloroplastic C16-fatty acids (Pohnert 2002; d'Ippolito et al. 2003; Cutignano et al. 2006; Fontana et al. 2007a). The first biosynthetic study in marine diatoms was in 2000, when Pohnert suggested that the production of aldehydes might derive from breakdown of C20 polyunsaturated fatty acids by lipoxygenase/hydroperoxide lyase (Pohnert, 2000). The first direct evidence of a mechanism involving lipoxygenases in oxylipin production was obtained with the characterization of the synthesis of octadienal and octatrienal from C16-fatty acids in *Skeletonema marinoi* and *Thalassiosira rotula* (d'Ippolito et al. 2002; d'Ippolito et al. 2003; d'Ippolito et al. 2005; d'Ippolito et al. 2006). To date there are very few studies on control and regulation of oxylipin biosynthesis in marine diatoms (Brownlee 2008; d'Ippolito et al.2009; Gallina et al. 2016; Ianora et al. 2015; Vardi et al. 2008; Vardi et al. 2006;

Vidoudez & Pohnert 2008) but none of them provides direct evidence. The presence of the complex network of oxygenated products in marine diatoms remains an unresolved question even if, in analogy with plants and animals, not a few authors correlate the occurrence of these products to response to physiological changes or environmental stress such as aging, herbivory, wounding, pathogen attack, osmotic shock, UV light and nutrient depletion.

1.7 Lipoxygenase

Fatty acids, the building blocks of triglycerides, phospholipids and glycolipids, are universally known as structural and storage molecules (Goold et al. 2015; Guschina & Harwood 2006; Nakamura & Li-Beisson 2016). However, many fatty acids play crucial roles in intracellular signaling pathways in prokaryote and eukaryote cells (Dave & Graham, 2012; Kenneth, 1996; León et al. 2001; Moore et al. 2013; Ryu 2004; Tsitsigiannis & Keller 2007; Weber 2002; Wymann & Schneiter 2008). The formation of fatty acid hydroperoxides (FAHs) may occur either by autoxidation or by the action of enzymes such as lipoxygenases (LOXs) or α-dioxygenase (α-DOX) (Feussner et al. 2001; Griffiths et al. 2000). Different metabolites collectively named oxylipins derive from FAHs by subsequent reactions.

LOXs are a large family of non-heme iron-containing dioxygenases (Brash 1999; Ivanov et al. 2010). They catalyze the insertion of molecular oxygen into the (1Z,4Z)-pentadiene system of polyunsaturated fatty acids (PUFAs) in a stereo– and regio-specific manner to produce the corresponding dienyl hydroperoxides (Brash 1999). Lipoxygenases are comprised of two domains; N-terminal and C-terminal domains. The N-terminal domain is a regulatory domain consisting mostly of β-barrels, while the C-terminal domain consists mostly of α-helices and contains the catalytic domain with the non-heme iron atom (Sigal 1991) (fig.8)

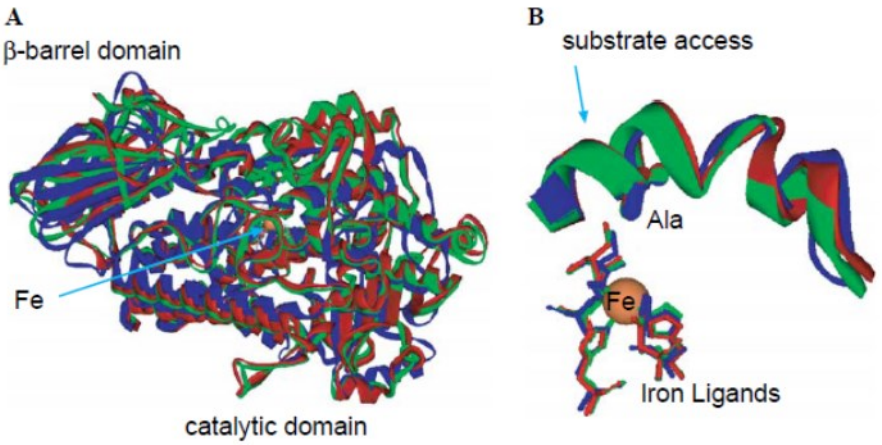

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Fig. 8 Structural conservation of lipoxygenases. **a** X-ray structures of Soybean LOX-1 (red), soybean LOX-3 (green), and rabbit reticulocyte 15-LOX-1 (blue). **b** Position of the Iron (orange), in the structure (Coffa et al. 2005).

The concept of a lipoxygenase activity was proposed in 1932 when André et al. suggested the presence of a lipid oxidase in soya beans. Lipoxygenases are found widely in plants, fungi, and animals (Grechkin 1994) and only recently they have been detected in coral, moss, algae and a number of bacteria as well (Andreou et al., 2009b) (fig. 9).

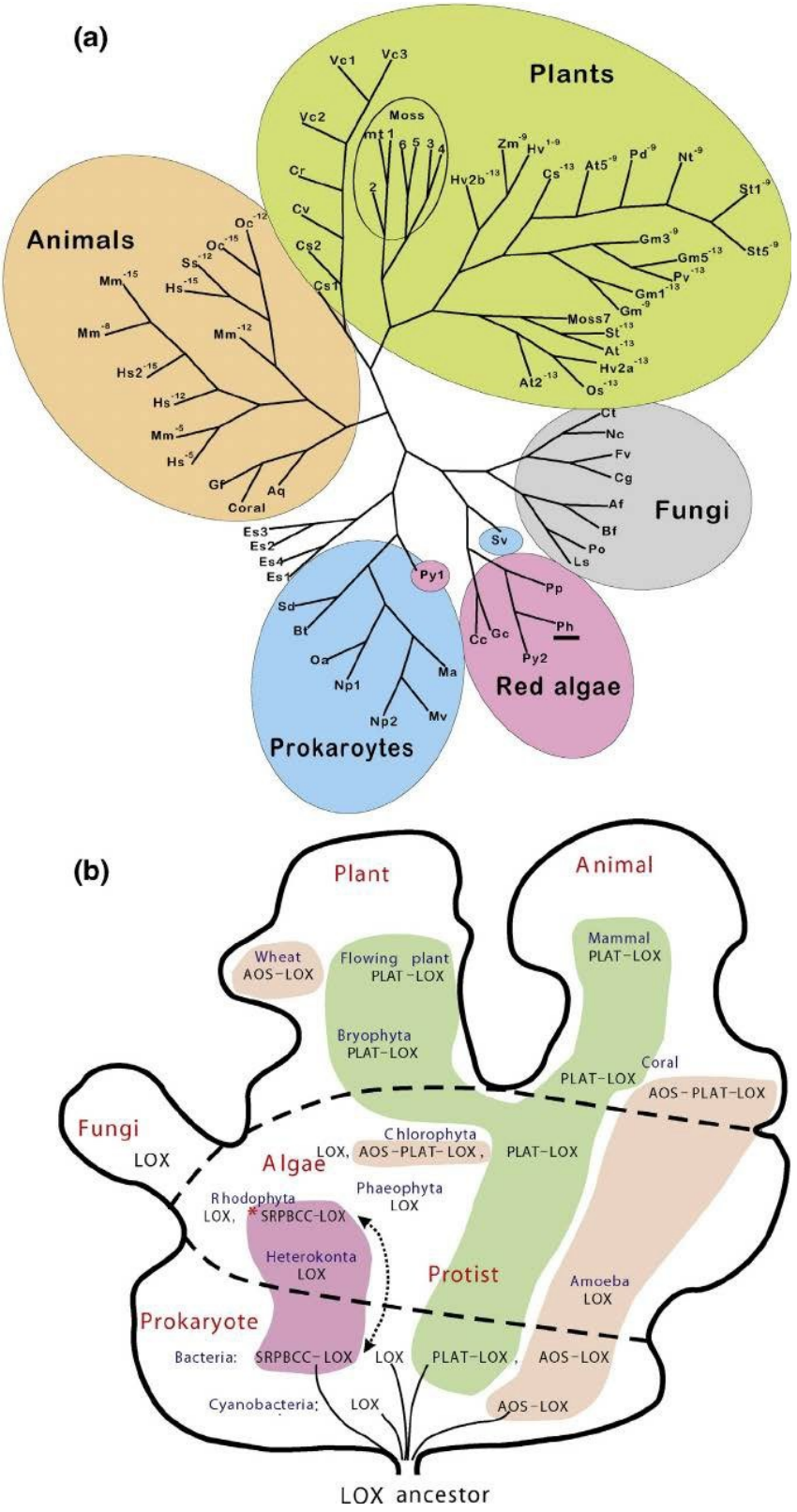


Fig. 9 LOXs phylogenetic tree. **a** The alignment was performed without the N-terminal domain of LOXs. **b** Structural evolution diagram of lipoxygenases (LOXs) according to N- and C-terminal domains (Chen et al. 2015).

The LOX reaction consists of three consecutive steps (fig. 10) starting from the removal of a hydrogen atom from a methylene unit between double bonds (hydrogen abstraction). The resulting radical is stabilized by electron delocalization through the double bonds and undergoes to addition of molecular at +2 or –2 position from the original radical carbon. Form this reaction arises a conjugated trans,cis-diene peroxy radical that is hydrogenated by water to form the hydroperoxide as final enzymatic product.

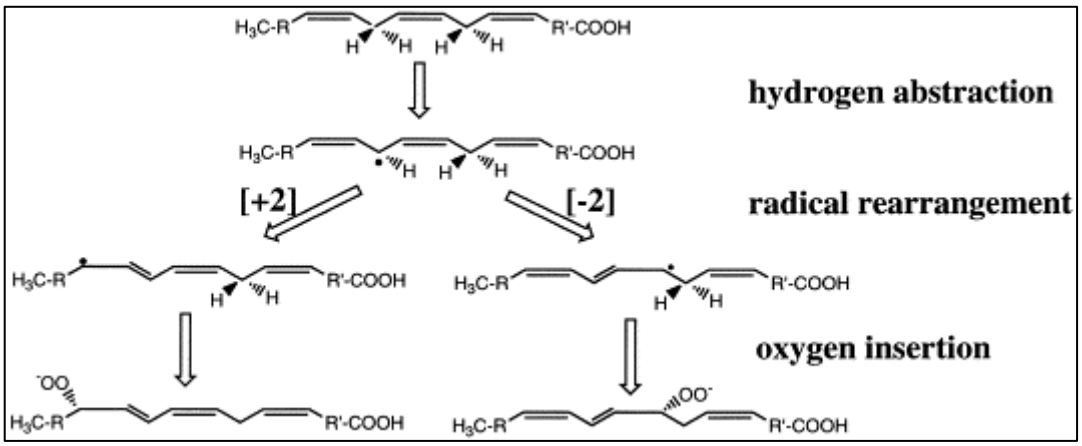


Fig. 10 LOX reaction divided in three steps (Kuhn and Thiele 1999).

LOXs are classified according to their positional specificity of the fatty acid oxygenation, which can take place at several different positions of the carbon chain. Generally speaking, lipoxygease sites vary in animals and plants even if there are position more common than other others. Besides the high regiospecificity, the insertion of oxygen exhibits also high stereospecificity that depends on the primary sequence of the enzyme, which is predicted to determine the orientation and depth of substrate penetration into the active site (Feussner and Kühn, 2000; Schneider et al., 2007). According to the current model of LOX catalysis, positional specificity of this class of enzymes is determined by the space within the active site and the orientation of the substrate. Different critical amino acids, identified at the active site of selected

LOX isoforms, influence the regio-specificity of the final products (Liavonchanka and Feussner 2006; Schneider et al. 2007).

1.7.1 Human lipoxygenase

The dominant LOX substrate in animals is arachidonic acid (AA) (20-carbon fatty acid) that is released from the membrane phospholipids by phospholipase A2 in response to various inflammatory stimuli including cytokines, peptides, and growth factors (Lambeau and Gelb 2008; Natarajan and Nadler 2004). There are three families of enzymes involved in the oxidative metabolism of AA. These include the lipoxygenases, which produce leukotrienes (LT), hydroperoxyeicosatetraenoic acids (HPETEs), hydroxyeicosatetraenoic acids (HETEs), and hydroxyoctadecadienoic acids (HODEs); the cyclooxygenases (COX-1 and COX-2) which produce prostaglandins including G2 and H2 as well as thromboxanes; and cytochrome P-450 monooxygenases that produce epoxides and HETEs (fig. 11)

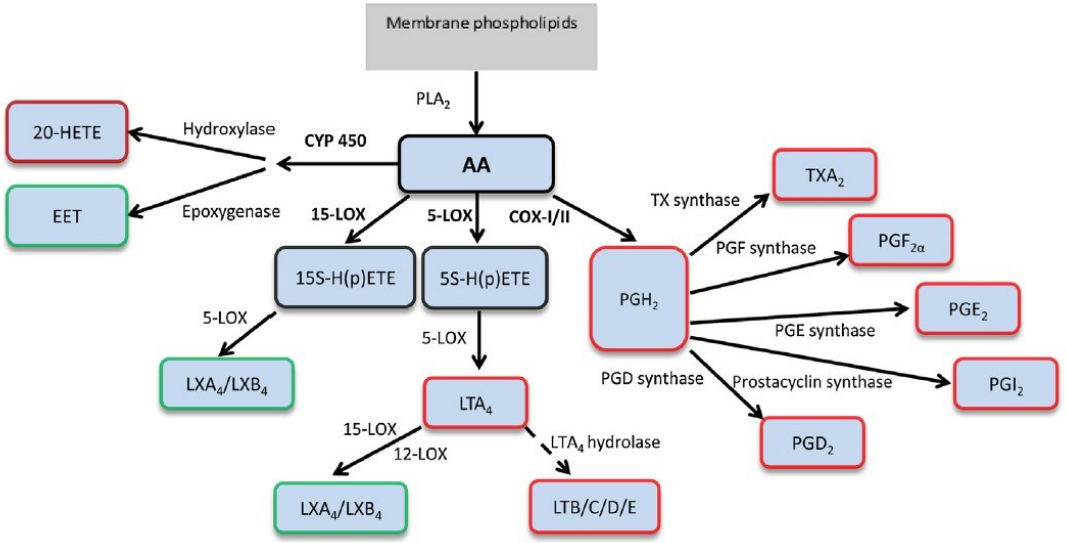


Fig. 11 Arachidonic acid metabolism (Klil-Drori and Ariel 2013).

For a long time it was believed that LOXs did not occur in animals but in 1974 the formation of (12*S*,5*Z*,8*Z*,10*E*,14*Z*)-12-hydroxyeicosa-5,8,10,14-tetraenoic acid (12-HETE) was described, when human thrombocytes were incubated with exogenous

arachidonic acid (Hamberg & Samuelsson 1974). This discovery marked the starting point of animal LOX research and over the following years more and more LOX isoforms were discovered. LOXs have been found in many organs and tissues, they are mainly cytosolic and active in the submembrane architecture of the cell. The products of their catalytic reaction, collectively known as eicosanoids, are potent signaling molecules that play an important regulatory role in the immune responses and other physiological processes.

In mammalians, lipoxygenases catalyze the formation of hydroperoxy eicosatetraenoic acids (HPETEs) from arachidonic acid. These HPETEs are subsequently reduced and transformed to form eicosanoids. Alternatively, LOX-dependent oxidation of EPA or docosahexaenoic acid (DHA) generates resolvins, oxygenated bioactive products (Serhan et al. 2002). These compounds are involved in many diseases such as in the regulation of inflammatory responses by generation of pro-inflammatory mediators known as leukotrienes or anti-inflammatory mediators known as lipoxins. Furthermore, they were found to be involved in many aspects of human cancer, such as angiogenesis, chronic inflammation, metastasis formation, and direct and indirect tumor suppression, and in cardiovascular and kidney diseases, neurodegenerative disorders and metabolic syndromes (Table 1).

New different therapeutic approaches for numerous inflammatory diseases and cancer have been developed using lipoxygenase small molecule inhibitors.

Lipoxygenase	Substrate	Product	Physiologial function
5-lipoxygenase (5-LOX)	arachidonic acid	5(S)-HPETE, Leukotriene A4	Pro-inflammatory mediator
	γ-linoleic acid	Dihomo-γ-linoleic acid (DGLA)	Inhibition of arachidonic acid conversion
	Eicosapentaenoic acid (EPA)	Leukotriene A5	Anti-inflammatory mediator/inhibitor LTA4 hydrolase
Platelet 12-lipoxygenase (p12-LOX)	arachidonic acid Dihomo-γ-linoleic acid (DGLA) Eicosapentaenoic acid (EPA) α-linoleic acid	12(S)-HPETE 12(S)-HPETrE 12(S)-HPEPE 12(S)-HPOTrE	Modulation of platelet aggregation
12R-lipoxygenase (12R-LOX)	arachidonic acid Linoleyl-ω-hydroxy ceramide	12(R)-HPETE 9(R)-hydroperoxyllinoleoyl-ω-hydroxy ceramide	Epidermal barrier acquisition
epidermis LOX3 (eLOX3)	9(R)-hydroperoxyllinoleoyl-ω-hydroxy ceramide	9(R)-10(R)-trans-epoxy-11E-13(R)-hydroxylinoleoyl-ω-hydroxy ceramide	
15-lipoxygenase-1 (15-LOX1)	linoleic acid	13(S)-HPODE	modulation of MAP kinase signaling pathways
	arachidonic acid	15(S)-HPETE	modulation of leukotriene B4, pro-inflammatory mediators
15-lipoxygenase-2 (15-LOX2)	arachidonic acid	15(S)-HPETE	negative cell cycle regulator and tumor supressor

Table 1. Human lipoxygenases and their most important substrates, products, and functions (Modified from Wisastra and Dekker 2014).

1.7.2 Plant Lipoxygenase

In plants the major substrates for LOX are linoleic acid (LA) and α-linolenic (α-LeA) (Mueller et al. 2006). A first plant LOX classification is based on the comparison of their primary structure due to the presence of signal peptide. They are divided in two groups: type 1-LOXs do not possess transit peptide and show a high sequence similarity (>75%), and type 2-LOXs that carry a chloroplast transit peptide sequence and have only a moderate sequence similarity (~35%). A second way to classify plant LOXs is with respect to their positional specificity of substrate oxygenation. Molecular oxygen can be introduced either at carbon atom 9 (9-LOX) or at carbon 13 (13-LOX) of the fatty acid hydrocarbon backbone leading to the formation of two groups of compounds, 9- and 13-hydroperoxy derivatives of linoleic (9- and 13-HPOTE) and linolenic (9- and 13-HPODE) acids. Plant LOX is a monomeric protein of about 95-100 kDa. The N-terminal domain of about 25–30 kDa is a β-barrel domain that is structurally related to so called C2 domains (Corbin et al. 2007). Its exact function is yet unknown. The C-terminal domain of about 55–65 kDa consists primarily of α- helices and contains the catalytic site of the enzyme (Schneider et al. 2007). According to substrate orientation model, a substrate fatty acid may penetrate the active site with its methyl end ahead, leading to linoleate 13-lipoxygenation. Alternatively, the substrate may enter the active site in an inverse ‘‘head-to-tail” orientation leading to linoleate 9- lipoxygenation (Andreou and Feussner 2009) (fig. 12).

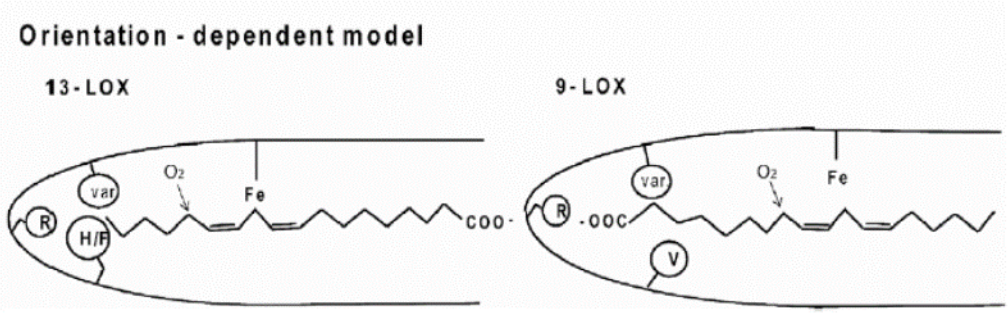


Fig. 12 Substrate orientation model. Straight- and inverse-substrate orientation lead to linoleate 13- and 9-lipoxygenation (Andreou and Feussner 2009).

The hydroperoxy fatty acid products of the LOX reaction can be further converted to different compounds through the action of enzymes participating in different pathways. Four major metabolic pathways have been well characterized. The peroxygenase (POX) pathway converts fatty acid hydroperoxides to epoxy- or dihydrodiol polyenoic fatty acids (Blée 1998; Andreou et al. 2009). Allene oxide synthase (AOS) is the first enzyme in the branch pathway leading to the biosynthesis of jasmonic acid (JA), and catalyzes the production of unstable allene epoxides that cyclize to form cyclopentenone acids, the precursors for JA (Schaller et al. 2004; Stenzel et al. 2003; Turner et al. 2002). The hydroperoxide lyase (HPL) enzymes catalyze the oxidative cleavage of the hydrocarbon backbone of fatty acid hydroperoxides (Howe et al. 2000; Matsui et al. 2000; Noordermeer 2001). This leads to the formation of short chain aldehydes (C6- or C9) and the corresponding C12- or C9-v fatty acids. The divinyl ether synthase (DES) pathway forms divinyl ethers (Hamberg 1999; Yu et al. 2003) (fig.13). There are other reactions less well characterized but not less important that include: (a) the LOX-catalyzed hydroperoxidase reaction of homolytic cleavage of the O–O bond to form ketodienes (Kühn et al. 1992); (b) the epoxy alcohol synthase (EAS) pathway that forms epoxy hydroxy fatty acids by intramolecular rearrangement of hydro(pero)xy fatty acids (Hamberg 1999); (c) the reductase dependent-reaction responsible for

the conversion of hydro(pero)xy fatty acids to their corresponding hydroxy derivatives (Feussner et al. 1998) (fig.13).

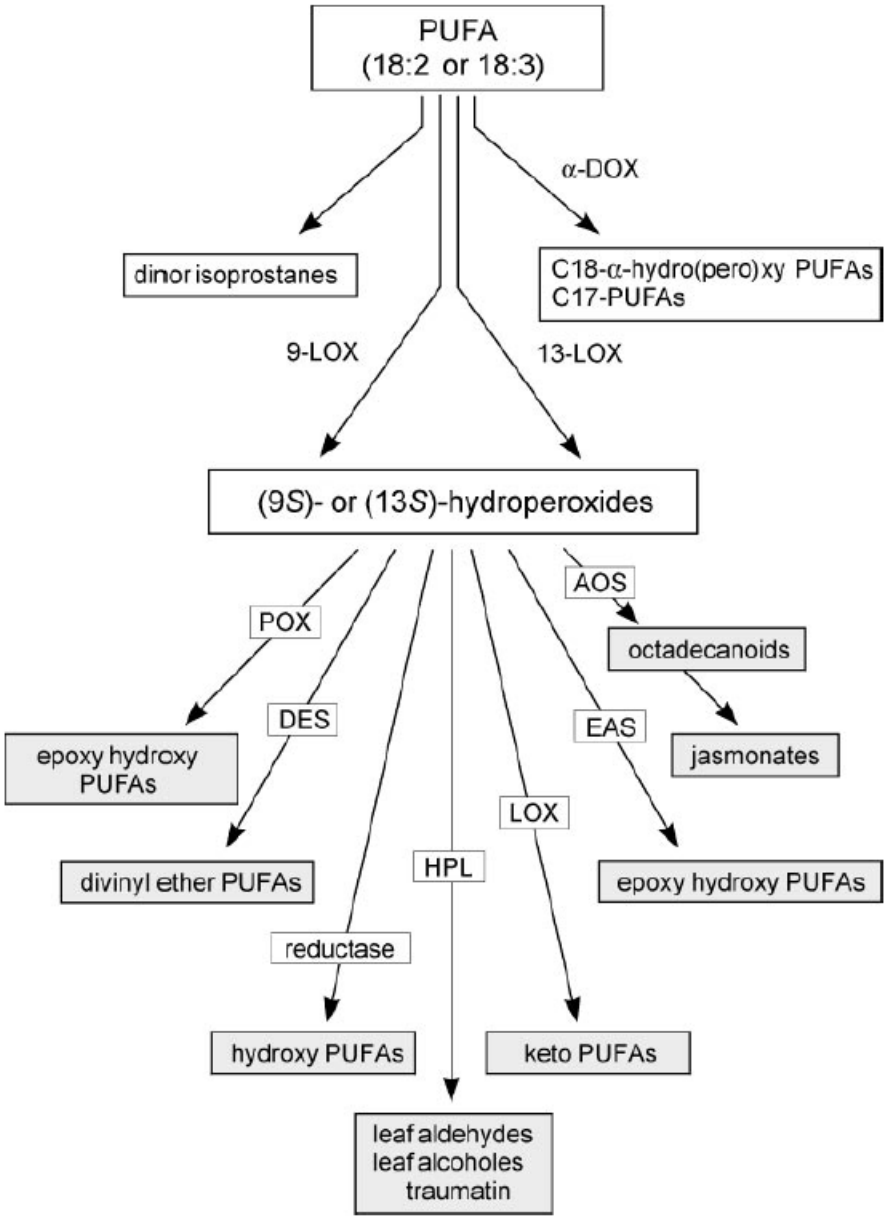


Fig.13 PUFAs metabolism in plants.

LOX proteins play important roles in lipid peroxidation under biotic and abiotic stress, and are involved in a number of developmental stages (Siedow 1991; Kolomiets et al. 2001). Some of them were shown to be involved in plant defense reaction such as a pathogen infection (Gomi et al. 2002) or wounding (Kim et al. 2003). Figure 14 summarizes the role of LOX pathways in higher plants.

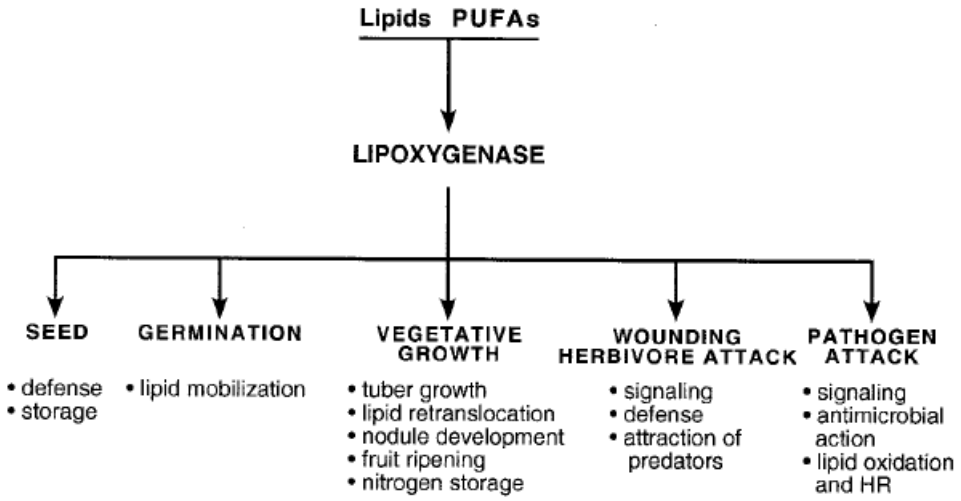


Fig.14 Different roles of LOX in plant lipid metabolism (from Porta and Rocha-Sosa 2002).

1.7.3 Diatom Lipoxygenase

Oxylipins act as chemical mediators in many ecological and physiological processes in marine and freshwater diatoms (Cutignano et al. 2011). Activation of the LOX pathway is initiated by cell damage (Pohnert 2002) after that an uncharacterized lipolytic enzyme releases polyunsaturated fatty acids from phospholipids and glycolipids (Cutignano et al. 2006; d'Ippolito et al. 2004).

The major substrate of diatom LOX is eicosapentaenoic acid (EPA, C20:5) but synthesis of characteristic derivatives of C16 PUFAs has been reported from axenic cultures of *Skeletonema marinoi* and *Thalassiosira rotula* (d'Ippolito et al. 2005, Fontana et al. 2007b). A distinctive difference of LOX metabolism in diatoms is the almost complete absence of products derived from C-18 PUFA that form the bulky of oxylipins in algae and plants.

The differences in the oxygenation position catalyzed by LOX and the variability of the downstream reactions add great diversity to the types of oxylipins in diatoms. Different chemical studies have identified three major families that include hydroxy fatty acids, carbonyl-containing fatty acids, and epoxyalcohol fatty acids (fig.15). In addition, hydroperoxide lyases (HPLs) are important enzymes involved in the oxidative metabolism of lipids in Bacillariophyceae. LOX ⁄ HPL pathways are responsible for the synthesis of aldehydic compounds, such as PUAs and oxoacids, which are usually found in a few diatom species. Like in higher plants and animals, LOX products of diatoms appear to be involved in systemic defense mechanisms (Ianora et al., 1999 and 2015; Fontana et al., 2007; Barreriro et al., 2011), cell growth (d'Ippolito et al., 2009) and to control inter- or intracellular response to external stimuli (Ribalet et al., 2007; Vardi et al., 2006).

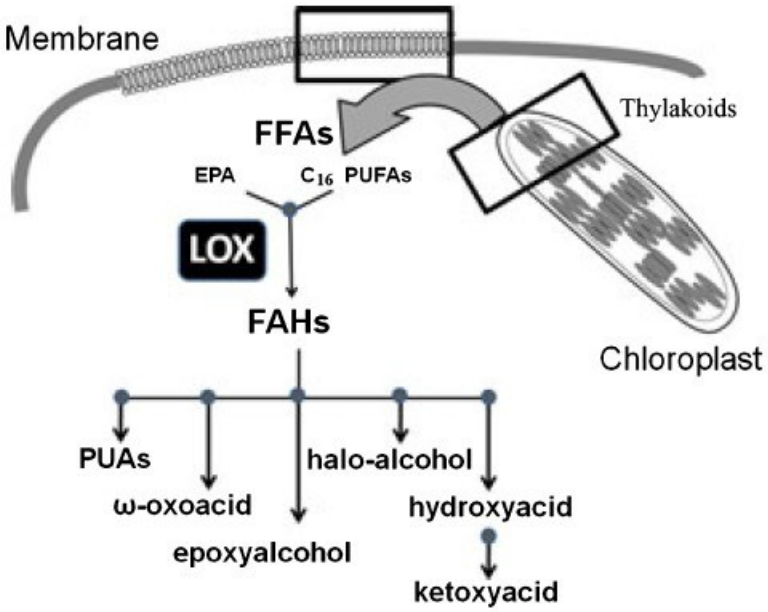


Fig.15 Lipoxygenase pathway in marine diatoms (Cutignano et al. 2011).

The primary products of LOX activities are fatty acid hydroperoxides (FAHs). FAHs compromise egg viability and larval development of copepods in a dose-dependent manner that requires concentrations lower than those reported for PUAs (Fontana et al. 2007). Activation of oxylipin pathways is also associated with oxidative stress and the synthesis of reactive oxygen species (hROS) (Fontana et al. 2007). In diatoms, the mix of hROS and oxylipins can lead to reproductive failure in copepods due to apoptosis and teratogenesis (Fontana et al. 2007). Diatom oxylipins may function as species-specific mediators of bloom control. It was demonstrated how diatoms can accurately sense a potent DD (2E,4*E/Z*-decadienal) and employ it as a signaling molecule to control diatom population sizes (Vardi et al. 2006).

In Lamari et al. (2013) the authors characterized the compounds derived from EPA in *Pseudo-nitzschia* species. These can be grouped in two major families containing

hydroxyl-eicosapentenoic acids (HEPEs) and hydroxyl-epoxy eicosatetraenoic acids (HEpETEs). *Pseudo-nitzschia* exhibits a rich and varied LOX metabolism of eicosapentaenoic acid (EPA). In *P. delicatissima* 5-LOX derived compounds were found, in *P. pseudodelicatissima* 8-LOX and in *P. fraudulenta* and *P. multistriata* 14-LOX.

In all *P. arenysensis* strains analyzed, Lamari et al. (2013) found the presence of 15-LOX-derived compounds, namely 15-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid (15- HEPE), 13-hydroxy-14-epoxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid (13,14- HEpETE); and 12-LOX-derived compounds, 12-hydroxy-5Z,8Z,10E,14Z,17Z- eicosapentaenoic acid (12-HEPE) and 10-hydroxy-11-epoxy-5Z,8Z,14Z,17Z- eicosatetraenoic acid (10,11-HEpETE). 15-oxo-5Z,9E,11E,13E-pentadecatetraenoic acid (15-OXO) was found only in some samples of *P. arenysensis*. The authors suggest that the observation that some strains possess both compounds and others just one of these could then be explained by the fact that diatoms are diploid and that some of the strains are 12-LOX or 15-LOX homozygous and others 12-LOX + 15-LOX heterozygous.

A *LOX* gene sequence, identified in *P. arenysensis* transcriptome (Keeling 2014) was characterized in the PhD thesis of Ida Orefice (Open University, 2010-2013). This sequence (MMETSP0329-20121206|11370) is 2230 nucleotides long and possesses an ORF (Open Reading Frame) that encodes for a protein of 704 amino acids with a molecular weight of about 79 kDa. The sequence alignment and the phylogenetic analysis performed in Orefice's thesis indicated that *P. arenysensis* LOX seems to be related to red algae, bacteria and animal 5- and 15-LOXs and distant from brown algae and plant LOXs.

2. AIM OF THESIS

The importance of diatoms in ecology and biotechnology prompt the development of new molecular tools to study gene function and their unique metabolic pathways. Diatoms produce oxylipins, oxygenated compounds that are involved in regulatory and signaling pathways in animals and plants. In diatoms, the role of oxylipins is unclear and the few pieces of evidence do not substantiate their function in natural systems. Many studies have so far described synthesis and occurrence of these molecules but there is a lack of information about control and regulation of the pathways.

The aim of this PhD thesis was the study of the oxylipin function in *Pseudo-nitzschia arenysensis* through the development of molecular tools. The genus *Pseudo-nitzschia* is responsible for blooms in coastal and oceanic waters and it shows a rich and varied lipoxygenase metabolism of eicosapentaenoic acid (EPA) (Lamari et al. 2013). On the other hand, the most widely used model diatoms do not produce oxylipins. Since molecular tools were not available for *Pseudo-nitzschia*, the first part of this work was dedicated to the setup of the molecular techniques in *Pseudo-nitzschia* such as the biolistic transformation.

The manipulation of the expression of single genes by genetic engineering technologies offers powerful ways of studying genes by reverse genetics. One of important and crucial application for elucidating gene function is the gene silencing, a mechanism that inhibits the expression of a gene of interest. In my case, the target was a lipoxygenase (*LOX*), encoding for the main enzyme responsible for oxylipins formation. For the identification of the encoding gene, I started this study from the unpublished results of a previous study carried out by Orefice in her PhD thesis on LOX occurrence and activity in diatoms. I also took advantage of RNAi, highly

used in different organisms to study gene function, to perturb the function of LOX. RNAi experiments to silence the *LOX* gene were performed: i) to understand if the RNAi strategy was effective in reducing the oxygenated products synthesis; ii) to understand the physiological effects that the reduction of oxylipins can have in diatoms. The overall scope was therefore to generate mutants of *Pseudo-nitzschia arenysensis* that were silenced for the *LOX* target genes.

3. MATERIALS AND METHODS

3.1 Cell culture

The strain of *Pseudo-nitzschia arenysensis* used for the biolistic transformation was B858 (Adelfi et al. 2014). The strain used for gene silencing was VS3, isolated on 5/12/13 from crosses performed in the laboratory shown in fig. 16. Both the clones were grown in seawater enriched with F/2 nutrients (Guillard 1975), incubated at 18°C under white light at approximately 80 µmol photons m^{-2} s^{-1} and 12:12h dark-light cycle. The cultures used for the experiments were always collected during their exponential growth phase.

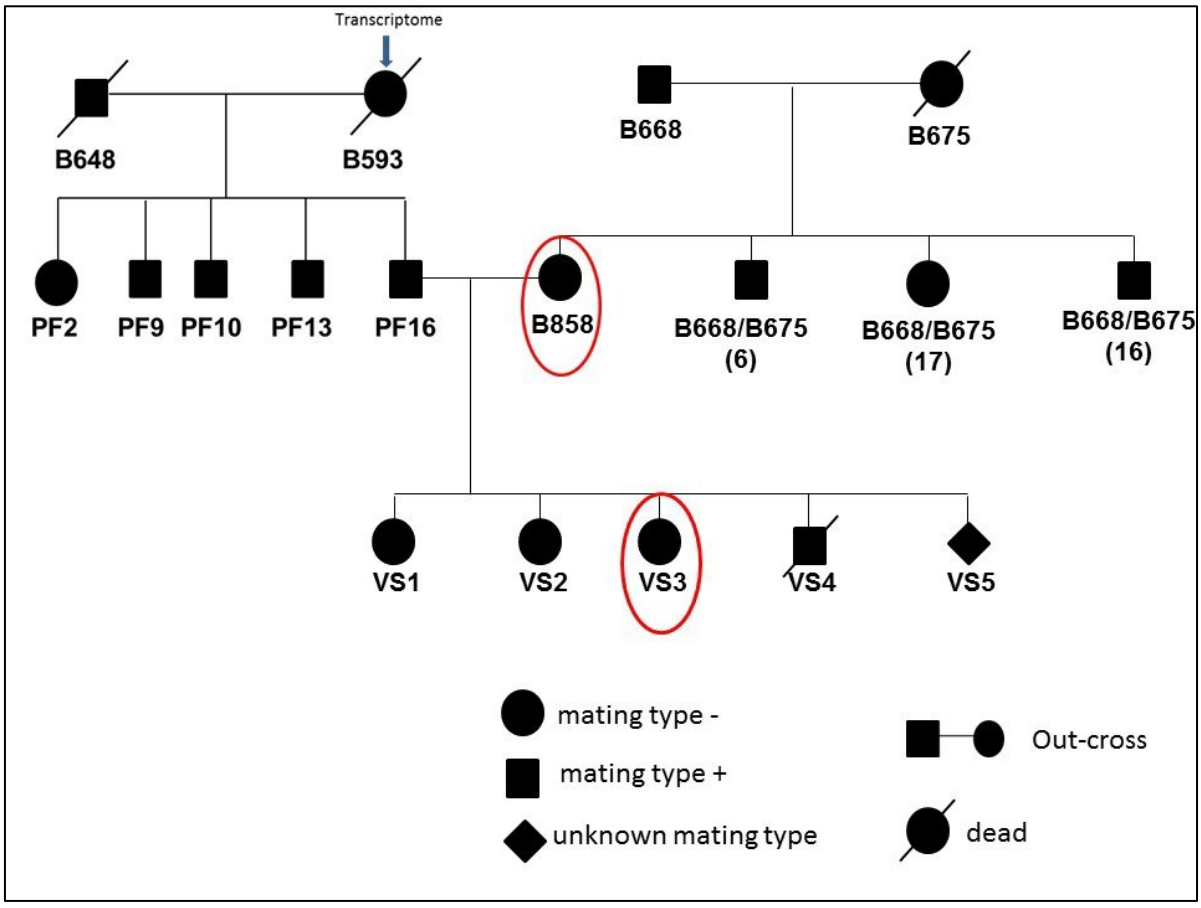


Fig. 16 Pedigree of *P. arenysensis* strains used in laboratory. The strains used in this work are circled in red.

3.2 Plasmids construction

The vector plasmid pFCPBp-Sh Ble (Falciatore et al. 1999) was used as the backbone transformation vector. The promoter sequence of the H4 gene, from base 663 to base 38 upstream of the H4 ATG, was amplified from *Pseudo-nitzschia multistriata* genomic DNA using the sense primer H4pKpnI for: 5′-GGCGGCCGGTACCGGCCATTTTGGAATTTGTCG-3′ and the antisense primer H4pKpnI rev: 5′- GGCGGCCGGTACCGCCGAAGACTGAGGTAGTCG-3′ (KpnI sites underlined). The resulting product was digested with KpnI generating a 625-bp fragment that was cloned replacing the fucoxanthin chlorophyll a/c-binding protein (FcpB) promoter to obtain the PmH4pShble plasmid. To construct the PmH4pH4N-GFP plasmid The H4 gene was amplified from *P. arenysensis* genomic DNA using the sense primer H4StuI for: 5′-GGCGGCCAGGCCTATGTCTGGAAGAGGAAAAGGAGG-3′ (StuI site underlined) and the antisense primer H4NotI rev: 5′-GGCGGCCGCGGCCGCTTATCCACCGAATCCGTAAAGG-3′ (NotI site underlined) The *P. arenysensis* Histone H4 gene was cloned replacing the Sh ble fragment in the PmH4pShble plasmid. For the amino-terminal fusion construct, the EGFP gene was amplified from the plasmid pEGFP-N1 (Clontech) by PCR using the sense primer GFPKpnI for: 5′-GGCGGCCGGTACCATGGTGAGCAAGGGCGAG-3′ (KpnI site underlined) and the antisense primer GFPStuI rev: 5′-GGCGGCCAGGCCTCTTGTACAGCTCGTCCATGCC-3′ (StuI site underlined).

The PmH4pGus plasmid was obtained by replacing the FcpB promoter in the FcpBp-GUSFcpA 3′ plasmid (De Riso et al. 2009) with the *P. multistriata* H4 promoter amplified using the sense primer H4pKpnI for and the antisense primer

H4pEcoRI rev: 5′-GGCGGCCGAATTCGCCGAAGACTGAGGTAGTCG-3′ (EcoRI site underlined).

The constructs for the *LOX* silencing were obtained using PmH4pShBle as backbone. Two fragments of the *LOX* gene were amplified by PCR from the *P. arenysensis* cDNA using the following primers: LOXNotI_F, LOXStuI_Rcorto and LOXStuI_Rlungo. A 180 bp fragment, corresponding to the *LOX* gene sequence from 1807 bp to 1986 bp, was obtained with LOXNotI_F and LOXStuI_Rcorto, while a 424 bp product, corresponding to the region from 1807 bp to 2230 bp, was generated using LOXNotI_F and LOXStuI_Rlungo. These two fragments have the first 180 bp in common. For the antisense construct, either the longer or the shorter *LOX* fragment were digested with *Not*I and *Stu*I and subsequently introduced in the antisense orientation, between the *Sh ble* gene and the FcpA terminator, into the PmH4pShble vector. For the inverted-repeat construct, both the longer and the shorter fragments were digested with *Not*I and *Stu*I and subsequently ligated to each other with the *Stu*I site and cloned, in sense and antisense orientations, into the PmH4pShble vector.

3.3 Biolistic transformation

The transformation of the B858 strain was performed by microparticle bombardment using the Biolistic PDS-1000/HE Particle Delivery System (Bio-Rad). The day before bombarding, cells were harvested at the mid-logarithmic phase. Approximately 5×10^7 cells were spread onto 0.4 % agarose plates containing F/2 medium and allowed to dry under a sterile hood. Plasmid DNA (5 µg) was coated onto M10 (0.7 µm, Bio-Rad) tungsten particles using the $CaCl_2$-spermidine method according to the manufacturer's protocol. The plates were positioned at a distance of 6 cm from the stopping screens and a burst pressure of 1550 psi was used. After

bombardment, the plates were incubated for 24 h at standard growth conditions. For each bombarded plate, the cells were then scraped from the agarose, resuspended in F/2 liquid medium (200 ml) containing 50 µg ml^{-1} zeocin (Invivogen) and incubated under standard growth conditions. Putative transformants were obtained after about 12 days of incubation. For co-transformation, cells were bombarded using conditions described above; particles for bombardment were coated with 3 µg of PmH4pShble plus 3 µg of PmH4pH4N-GFP or with 3 µg of PmH4pShble plus 3 µg of PmH4pGus. The same procedure was performed for the *LOX* silencing with VS3 strain.

3.4 PCR analysis on transformed cells

PCR was performed on genomic DNA from transformants, silenced and wild-type cells, obtained by centrifugation of the culture for 15 min at 2465 g at 16°C, followed by resuspension of the pellet in 500 µl of CTAB extraction buffer (Tesson et al. 2011). The thermal profile used was: 94 °C for 2 min (94 °C for 45 s, 55 °C for 45 s, 72 °C for 90 s)×30 cycles, 72 °C for 7 min. The primers used were: H4p 5′-GACTACTTCCGGGTCCATG-3’; Forble 5′-ACGACGTGACCCTGTTCATC-3’; Revble 5′-GTCGGTCAGTCCTGCTCCT-3’. The amplification with H4p and Revble produces a 889 bp fragment, the amplification with Forble and Revble produces a 250 bp fragment. Amplified fragments were analyzed on agarose gel.

3.5 Hoechst staining and fluorescence microscopy

Five microliters of 0.1 mg ml^{-1} Hoechst (33342, Molecular Probes, Invitrogen) solution was added to each milliliter of live sample and incubated for 15 min in the dark before observation. Live transformant cells were imaged using the Leica DMI6000B inverted epifluorescence microscope equipped with a DFC360FX

camera.

3.6 GUS assay

After about 10 days from the co-transformation, resistant *P. arenysensis* cells were isolated, expanded, and analyzed by histochemical GUS assay. The *P. tricornutum* Pt/GUS strain was grown and treated as described in De Riso et al. (2009). Histochemical assays were performed on cells plated on agarose in 5.5 cm Petri dishes containing 400 µl X-Gluc solution (0.1 % X-Gluc, Clontech); 100 mM Na-phosphate at pH 7.0; 0.5 mM $K_4Fe(CN)_6$; and 0.5 mM $K_3Fe(CN)_6$, placed in the dark and incubated at 37 °C overnight. For spectrophotometric assays, 50 ml of cells were collected in exponential phase and centrifuged at 2900 g for 15 min. The pellet was immediately frozen in liquid nitrogen and stored at −80 °C. Later, the cells were resuspended in 200 µl of fresh GUS extraction buffer (50 mM Na-phosphate pH 7.0; 10 mM β-mercaptoethanol; 0.1 % Triton X-100; 1 mM PMSF; 1× cocktail of protease inhibitors (Complete, Roche 11873580001, 1 tablet/2 ml)), frozen twice in liquid nitrogen and thawed at 37 °C. The samples were centrifuged at 15300 g for 5 min at 4 °C. GUS assays were performed by incubating 20 µg of total protein extract with the substrate p-nitrophenyl glucuronide (PNPG) at 1 mM final concentration. After a 4-h incubation at 37 °C, the reaction was stopped by adding 0.4 ml of 2.5 M 2-amino-2methyl-1,3 propandiol and absorbance was measured at 415 nm. Enzymatic activity was calculated on the basis of the OD recorded and the molar extinction coefficient of the GUS substrate p- nitrophenol.

3.7 Crosses and F1 cells isolation

To demonstrate inheritance of exogenous DNA from transformed cells to the next generation, transformed cells were crossed with sexually compatible wild-type

strains. Mating experiments were carried out by mixing a single resistant clone with a wild-type strain of opposite mating type in 6 well plates and incubating them at standard growth conditions. F1 generation initial cells, easily distinguishable by size difference, were observed after 2 to 3 days. Few days after, initial cells appeared, the mixed cultures were placed in zeocin selection (50 µg ml^{-1} zeocin). After 8 days, the surviving initial cells were serially re-isolated (two times) from the mating culture by micropipette under inverted microscope, and each initial cell was placed into a separate well of a 24-well plate containing sterile seawater enriched with F/2 nutrients. The initial cells were subsequently grown under standard growth conditions and later genotyped for the incorporation of *Sh ble* gene by PCR analysis and Southern blot analyses.

3.8 Genomic DNA extraction and southern blot hybridization

For genomic DNA preparation *P. arenysensis* cultures with cell density of 1×10^5 cells ml^{-1} were harvested by filtration onto 1.2 µm pore size filters (RAWP04700 Millipore). Cells were disrupted by vortexing in the presence of 0.4 g glass beads 0.2–0.3 mm diameter (G1277, Sigma-Aldrich), 0.5 ml TE buffer, and 0.5 ml buffered phenol. Following steps of DNA extraction and purification were performed as reported in Vanstechelman et al. (2013). Digoxigenin (DIG)-based Southern blot was performed on 5 µg of genomic DNA digested with HindIII. Only one site for this restriction enzyme exists in the antibiotic resistance expression vector used for transformation, and no site was present in the hybridization probe. The digested DNA was separated on 0.9% agarose gel, and then transferred to a positively charged nylon membrane (Roche) according to Sambrook and Russel (2001). The DIGlabeled Sh ble DNA probe was prepared by PCR DIG Probe synthesis kit (Roche), according to the manufacturer's instructions, using the primers Forble and

Revble (see BPCR Analysis on Transformed Cells^). Hybridization and autoradiography were performed according to the DIG Application Manual for filter hybridization (Roche).

3.9 Signal peptide prediction

The signal peptide potential for the protein sequence was analyzed using two different commonly used prediction algorithms. SignalP 3.0 (Bendtsen et al. 2004) consists of two different predictors based on neural network and hidden Markov model algorithms. ASAFind (Gruber et al. 2015) identifies nuclear-encoded plastid proteins in algae with secondary plastids of the red lineage based on the output of SignalP and the identification of conserved “ASAFAP”-motifs and transit peptides. A further prediction program used was ChloroP (Emanuelsson et al. 1999)

3.10 FOX2 assay

The measurement of fatty acid hydroperoxide levels was performed with the method described in Orefice et al. (2015). The reagent A was prepared by dissolving 2.5 mM ferrous sulfate with 1.1 M perchloric acid in dd water. In the reagent B 167 µM xylenol orange and 4.4 mM butylated hydroxytoluene were mixed with methanol. One volume of reagent A was added to 9 volumes of reagent B to form the Fox2 reagent. Cell pellets were resuspended in 1 ml of 50 mM Tris-HCl pH8, 0.5 M NaCl buffer. Cells were sonicated for 1 min, carefully avoiding foam formation and incubated at room temperature for 10 min. Part of these samples was taken for protein quantification. Three aliquots of VS3 control sample and of sample 5.5 corresponding to different cell concentrations were combined with 50 mM Tris-Hcl pH8, 0.5 M NaCl to obtain a final volume of 200 µl. Then 800 µl of Fox2 reagent was added to each sample and incubated for 15 min at room temperature. Samples were

centrifuged at 12000 g for 4 min at 16°C and the absorbance was measured at 560 nm (Beckman DU 530). Fox2 assay was performed with biological triplicate samples and all data points were run in duplicate. Statistical analysis were carried out with GraphPad Prism 4.00 (GraphPad Software, Inc.). The fatty acid hydroperoxide levels were normalized to protein content. The protein concentration was measured according to Bradford method (1976). Bovine serum albumin (BSA) was used as standard to establish calibration curves for protein quantification. Different volumes of BSA were mixed with 50 mM Tris-Hcl pH8, 0.5 M NaCl to a final volume of 100 µl, all samples were prepared in duplicate, 1 ml of Bradford was added to each sample. After an incubation time of 5 min at room temperature, absorbance was measured at 595 nm. To determine protein sample concentrations, three different volumes, 5, 10 and 20 µl, were mixed with 50 mM Tris-Hcl pH8, 0.5 M NaCl to a final volume of 100 µl, then 1 ml of Bradford was added to each sample. Samples were processed as described for BSA samples.

3.11 Oxylipins analysis

To profile oxylipins (Cutignano et al. 2011), frozen cell pellets were suspended in 1 ml of sea water (pre-filtered through 0.22 µm nylon filter) and sonicated for 1 min in a beacker with ice and water. The suspension was kept at room temperature for 30 min, then one aliquot was taken for protein assay. Acetone (1 ml) and 2 µg of 16-hydroxyhexadecanoic acid were added as internal standard. The samples were sonicated for 1 min again and then centrifuged for 5 min at 2830 g at 4°C. The supernatants were transferred to a separatory funnel and the residual pellets were extracted again with 2 ml of a mixture of distilled water:acetone (1:1 v/v), sonicated for 1 min and centrifuged again. The supernatants were combined and extracted with dichloromethane (twice, equal volume with aqueous phase). The organic layers

were combined, dried over anhydrous Na_2SO_4, filtered on paper and evaporated under reduced pressure with a rotary evaporator (Buchi, Rotovapor R-200). The extracts were dissolved in methanol to a final concentration of 1 µg/µl and directly analyzed by LC-MS according to Cutignano et al., 2011. Analysis were performed in triplicate.LC-MS analysis was performed on a microQuadrupole time-of-flight (micro-QToF) Mass spectrometer (Water Spa, Milan, Italy) equipped with electrospray ionization (ESI) source (negative mode) and coupled with a Waters Alliance HPLC system. For ESI-QToF-MS/MS experiments, argon was used as collision gas at a pressure of 22 mbar.

3.12 Protein assay for biochemical analysis

The protein content was determined by using the Lowry assay following the manufacturer's instructions (Bio-Rad) with BSA as standard. All measurements were performed in triplicates and data are presented as means ± SD. A 50 µl aliquot of culture was mixed with 10 µl of Triton 25%. Samples were then sonicated in ice 5 minutes in an ultrasonic bath at 100 MHz (UltraSONIK NDI) and incubated 15 min at room temperature. 190 µl of phosphate buffer 50 mM pH 7 was added to each sample and they were centrifuged at 4°C 5 minutes at 10620 g. Two different concentrations of samples were assayed.

3.13 Protein extraction and western blot analysis

Total proteins were extracted from 30 ml of cells in exponential phase. Cell extracts were prepared by incubating the cell pellets in 30 µl of lysis buffer (Tris-HCl pH 6.8, 50 mM SDS 2%). Cells were resuspended by vortex and incubated at room temperature for 30 min. After centrifugation at 4°C for 30 min at 17950 g the supernatant was kept at -80°C. Proteins were measured with BCA assay protocol

(Thermo Scientific) and then were denatured adding Laemly buffer 6 x and boiling at 100°C for 5 min. 50 µg of protein extract from wild type and transformed clones were resolved on 10% SDS-PAGE gels. After electrophoresis, the proteins in the gel were transferred on Immobilon-P PVDF membrane (Millipore) using a sandwich system consisting of 3MM paper (2 layers), membrane, electrophoresed gel, and two layers of paper soaked in 1 x transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol). The transfer was performed at 100 V for 1 h and 15 min in a cold room. Proteins transferred onto PVDF membrane were visualized by staining with Ponceau S solution (Sigma) for 30 seconds. Membrane was then washed three times with deionized water to visualize proteins, and washed in TBS-T (0.1% Tween-20, 150 mM NaCl, 50 mM Tris, HCl to adjust pH 7.4) to remove the remaining stain. Membrane was blocked for 1 h in a solution of 1 x TBS, 0.1% Tween-20, 5% powdered milk at room temperature. The membrane was incubated with anti-LOX primary antibody (Primm), diluted 1:1500, overnight in a cold room and after 3 washing steps incubated with the Anti-Rabbit secondary antibody (Promega) for 1 h. After the washing steps the membrane was exposed to chemiluminescent substrates for detection. After the detection the membrane was washed with TBS-T and incubated in a stripping buffer solution (25 mM glycine pH 2.2, 0.1% SDS, 1% Tween-20) for 45 min-1 h reprobing with the AtpB control antibody (Agrisera).

3.14 Growth curve

Strains of *P. arenysensis*, one control clone VS3 and two silenced clones, 5.4 and 5.5, were cultured in F/2 + Si (Guillard 1975) with (5.4 and 5.5) and without (VS3) zeocin antibiotic at the same conditions already described. For each strain, 1 x 10^4 cells ml^{-1} were inoculated into flasks containing 100 ml of culture medium and incubated for 8 days. Three replicates were used. The growth was monitored daily by cell counting under a Zeiss inverted microscope and using Malassez chambers of 100 µl capacity.

3.15 Determination of the photochemical efficiency of the photosystem II

To assess the photosynthetic capacities and the photophysiological state of phytoplankton cells, measurements of active chlorophyll a fluorescence were performed on 5.5 and VS3 samples using a DUAL-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany). The pulse amplitude modulation (PAM) measurement is based on the selective amplification of the fluorescence signal emitted by the Chl *a* after excitation by an intense and very short light pulse. Measurements of the photosystem II (PSII) photochemical efficiency were performed on 15-minutes dark-acclimated samples according to Brunet et al. (2014). These experiments were conducted in collaboration with the laboratory of Christophe Brunet at the Stazione Zoologica “A. Dohrn”.

4. RESULTS

4.1 Identification of the *Pseudo-nitzschia multistriata H4* gene promoter

The choice of an active promoter is the first critical step to achieve efficient transformation. The *H4* promoter has been identified as a good candidate to drive constitutive expression of transgenes (De Riso et al. 2009). After the sequencing of the *P. multistriata* genome (Basu et al. 2017) in our laboratory, it has been possible to use a species-specific promoter. A BlastN search against the draft genome sequence of *P. multistriata* with the nucleotide sequence of the *Phaeodactylum tricornutum* histone *H4-1a* retrieved two hits on scaffold 41 (at positions 47527–47838 and 151143–150832) with an identity of 87.50 and 87.18 %, respectively. These two sequences differ between them for two nucleotides only. At both these two loci, a homologue of the *H3* gene is present upstream of the *H4* gene, with a reverse orientation. The intervening sequences between the *H3* and *H4* copies and the sequences downstream of the genes at both loci are not conserved, indicating that the two loci represent a duplication and not an assembly error. A 625-bp sequence was cloned upstream of the *H4* gene present in the second locus on scaffold 41 (151143–150832) and placed upstream of *Sh ble* (Falciatore et al. 1999) to obtain the PmH4pShble plasmid (fig.17). In order to verify whether the selected sequence could act as a promoter and could drive expression of the *Sh ble* gene, we first used this construct to transform *P. tricornutum*. We obtained resistant clones and were able to confirm the presence of the transgene by PCR.

4.2 Genetic transformation of *Pseudo-nitzschia multistriata* and *Pseudo-nitzschia arenysensis*

In order to find an efficient mechanism of transformation for *P. multistriata* and *P. arenysensis*, a protocol for *P. tricornutum* transformation (Apt. et al 1996, Falciatore et al. 1999) was modified. The *Sh ble* gene from *Streptoalloteichus hindustanus* confers resistance to the antibiotics phleomycin and zeocin. Independent experiments, to test the sensitivity of *Pseudo-nitzschia* cells to these antibiotics were performed. Three different zeocin concentrations, 25, 50 and 75 μg ml^{-1} were tried. The 50 and 75 μg ml^{-1} concentrations resulted efficient and 50 μg ml^{-1} was chosen for the selection. A growth test on plates with solid medium was performed at different concentrations of agarose and agar. It was observed that *P multistriata* and *P. arenysensis* cells grew in 0.4% agarose better that in agar plate, however the liquid medium is the best method to maintain cells. At the beginning the pFCPBp-Shble plasmid (Falciatore et al. 1999) was used to transform both the species, but no live cells were found in any of them. I therefore decided to test the *P. multistriata H4* promoter. In the first step of cloning, the *P. tricornutum FcpB* promoter was replaced with the *P. multistriata H4* histone promoter to drive the *Sh ble* gene expression into cells (fig.17). In order to find an optimal transformation condition both M10 and M17 tungsten particles were used, but were not found live cells in the experiments in which M17 particles were used. For this reason next experiments were performed only with M10 particles.

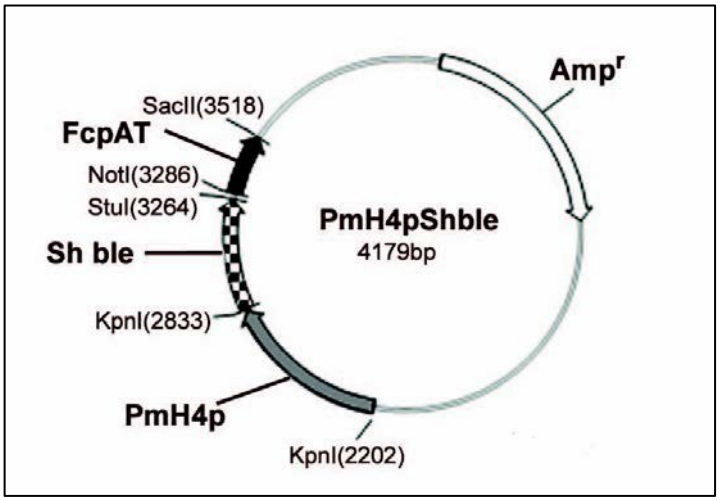


Fig. 17 PmH4pShble plasmid. It contains the *P. multistriata* H4 promoter, the *Sh ble* gene that confers resitance to the cells and the *P. tricornutum* FcpA terminator.

The day before the transformation, 5x10^7 cells were spread on plates and incubated at 18°C. The day after transformation cells were transferred from solid to liquid medium where zeocin was added (fig.18).

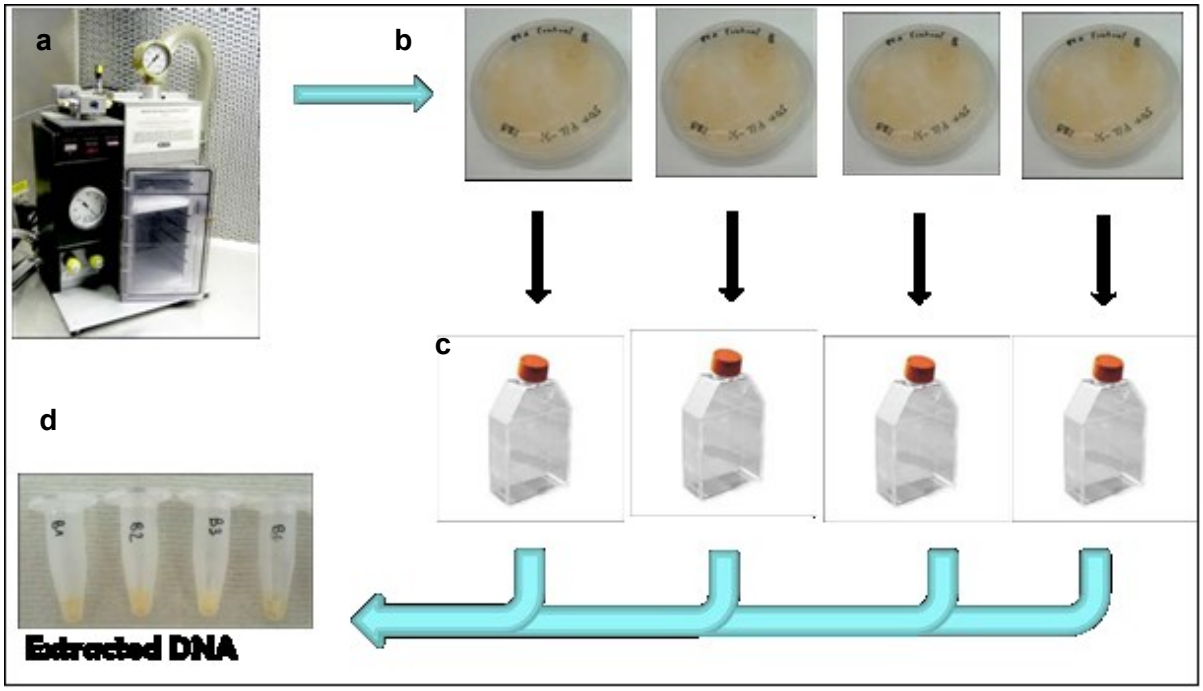


Fig.18 Workflow chart of gene gun experiments. **a** gene gun machine; **b** transformed cells, spread on agarose plates; **c** flasks with liquid medium in which the cells were transferred the day after the bombardement; **d** cell pellets from positive zeocin flasks from which genomic DNA can be extracted.

After about 10-12 days all the cells in control flasks, containing no transformed cells, were dead. Zeocin resistant cells, from different flasks, were collected, their DNA was extracted and analyzed by PCR. Primers to amplify the *H4* promoter and *Sh ble* gene were used. In most of the antibiotic-resistant clones, a 900-bp fragment was amplified, whereas in wild-type cells, no amplification product was obtained. (fig. 19). Transformed cells were obtained with variable efficiency in the experiments performed most likely due to the variable state of the starting culture (Table 2). The highest number of positive clones was obtained when cells was collected in the mid-exponential phase of growth, whereas cells collected later along the growth curve led to a lower number of positive clones.

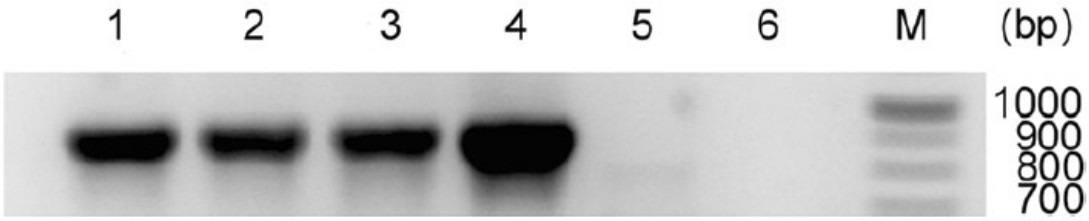


Fig. 19 PCR analysis performed on genomic DNA of *P. arenysensis* transformant clones to amplify *H4* promoter and *Sh ble* gene. Lanes 1–3, three independent zeocin-resistant strains (B858R1, B858R2, B858R3); lane 4, PmH4pShble plasmid; lane 5, wild-type strain B858; lane 6, blank; M 100 bp ladder.

Experiment	Plasmid	Number of bombardments	Flasks[a] positive for antibiotic resistance
1	PmH4pShBle	14	12
2	PmH4pShBle	6	3
3	PmH4pShBle	6	3
4	PmH4pShBle	7	3
5	PmH4pShBle	5	4

[a] One flask was established from each bombardment

Table 2 Summary of the transformation experiments for *P. multistriata*

4.3 Overexpression and protein localization studies

Co-transformation was performed in *P.arenysensis* to demonstrate the feasibility of the overexpression, gene tagging and localization in this species. In co-transformation two plasmids were introduced together into the cells, usually one contains a resistance gene and the other one contains the gene for the overexpression. I constructed the plasmid PmH4pH4N-GFP, containing the *P. arenysensis H4* histone gene fused with a GFP (fig. 20), and inserted this together with PmH4Shble in the cells by the biolistic method.

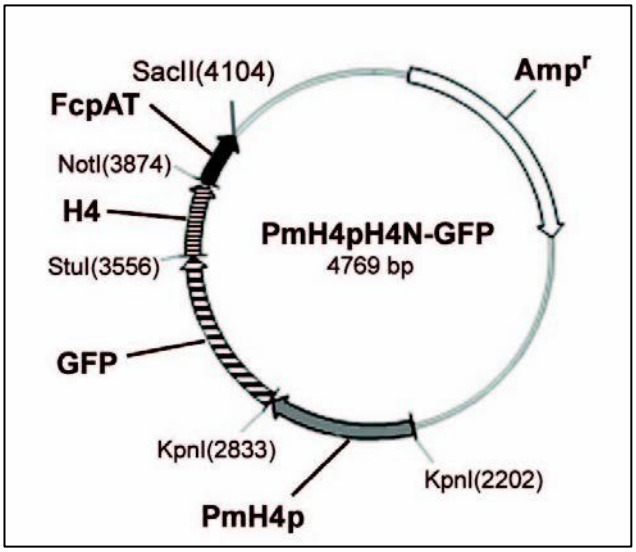


Fig. 20 PmH4pH4N-GFP plasmid. It contains the *P. multistriata H4* promoter, the GFP coding sequence fused to the histone *H4* gene that encodes for a nuclear protein and the *P. tricornutum* FcpA terminator.

After about 10 days of selection, the cells were observed under a fluorescent microscope and from three independent experiments five out of seven zeocin positive clones showed to be positive also to the GFP. In order to demonstrate that the GFP signal was localized to the nuclei a live nuclear stain with Hoechst was performed (fig. 21).

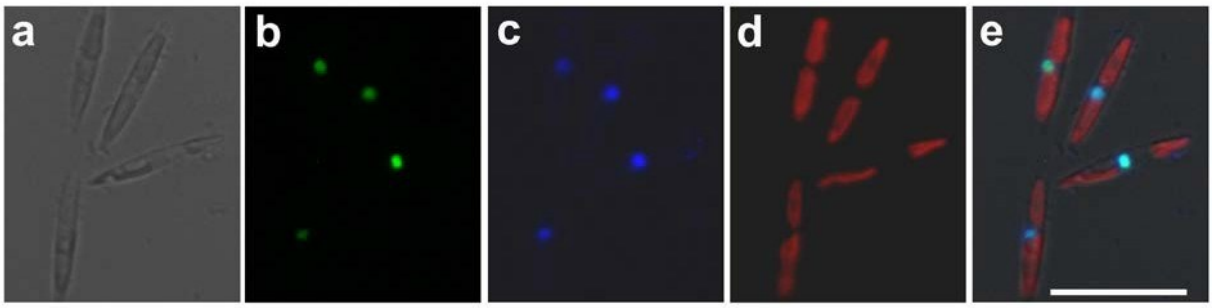


Fig. 21 Four *P. arenysensis* cells expressing the fusion protein H4-GFP. **a** Bright field. **b** GFP fluorescence. **c** Hoechst staining. **d** Chlorophyll auto-fluorescence. **e** Merge of the previous four images. Scale bar, 20 µm.

Another co-transformation was performed with PmH4Shble and PmH4GUS plasmids. I constructed the PmH4GUS plasmid (fig. 22) which contains a *GUS* gene that encodes for β-glucoronidase enzyme, this is one of the most widely used reporter genes in plant molecular biology. The presence of this reporter gene in the cells is visualized by a colorimetric assay. The co-transformation experiments have a different transformation efficiency (Table 3). After co-transformation, qualitative and quantitative GUS assays were performed on zeocin positive cells.

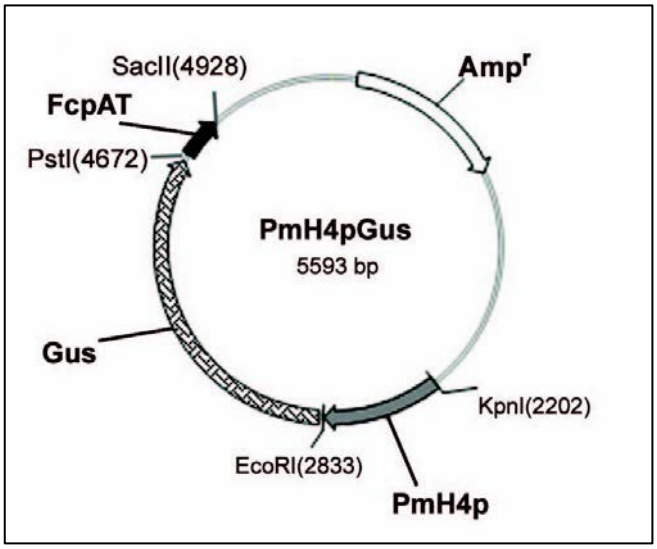


Fig. 22 PmH4pGUS plasmid. It contains the *P. multistriata H4* promoter, the *GUS* gene that encodes for β-glucoronidase protein and the *P. tricornutum* FcpA terminator.

The colorimetric assays were performed on B858 wild type, Pt/GUS positive control, B858GUS2 and B858GUS12 clones. For the qualitative assay, the cells of these four samples were plated on agarose and incubated with X-gal substrate. The X-gal substrate permits the formation of a blue color that appears if the cells in contact with the substrate express the *GUS* transgene. As shown in fig. 23c-d on both B858GUS2 and B858GUS12 samples a blue color was formed, similarly to the Pt/GUS positive control (fig. 23b), while in B858 wild type (fig. 23a no color appeared.

The quantitative GUS assay analyzes the activity of the promoter that drives the gene expression by a spectrophotometric assay. The measure of the enzymatic activity of GUS for two clones was reported as percent of the *P. tricornutum* Pt/GUS strain (De Riso et al. 2009), used as reference value. The B858GUS2 and B858GUS12 clones showed a level of GUS activity ranging from 70 to 92 % with respect to the reference strain (fig. 23e).

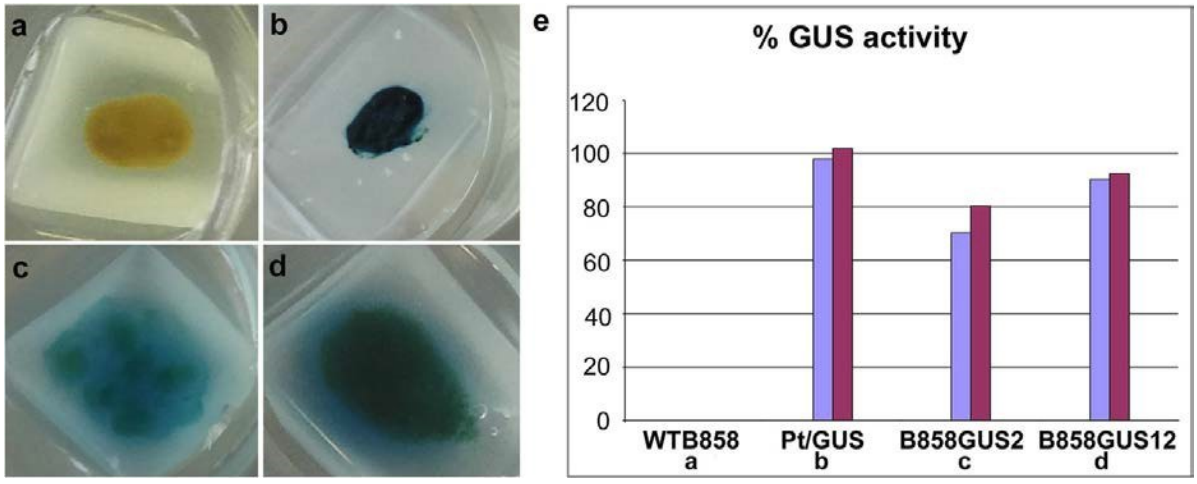


Fig. 23 GUS assay on *P. arenysensis* cells. **a-d** Images of cells plated on agarose stained for GUS activity. **a** *P. arenysensis* wild-type B858. **b** *P. tricornutum* Pt/GUS. **c** *P. arenysensis* B858GUS2. **d** *P. arenysensis* B858GUS12. **e** GUS activity of the clones shown in the previous panels, normalized to the GUS activity of the Pt/GUS strain (100 %). Violet and purple bars represent two independent measurements made for each clone.

Experiment	Plasmid	Number of bombardments	Flasks[a] positive for antibiotic resistance	Flasks[a] positive for second transgene
1	PmH4pShBle	2	2	
2	PmH4pShBle	3	1	
3	PmH4pShBle	6	5	
4	PmH4pShBle	10	2	
5	PmH4pShBle	10	2	
6	PmH4pShBle	10	3	
7	PmH4pH4N-GFP+PmH4pShBle	8	3	1
8	PmH4pH4N-GFP+PmH4pShBle	5	1	1
9	PmH4GUS+PmH4pShBle	12	3	2
10	PmH4pH4N-GFP+PmH4pShBle	10	3	3

[a] One flask was established from each bombardment

Table 3 Summary of the transformation experiments for *P. arenysensis*

4.4 Inheritance of the transgene

P. arenysensis, unlike most model diatoms, can have sexual reproduction. In order to see if the transmission of exogenous DNA to the progeny was possible, crosses between resistant and wild type strains with opposite mating type were performed. Transgenic cells with mating type minus that contained the *P. arenysensis* H4 gene fused with green fluorescent protein (PmH4pH4N-GFP) and that showed fluorescence in the nuclei under microscope (fig. 21) were put in contact with wild type cells with mating type plus. After a few days initial cells, which can be easily recognized for the length, were observed in the cultures. To isolate resistant initial cells zeocin was added to the cultures, in this way after about 10 days all the non-transformed parental cells and all the non-resistant initial cells died. The resistant initial cells were checked under fluorescent microscope and they showed the presence of the transgene (fig. 24)

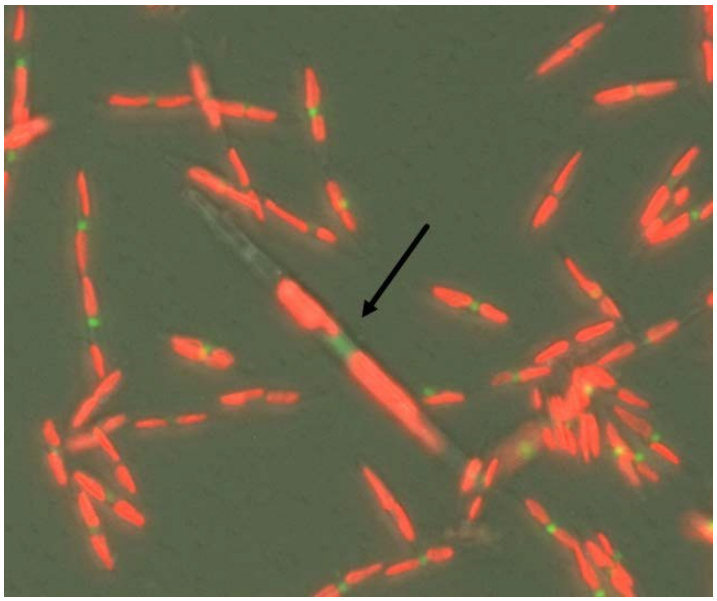


Fig. 24 Resistant *P. arenysensis* F1 cell. Fluorescent light micrograph of live *P. arenysensis* cells, the longest cell indicated by the arrow is an F1 initial cell; the other cells are the small parental cells, some of which are transgenic (green nuclei, MT-) and some wild type (non-fluorescent nuclei, MT+). The red color is due to chlorophyll autoflorescence.

Southern blot analyses were performed to assess the number of integration events in transformed strains and to confirm inheritance of the resistance gene in the F1 cells. Genomic DNA was digested with *Hind*III, a unique site in the PmH4pShble plasmid cutting in the terminator, and a 250-bp probe was designed against the Sh ble sequence (fig. 25a).

Three independent *P. arenysensis* resistant strains showed different patterns of hybridization (fig. 25b, lanes 1, 5, and 8), confirming independent events of integrations. A culture established after manual isolation of a single cell from the B858GUS12 culture was also included (fig. 25b, lane 6) to verify whether multiple bands derived from independent integrations in the same genome or from the presence of different transformed genotypes in the original resistant culture. As the same bands appear in lanes 5 and 6, it appears that a single genotype with multiple integration events for the resistance cassette was present in the original B858GUS12 culture. F1 generation cells obtained by crosses of either B858GUS2 or B858GUS12 with a wild type strain were also analyzed. The same pattern of the parental strain was observed in two siblings of B858GUS2 (fig. 25b, compare lanes 3 and 4 with lane 1), whereas two bands were not detectable for the third sibling of B858GUS2 (fig. 25b, compare lane 2 with lane 1) and one band was absent for the only sibling of B858GUS12 analyzed in this study (fig. 25b, compare lane 7 with lane 5), suggesting in the latter two cases that some of the Sh ble copies from the parental strain had not been inherited.

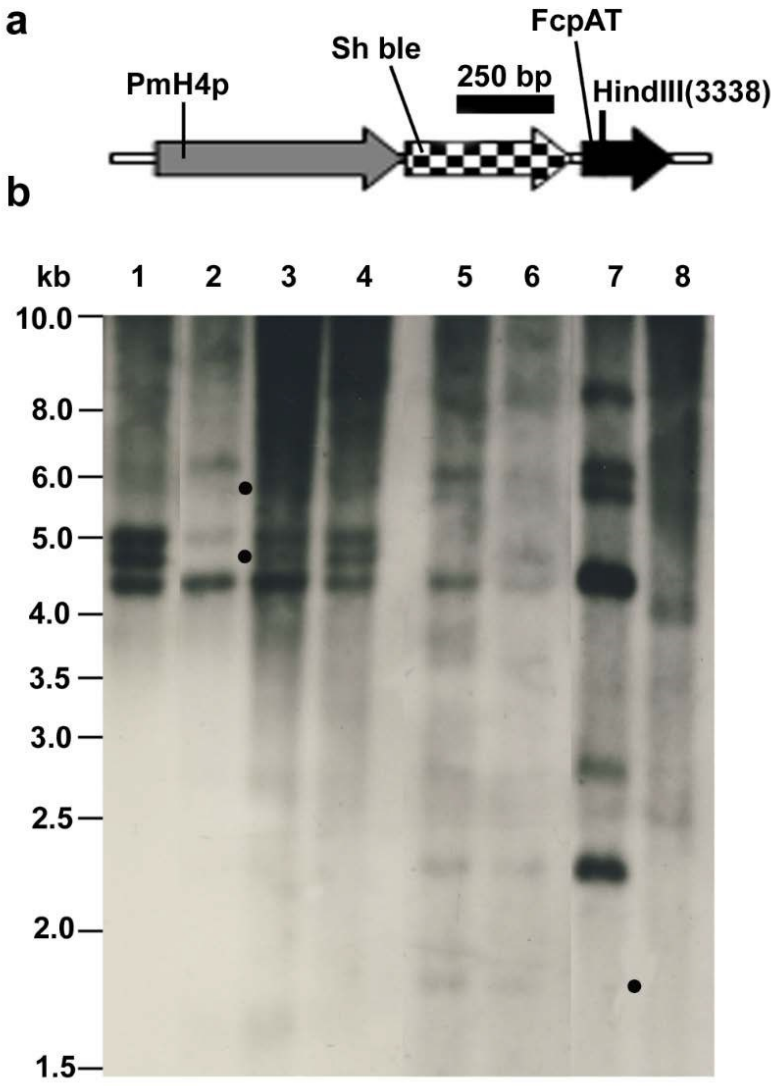


Fig. 25 Southern blot analysis of *P. arenysensis* transformants and F1 siblings. **a** Schematic representation of the expression cassette for the antibiotic resistance. The black bar represents the probe used for hybridization; the position of the HindIII restriction site used to digest DNA is indicated. **b** Southern blot autoradiography. Lane 1, strain B858GUS2; lanes 2–4, three independent F1 clones, C2, C3, and C5, deriving from the cross B858GUS2×B947; lane 5, strain B858GUS12; lane 6, clonal B858GUS12 (established from a manually isolated single cell); lane 7, one F1 clone deriving from the cross B858GUS12×B947; lane 8, strain B858T2A1. Black dots on the right side of a lane indicate bands that are missing in some of the samples. Molecular weights are indicated on the left.

4.5 Identification of sequences for the RNAi machinery in *P. arenysensis*

In order to know if it is possible to perform silencing experiments in *P. arenysensis*, key RNAi components were searched in the MMETSP *P. arenysensis* transcriptome (Keeling et al. 2014). Dicer, Argonaute-Piwi and the DEAD/HELICASEc domain are the major enzymes involved in the RNAi mechanism. In diatoms, a canonical Dicer sequence is absent (De Riso et al. 2009). The only universal feature of the Dicer family is the presence of two RNase III domains.

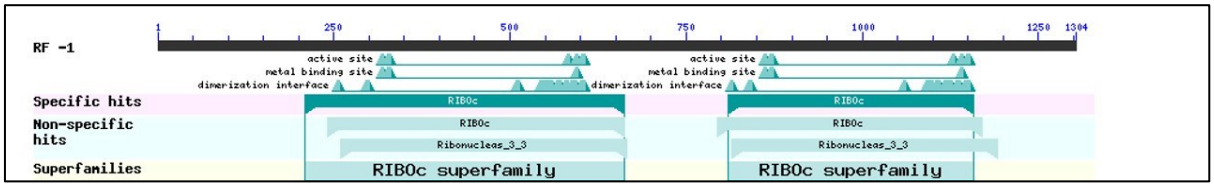
The *P. arenysensis* transcriptome showed a transcript encoding a protein with two RNase III domains (sequence ID: MMETSP0329-20121206|1584) (fig. 26a), found by tblastn with the *Arabidopsis thaliana* dicer-like 1 (DCL1) mRNA (sequence ID: NM_001197952.1).

The Argonaute proteins involved in RNAi pathways contain distinct functional domains: a variable N-terminal domain and conserved C-terminal PAZ, MID and PIWI domains (Hutvagner and Simard 2008). Putative proteins containing these domains were identified in the *P. arenysensis* transcriptome (sequence ID: MMETSP0329-20121206|10545) (fig. 26b) starting from the *Arabidopsis thaliana* protein (sequence ID: NP_001322306.1).

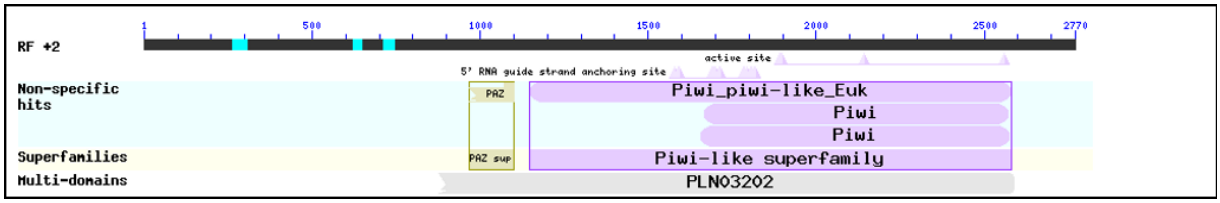
A *P. arenysensis* protein (sequence ID: MMETSP0329-20121206|6839) (fig. 26c) with a DEAD/HELICASEc domain was found by tblastn using as query again an *Arabidopsis thaliana* protein (sequence ID: NP_175911.1).

I looked in other diatom genomes and found that *P. arenysensis* RNAi related protein sequences are closed to hypothetical proteins of *Fragilariopsis cylindrus* and *Thalassiosira oceanica* with a high query cover and with an identity of about 90% (data not shown).

a



b



c

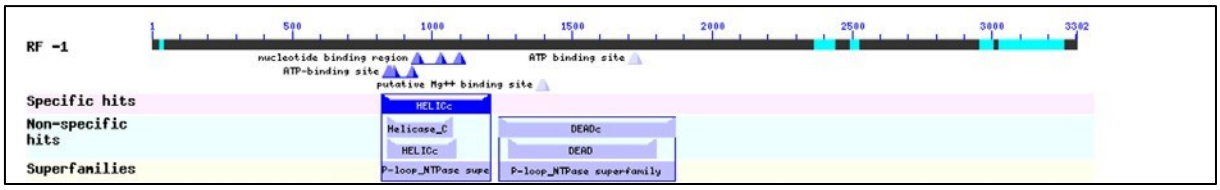


Fig. 26 RNAi domains found in the *P. arenysensis* transcriptome and blasted on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to show the domains identified. **a** Dicer domain. **b** Argonaute domain. **c** DEAD/HELICASEc domain.

4.6 Identification of transit peptide in *P. arenysensis* LOX protein

The *P. arenysensis* LOX sequence (MMETSP0329-20121206|11370) was identified in the *P. arenysensis* transcriptome by Orefice (PhD thesis 2013). In plants there are two LOX families: type 1-LOXs and type 2-LOXs. They differ for the presence of a transit peptide. The type 2-LOXs act in the chloroplast. In order to know where LOXs localize in diatoms, the LOX sequence found in *P. arenysensis* was analyzed by prediction tools.

The signal peptide of plastid-targeted proteins in diatoms and other organisms with secondary plastids can be identified via the prediction program SignalP (Nielsen et al. 1997; Emanuelsson et al. 1999). In diatoms, the signal peptide cleavage site contains the 'ASAFAP' motif (Kilian and Kroth 2005; Gruber et al. 2007)

The software utilized for the LOX sequence analysis were: **SignalP 3.0** (http://www.cbs.dtu.dk/services/SignalP-3.0/), that employs either a neuronal network (NN) or a hidden Markov (HMM) model to identify the signal peptide (Nielsen and Krogh 1998; Bendtsen *et al*. 2004); **ASAFind** (http://rocaplab.ocean.washington.edu/tools/asafind), that identifies nuclear-encoded plastid proteins in algae with secondary plastids of the red lineage based on the output of SignalP and the identification of conserved 'ASAFAP' motifs and transit peptides (Gruber et al. 2015), and **ChloroP** (http://www.cbs.dtu.dk/services/ChloroP/) that predicts the presence of chloroplast transit peptides (cTP) in protein sequences and the location of potential cTP cleavage sites.

In all these prediction programs, the *P. arenysensis* LOX sequence resulted to possess the signal peptide. The SignalP 3.0 NN prediction was positive and identified the position with the highest cleavage site score (fig. 27). The ASAFind

predicted cleavage site coincided with the SignalP prediction and the transit peptide score resulted higher than 2, for this reason the sequence was defined as ‘plastid, high confidence’ (fig. 28). In addition, ChloroP indicates the same result of the other prediction programs (fig. 29)

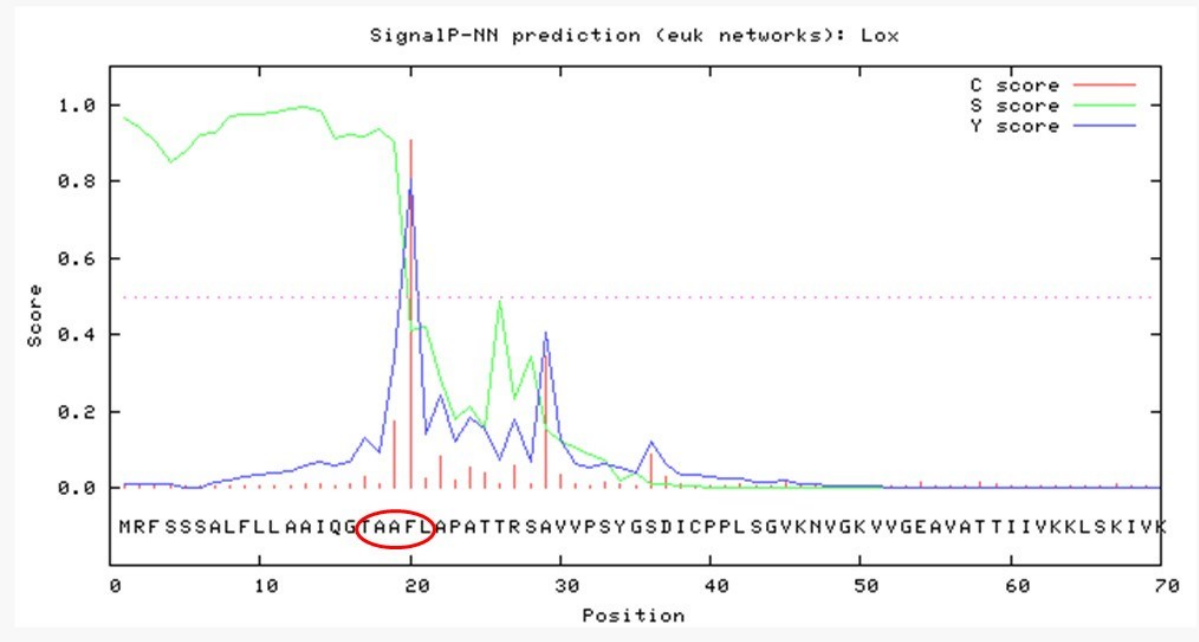


Fig. 27 The graphical output from SignalP 3.0 indicated the presence of signal peptide and that most likely cleavage site is between positions 19 and 20: TAA-FL (circled in red).

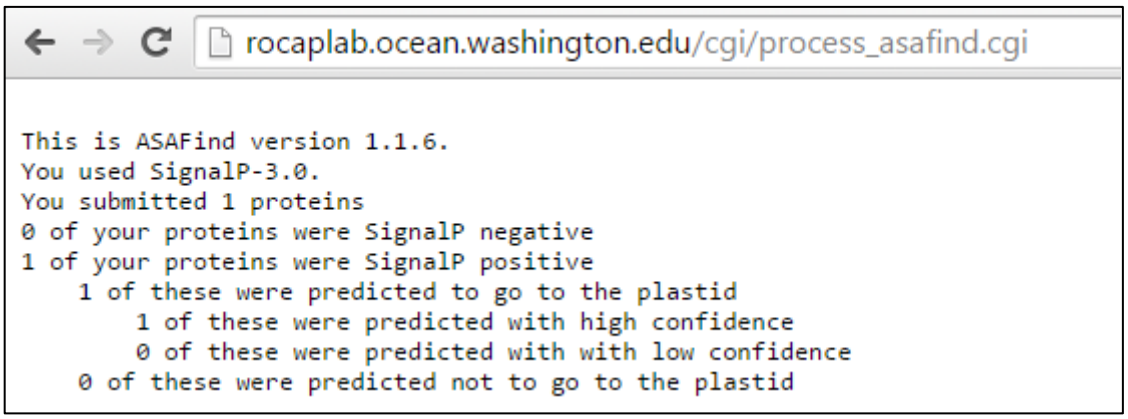


Fig. 28 The ASAFind output indicated the presence of the signal peptide and the high score predicted the plastid localization with high confidence.

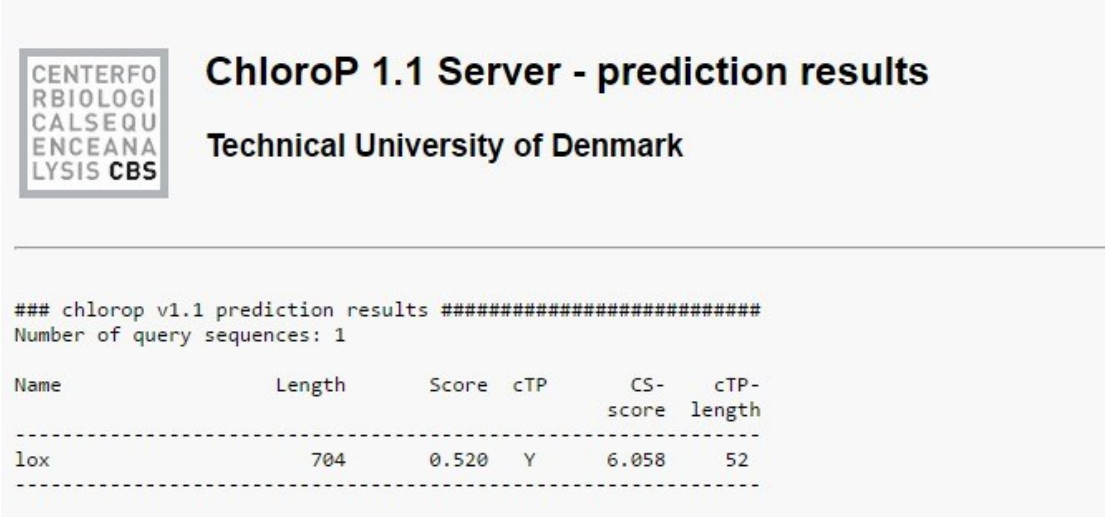


Fig. 29 ChloroP output indicates the presence of chloroplast transit peptide cTP: "Y" means that the sequence *is* predicted to contain a cTP.

4.7 Silencing of *LOX* gene in *P. arenysensis*

In order to study the function of LOX in diatoms a gene silencing method was established for *P. arenysensis*. Since this technique is little explored in diatoms, different constructs were generated containing either anti-sense or inverted repeat fragments of the *LOX* gene (fig. 30). The *LOX* fragments were cloned as part of a transcriptional fusion downstream of the selectable *Sh ble* gene, which confers resistance to the antibiotic zeocin. This was done to increase the number of silenced transformants and to make the screening more efficient. To determine whether anti-sense RNA length could also affect silencing efficiency, different anti-sense constructs containing *LOX* fragments of 180 and 424 bp were generated. The constructs containing the inverted repeat *LOX* fragments were expected to encode for RNAs that fold into hairpin structures.

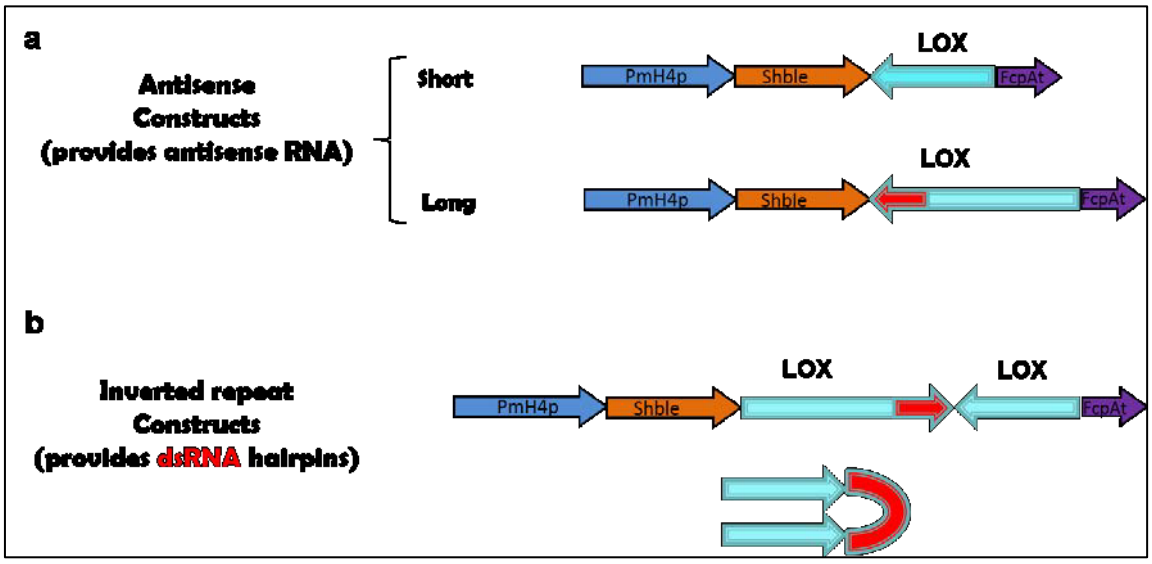


Fig. 30 Schematic maps of the anti-sense and the inverted repeat constructs. **a** Anti-sense constructs: *LOX* fragments of 180 bp (short) or 424 bp (long) cloned between the stop codon of the selectable *Sh ble* gene and the diatom FcpA terminator region. **b** Inverted-repeat construct: the *LOX* fragments were cloned in sense (long) and anti-sense orientation (short). The region of self-complementarity is shown in light blue, whereas the non-complementary region (corresponding to the spacer) is indicated in red.

The different constructs were introduced in VS3 *P. arenysensis* cells and transformants expressing the silencing construct were first selected on zeocin. In the first experiment 5 out of 9 clones resulted positive to the selection with zeocin, three (CA1, CA2 and CA4) obtained with the short antisense construct and two (LA1 and LA4) with the long antisense construct. From these clones, genomic DNA was extracted and analyzed by PCR to verify the presence of the construct (fig. 31). All five samples showed to possess the transgene. I could not obtain any resistant strain with the inverted-repeat construct in the first set of experiments, and for this reason only the antisense constructs were used for the subsequent experiments.

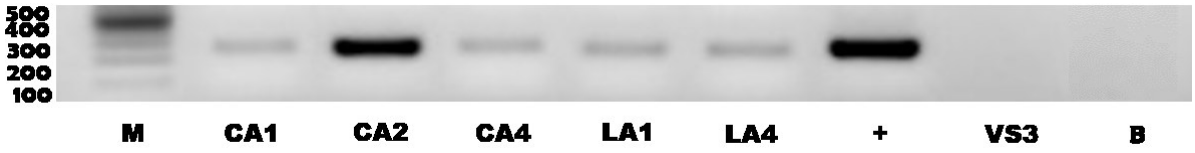


Fig. 31 PCR analysis of *Sh ble* gene on five silenced clones, the wild type VS3 and on a positive control sample (+). B is blank. M is 100bp DNA marker.

4.8 Western blot analyses detecting LOX protein reduction in *P. arenysensis* silenced cells

In order to investigate if there was a reduction of the LOX protein in the cells due to the *LOX* silencing, a polyclonal LOX antibody was produced by Primm company. This antibody was directed against a mix of three synthetic peptides of the *P. arenysensis* protein sequence. Different western blot analyses were performed on zeocin positive clones. As control antibody I used the beta subunit of ATP synthase AtpB (Agrisera) already used for *P. tricornutum* (Fortunato et al. 2016). LOX Ab binds a protein of about 79 kDa, while AtpB Ab binds a control protein of about 54 kDa.

In the first experiment, four zeocin-positive samples (1.1, 2.2, 5.2 and 5.4) were analyzed by immunoblot but only the 5.4 sample showed a protein reduction of about 30% compared to VS3 wild type (fig. 32).

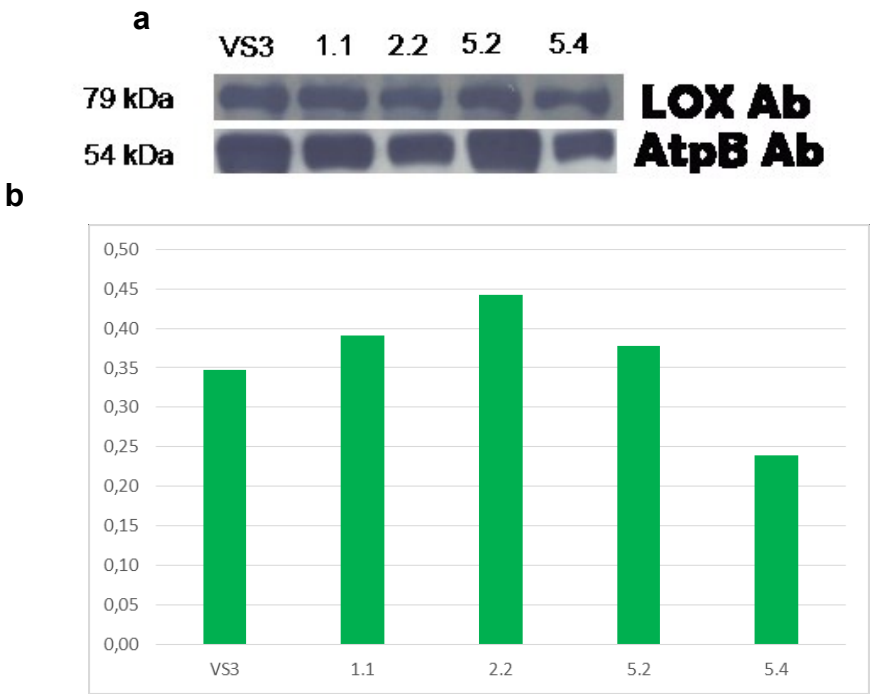


Fig. 32 a Western blot analysis on VS3 wild type and four silenced samples. **b** The protein reduction level of sample 5.4 is around 30% Analyses were performed with the BioRad Quantity One 1-D Analysis Software.

In a second experiment, four other zeocin-positive samples (1.2, 1.3, 5.1 and 5.5) were analyzed by western blot. After quantification the 5.5 silenced sample showed a protein reduction level of about 70% compared to the control (fig. 33).

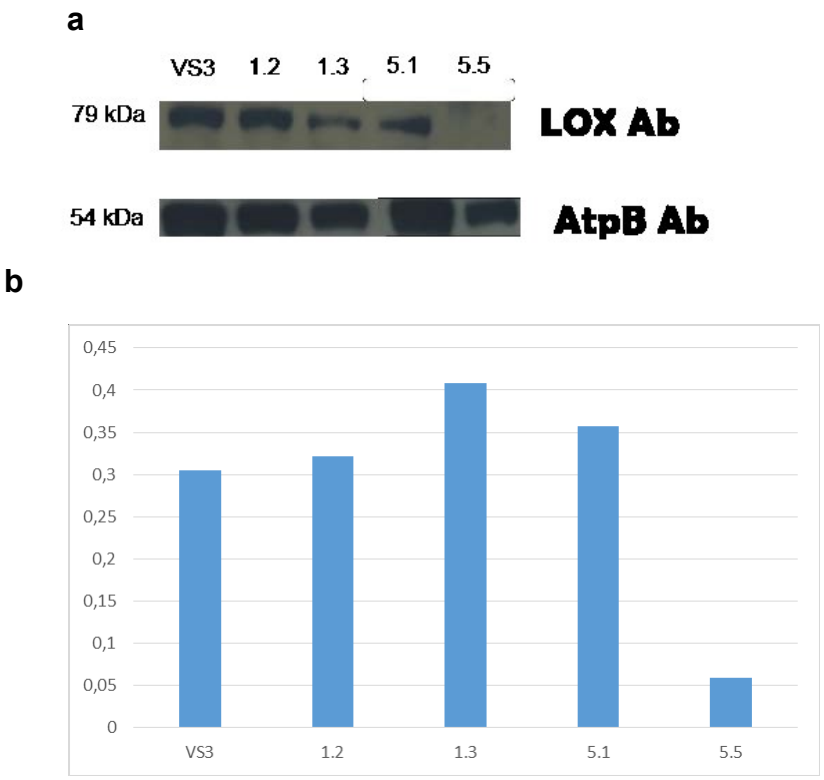


Fig. 33 a Western blot analysis on VS3 wild-type and four silenced samples. **b** The protein reduction level of 5.5 sample is around 70%. Analyses were performed with Quantity One 1-D Analysis Software Bio-Rad.

4.9 Characterization of LOX pathway and oxylipin profiling in *P. arenysensis*

With the aim of understanding if the LOX silencing affected the fatty acid hydroperoxides (FAHs) and oxylipin contents, chemical analyses were performed on different silenced samples.

FOX2 assay (Orefice et al. 2015) was used to assess FAH levels in diatoms cells. This colorimetric method measures the reduction of LOX direct products in the silenced clones compared to the control VS3. The graphic in figure 34a shows that the reduction of FAH levels occurred in all five samples (LA1, CA2, LA4, CA4 and CA1) compared to VS3 control. In particular, the CA1 sample reduced its FAH level of about 65%.

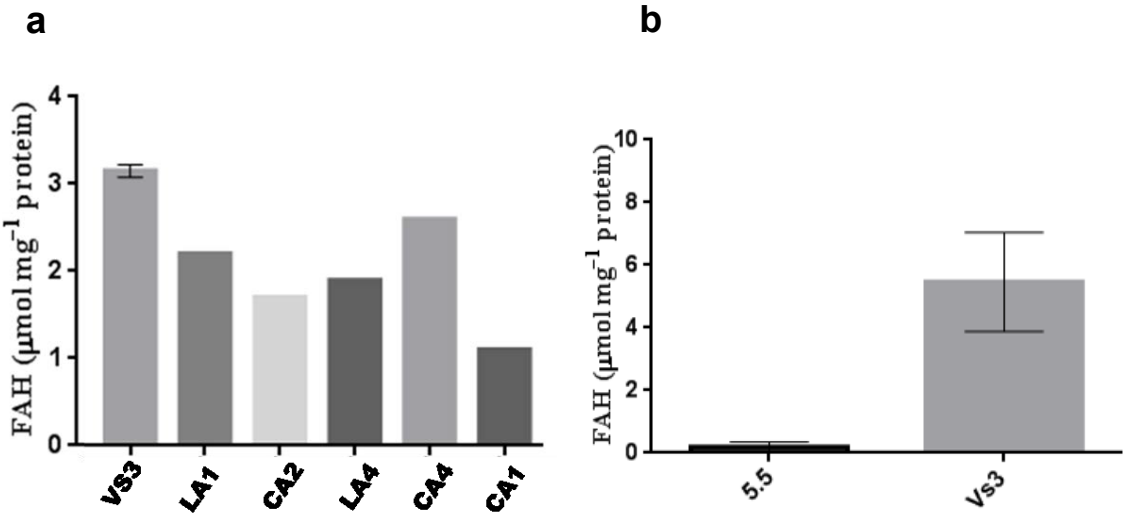


Fig. 34 Fatty acid hydroperoxide levels measured with FOX2 assay. **a** VS3 is the wild type sample; LA1, CA2, LA4, CA4, CA1 are the silenced samples. **b** 5.5 silenced clone and VS3 sample. The FAH levels were normalized to protein content.

When this experiment was performed, the LOX antibody for the western blot analysis was not yet available, and therefore the LOX protein levels for samples LA1, CA2, LA4, CA4 and CA1 were not determined.

In a second independent experiment, FOX2 assay was performed on the 5.5 silenced clone and VS3 sample. The test showed a reduction of FAH in 5.5 of

about 95% compared to the control VS3 (fig. 34b). These analyses were performed on three biological replicates.

The samples in which the FAH reduction is more evident compared to VS3 wild type sample were analyzed by liquid chromatography-mass spectrometry (LC-MS). This technique is used for the detection and characterization of oxylipins in marine diatoms. LOX metabolism is induced by damage of the diatom cells that is achieved by treatment of the cells with ultrasound. Oxylipins are revealed as sodium adducts with molecular ions and are inversely eluted according to the number of double bonds and length of the alkyl chain. Epoxy-alcohols from EPA are revealed by typical ion mass at m/z 371. Hydroxy-acids (HEPEs) from EPA are indicated by molecular ion at m/z 355. Biosynthesis of these metabolites from hydroperoxy precursors is specific and proceeds by preservation of linkage between carbon and oxygen (Chang et al. 1996). Oxylipins were detected in the CA1, CA2, samples that showed the highest FAH reduction, and VS3 by LC-MS analysis (fig. 35).

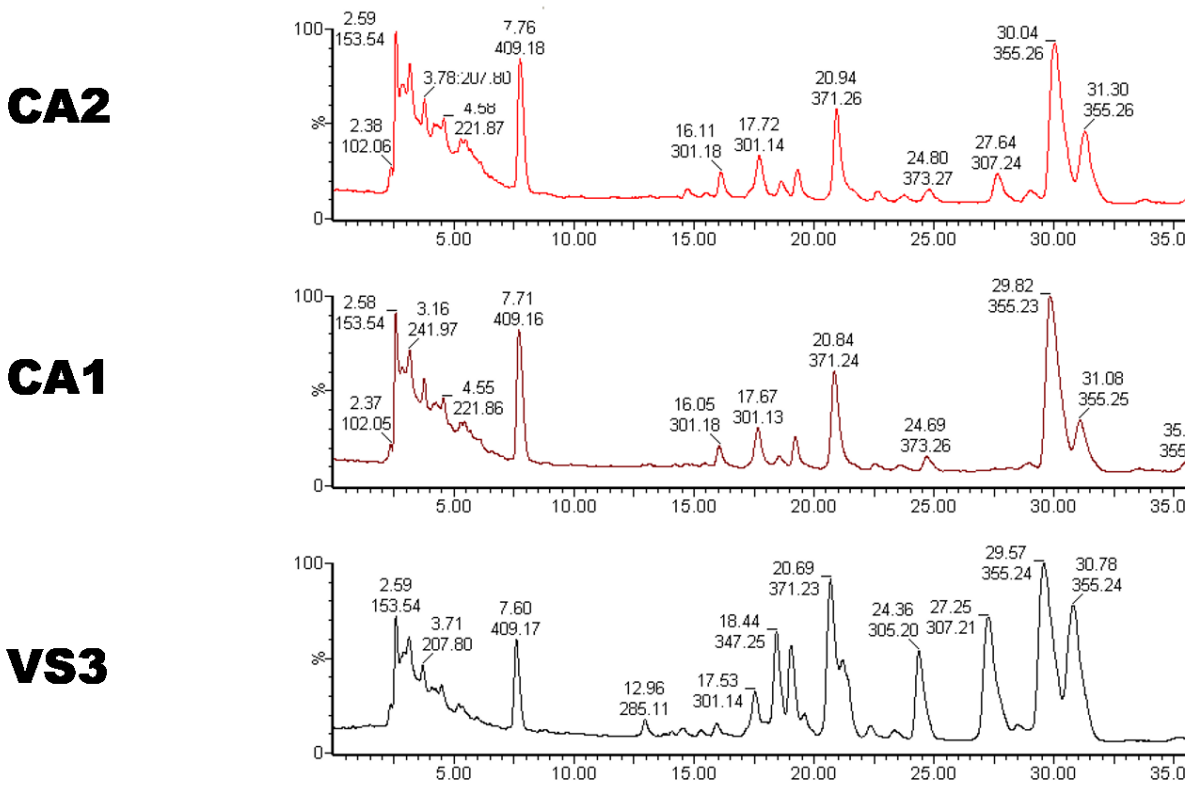


Fig. 35 Extraction ion (m/z 355, 371) profile for the oxylipin family derived from eicosapentaenoic acid. CA2 and CA1 are silenced samples and VS3 is wild-type sample.

In LC-MS profile of VS3 at 371 a double peak could be visualized; the CA2 sample at 371 showed one well defined peak and a very small peak as a small hump; in CA1 only one peak at 371 could be visualized.

In order to clarify the result obtained from LC-MS, an analysis of the fragmentation pattern of HEpETEs was performed by MS/MS analysis. It has been previously reported that *P.arenysensis* is characterized by 12-LOX and 15-LOX EPA-dependent pathways (Lamari et al. 2013). However the presence of these pathways is rather variable and strain dependent since they can be detected together or singularly in different strains. This feature is associated to positional specificity of LOX enzymes that is detectable by MS/MS analysis (Cutignano et al., 2011).

The MS/MS spectrum of oxylipins from VS3 and CA2 samples revealed the presence of 15- and 12-LOX-derived compounds namely 15-hydroxy-5Z,8Z,11Z,13E,17Z- eicosapentaenoic acid (15-HEPE), 13-hydroxy-14-epoxy-5Z,8Z,11Z,17Z- eicosatetraenoic acid (13,14-HEpETE) in red, 12-hydroxy-5Z,8Z,10E,14Z,17Z- eicosapentaenoic acid (12-HEPE) and 10-hydroxy-11-epoxy-5Z,8Z,14Z,17Z- eicosatetraenoic acid (10,11-HEpETE) in green (fig. 36).

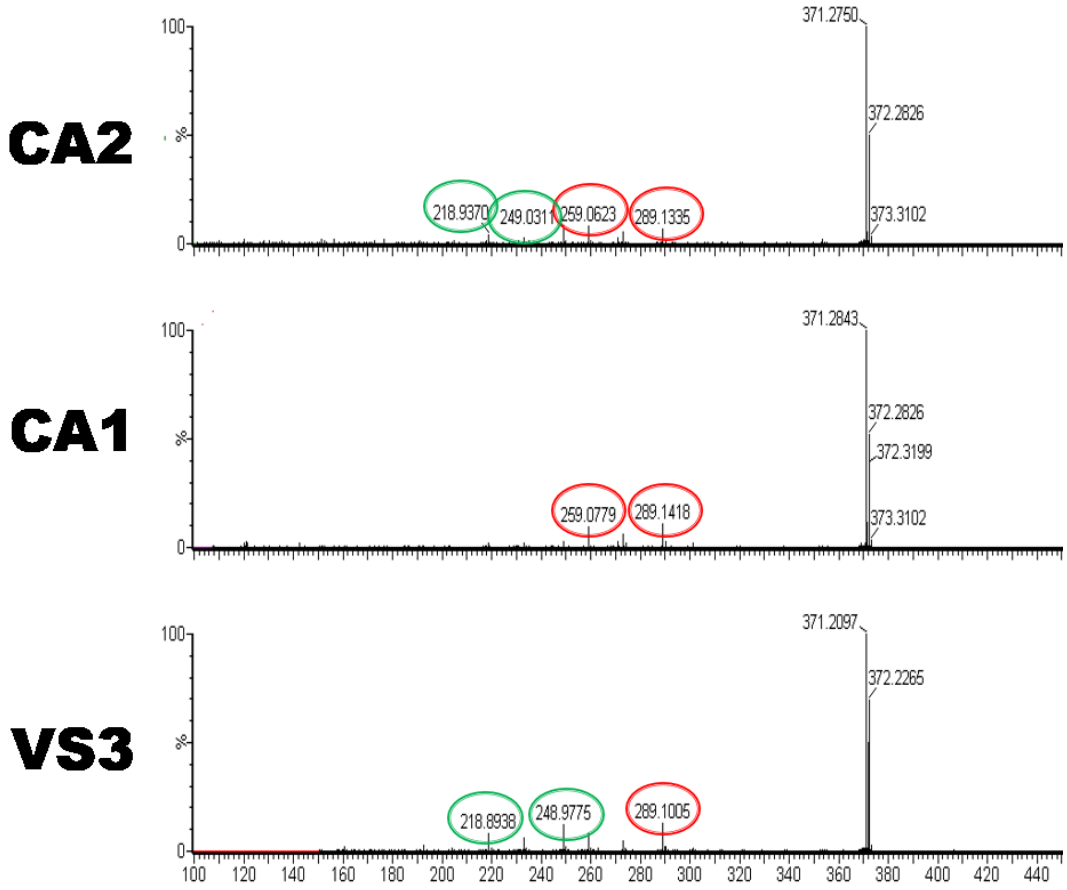


Fig. 36 MS/MS spectrum of 13-hydroxy-14-epoxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid (13,14-HEpETE) methyl ester shows fragments at m/z 289, 273, and 259 in red; MS/MS spectrum of 10-hydroxy-11-epoxy-5Z,8Z,14Z,17Zeicosatetraenoicacid (10,11-HEpETE) methyl ester shows fragments at m/z 249, 233 and 219 in green.

On the contrary, the MS/MS spectrum showed only 15-LOX-derived compounds (circled in red) in the CA1 sample.

From these evidences it seems that the LOX silencing in CA1 sample has led to

absence of products derived from 12-LOX while the 15-LOX was downregulated and the corresponding products reduced. In the CA2 sample both the products of the 15- and 12-LOX pathways were reduced.

A second independent analysis was carried out on the 5.5 silenced clone that showed LOX and FAH levels lower than the previous samples. As shown in fig. 37, LC-MS/MS analysis, the HEpETEs derived from 15- LOX were reduced by 84% and HEpETEs derived from 12-LOX by 63% in comparison to the wild type strain.

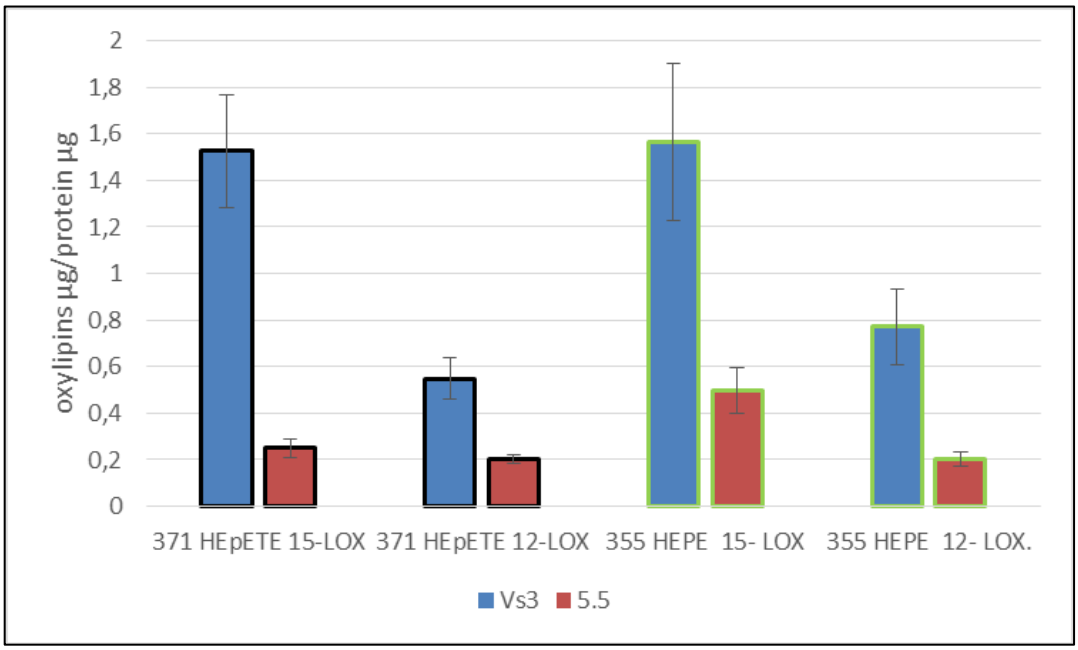
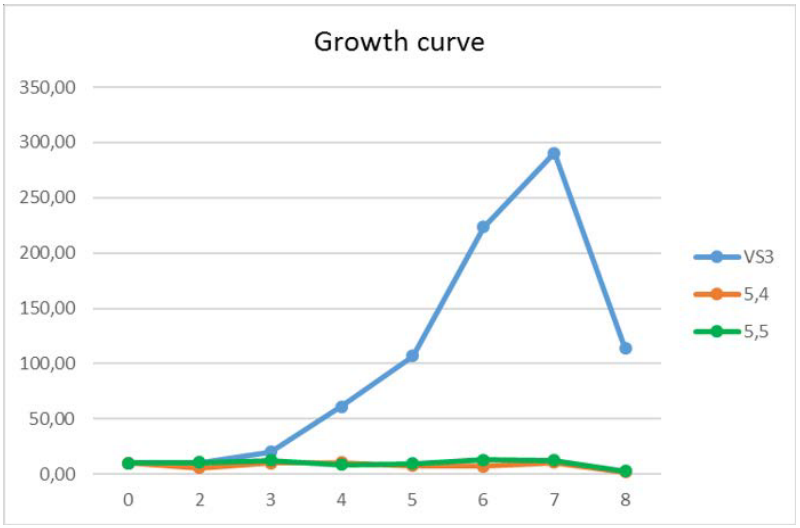


Fig. 37 Oxylipin analyses by LC-MS. Columns bordered in black represent HEpETEs derived from 15- and 12-LOX; columns bordered in green represent 15- and 12 LOX HEPEs.

4.10 *Lox* silencing affects growth in *P. arenysensis* cells

In plants, LOXs are involved in the growth (Stintzi and Browse et al. 2000; Kolomiets et al. 2001). In order to understand if this role is maintained also in diatoms, growth curves were performed in triplicate on the two strains, namely 5.4 and 5.5, that showed the strongest reduction of LOX expression, together with VS3 that was use as control. The growth was monitored daily for 8 days.

a



b

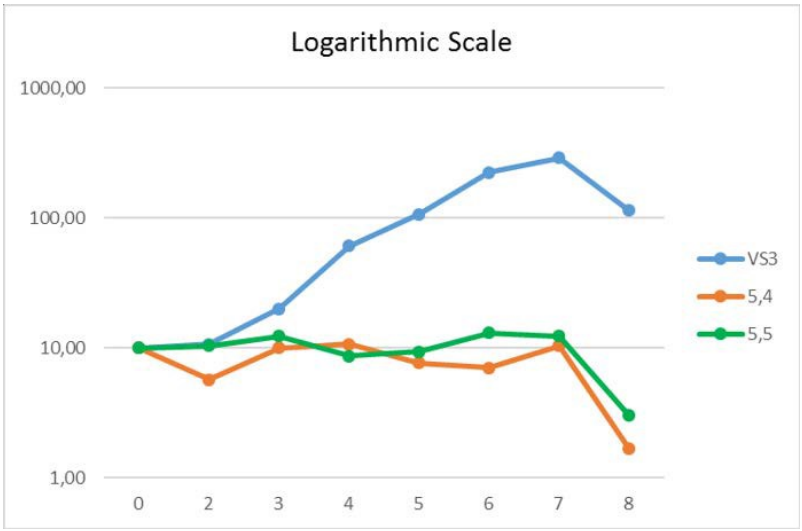


Fig. 38 Growth curve representation of VS3 (blue line), 5.4 (orange line) and 5.5 (green line). **a** linear scale **b** logarithmic scale.

As shown in fig. 38a, the highest number of VS3 cells (blue line) was recorded on the seventh day while on the eighth day the cells started dying. On the other hand, the growth for silenced clones 5.4 (orange line) and 5.5 (green line) strongly reduced in comparison to VS3. From the graph of the growth curve in logarithmic scale (fig. 38b), it is evident that *LOX* silencing in the two strain caused absence of growth with a number of cells that remained steady for seven days.

4.11 Preliminary PAM analyses on LOX silenced clone

In order to find the possible reasons of this growth phenotype in silenced clones, I focused on the photoprotective capacity of these cells. Diatoms developed some rapidly-regulated photoprotective mechanisms, such as the xanthophyll cycle activation (XC) and the non-photochemical chlorophyll fluorescence quenching (NPQ), to protect themselves from photooxidative damages caused by excess photon flux density (PFD). Since the 5.5 clone showed problems in the growth compared to VS3 control, a preliminary experiment was performed with Phyto-PAM fluorometer on these two samples. The 5.5 sample showed a marked photoinhibition and a NPQ much lower than VS3 control (data not shown). These interesting results will be further investigated in future.

5. DISCUSSION

Diatoms are very interesting organisms for different reasons, including their ecological relevance, their biotechnological role and their peculiar metabolic pathways. Lipoxygenase products, named oxylipins, are known ecological and physiological mediators in many diatom species. This study aims at understanding the role of oxylipins in the diatom *Pseudo-nitzschia.* This work is innovative and important because:

- molecular tools as the genetic transformation and loss of function studies were performed for the first time in the planktonic diatoms *Pseudo-nitzschia arenysensis* and *Pseudo-nitzschia multistriata*, members of the widely distributed and ecologically important genus *Pseudo-nitzschia*;
- it was possible to show inheritance of the transgene from a transformed parental strain to the progeny through sexual reproduction. This is not currently possible in any other model diatom and opens the way to the use of *Pseudo-nitzschia* as a genetic system;
- it shows for the first time a correlation between the LOX reduction and oxypilins synthesis reduction in diatoms, providing direct evidence that the reduction of lipoxygenase expression levels are correlated to a marked physiological effect.

5.1 Biolistic transformation is now possible in the *Pseudo-nitzschia* genus

In contrast to the wide interest in diatom biology, molecular tools, such as genetic manipulation, to understand the gene function and the metabolic pathways in diatoms are not well developed in many species.

In order to perform an efficient genetic transformation, the application of an

appropriate promoter able to drive the expression of the gene of interest is the first critical step. Different promoters have been reported to drive the expression of an exogenous gene in diatoms. The use of a heterologous promoter in diatoms was not always successful for expression of exogenous DNA. Initial tests with the *P. tricornutum FcpB* promoter that drives *Sh ble* (exogenous) gene have not succeeded in either *P. multistriata* or *P. arenysensis* species. The *H4* promoter has been identified as a good candidate to drive constitutive expression of transgenes (De Riso et al. 2009). With the replacement of the *FcpB* promoter with *P. multistriata H4* promoter, the *Sh ble* expression in cells took place successfully. The availability of the *P. multistriata* genome allowed easily the identification and cloning of the promoter of a histone gene, allowing the implementation of transformation in this species. The *H4* promoter allowed generating zeocin-resistant strains. Differently to what is done in other diatoms, selection with zeocin antibiotic was performed in liquid rather than solid medium because it appeared faster and more appropriate for *Pseudo-nitzschia* cells growth. A potential drawback of liquid selection is that multiple independent transformation events can occur in one shot, the resulting resistant culture will be a mixture of different transformed strains with different genotypes. This problem was overcome by aliquoting cells recovered after each bombardment in small wells with antibiotics and by manually isolating a single cell. In this way, monoclonal transformed cultures can be obtained.

The *P. multistrata H4* promoter was also used to overexpress a fusion protein, Histone H4-GFP, to obtain *P. arenysensis* cells with fluorescent nuclei. This experiment provided a proof of principle for the feasibility of protein tagging and in addition, fusions with fluorescent tags help in determining the sub-cellular localization of a protein which further helps in defining a function in *Pseudo-nitzschia* species. The fact that some transformants obtained from the co-transformation

experiment did not show green fluorescence under microscope could be attributed to the fact that only the PmH4pShBle plasmid was integrated in the genome. An alternative explanation could be that the plasmid PmH4pH4N-GFP was indeed introduced but the GFP transgene might have been truncated or might have integrated in a region of the genome in which transcription is suppressed, becoming nonfunctional. The introduction of PmH4pGUS plasmid in cells represented an independent confirmation of the feasibility of co-transformation and a further evidence of strong activity of the *H4* promoter. *P. arenysensis* GUS-overexpressing strains were compared with the *P. tricornutum* Pt/GUS strain (De Riso et al. 2009), and their GUS activity ranged between 92 and 70 % with respect to the Pt/GUS strain.

5.2 Possibility to transmit the transgene by sexual reproduction

An important result of this study is the possibility to transfer the transgene from a transformed parental strain to the progeny by sexual reproduction. This is not currently possible in any other model diatom such as *T. pseudonana* and *P. tricornutum* because they have not been observed to reproduce sexually under laboratory conditions. This could particularly be helpful to study multiple combinations of targeted proteins or could be exploited to obtain double mutants. The possibility to transmit a given character to the progeny is also desirable in species in which cell size reduction eventually leads to death of the culture, as this allows to preserve interesting properties, that otherwise would be lost, over a long period through generations. Moreover, different combinations of double transgenes could be created by crossing parental strains obtained from different experiments. F1 resistance cells, obtained applying selection to cultures containing crosses of transformed and wild type cells, were further analysed to obtain more information on

the process. Visualization under fluorescence microscopy and Southern blot analyses demonstrated that the resistant F1 can inherit one or more copies of a functional transgene from the parental strain. We observed that sexual reproduction in some transformed strains was less efficient compared to sexual reproduction occurred among cells not transformed. This could depend on the number of insertions of exogenous DNA that could lead to a disturbance in the meiosis process. Probably the variation of the amount of DNA inserted in the cell could further improve the yield of transgenic F1 strains. Lowering the antibiotic concentration could help in selecting transformants and F1 clones that have a single integration rather than multiple integrations.

5.3 Establishment of *LOX* silencing in *P. arenysensis* cells

A wide fraction of diatom genes cannot be associated to a function by homology-based methods (Armbrust et al. 2004; Bowler et al. 2008). The generation of loss of function mutants by insertional or chemical mutagenesis may be challenging in diploid organisms with unknown sexual cycles such as some diatoms (Parker et al. 2008). As an alternative, RNA-mediated silencing is becoming a promising tool for targeted gene knockdown in algae having a functional RNAi machinery (Cerutti et al. 2011). The important results of this thesis have opened the way to the study of traditional loss of function approaches that are only used in *P. tricornutum* (De riso et al. 2009; Lavaud et al. 2012). Biochemical and genetic studies of different eukaryotes have led to the identification of key components of the RNAi machinery. In the *P. arenysensis* transcriptome, alignment programs revealed RNase III-like endonuclease named Dicer and Argonaute-Piwi proteins. DICER processes hairpin and long dsRNAs into sRNAs while Argonaute contains a PAZ (Piwi-Argonaute-

Zwille) domain that functions as sRNA-guided endonuclease that cleaves complementary transcripts. The presence of key components of the RNAi machinery suggested that RNA-mediated silencing may play regulatory roles and may be used as a reverse genetics tool in *P. arenysensis*.

Lipoxygenases are enzymes that catalyze the synthesis of oxylipins. In order to study the oxylipins function in *P. arenysensis* we performed an efficient lipoxygenase silencing. Because only few silencing studies have been so far carried out on diatoms, we generated different constructs containing either anti-sense or inverted repeat fragments of the target *LOX* gene. The initial positive results obtained with antisense constructs, brought us to use these for the subsequent experiments. We cannot provide data on the functionality of the inverted construct at this stage because these were only used once in an experiment in which we could not obtain transformed strains. This could have happened by chance and the experiment should have been repeated, since however I had obtained tranformants with the other contructs I preferred to focus on those and proceed to the functional studies. The clones resulting from the introduction of interference constructs, selected for the zeocin resistance, showed the presence of a transgene by PCR analysis. The main proofs of successful *LOX* silencing were obtained from western blot analyses and FOX2 assay.

The use of a LOX antibody that binds the *P.arenysensis* protein allowed us to understand to what extent the *LOX* silencing had led to reduced levels of protein by western blot analyses. Some clones showed from 30 to 70% of LOX protein reduction compared to the wild type clone, while by FOX2 assay, these clones also showed a reduction of highly reactive hydroperoxides (FAHs) levels from about 20 to about 95% compared to the wild type.

The FAHs are the first products of LOX and are converted spontaneously or

enzymatically in different oxylipins.

5.4 Two LOX pathways catalyze oxylipins production

LOXs are the entry point to pathways that provide a variety of oxylipin molecules involved in signaling for stress responses. LOX enzymes oxygenate polyunsaturated fatty acids in a position-specific manner. The differences in the oxygenation site catalyzed by LOX and the variability of the downstream reactions add great diversity to types of oxylipins in diatoms. Differently from plants and mammalians, the composition and level of these compounds in diatoms are highly unpredictable, differing not only between species, but also at strain level and in consequence of the physiological status of the same diatom culture (Pohnert 2002; Ribalet et al. 2007; d'Ippolito et al. 2009). For this reason we analyzed the VS3 wild type and other silenced clones by LC-MS procedure (Cutignano et al. 2011) to detect and characterize the oxylipins in *P. arenysensis*. LC-MS/MS profiling of wild type and silenced clones showed the presence of hydroxy-eicosapentaenoic acid (EPA) derivatives, namely hydroxy-fatty acid (HEPEs) and epoxyalcohol (HEpETEs), produced by 12- and 15-LOX (fig. 33 and 34). As reported in fig.37, the analysis identified in VS3 wild type both product series with 15-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid (15-HEPE), 13- hydroxy-14-epoxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid (13,14-HEpETE), 12- hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoicacid (12-HEPE) and 10-hydroxy-11-epoxy-5Z,8Z,14Z,17Z-eicosatetraenoic acid (10,11-HEpETE).

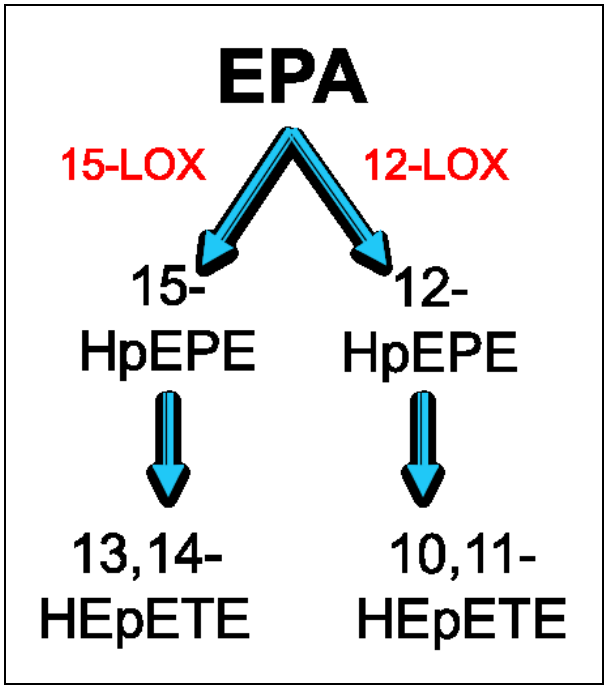


Fig. 39 Schematic representation of the lipoxygenase pathway identified in this study in the *P.arenysensis* VS3 strain. EPA, eicosapentaenoic acid; HpEPE hydroperoxy-eicosapentaenoic acid HEpETE, hydroxy-epoxy-eicosatetraenoic acid; HEPE, hydroxy-eicosapentaenoicacid. In red two LOX enzymes.

In all silenced clones was demonstrated that both 15- and 12-LOX derived products reduce their levels compared to the VS3 wild type. In the CA1 sample the 12-LOX products totally disappeared while the 15-LOX products were reduced. After silencing, the 5.5 sample showed a reduction of HEpETEs from 63% to 84% and a HEPEs reduction from 68% to 73%.

The incosistency between the presence of only a single sequence coding for 15S-LOX in the transcriptome and the evidence of two LOX pathways in wild type strain used to produce the silenced strains can be explained by the very high variability of LOX pathways in the genus (Lamari et al. 2013). Diatoms are diploid, some *P. arenysensis* strains have been shown to possess either 12-LOX or 15-LOX products while others both 12-LOX and 15-LOX products (Lamari et al. 2013). Thus it is very

likely that the VS3 silenced strain possess two genes that encode for 12- and 15S-LOX, whereas the original B593 strain that was used for the transcriptome analysis only the gene for 15S-LOX. It is known that LOX sequences can have high similarity (Sloane et al. 1995), therefore we believe that the RNAi-dependent silencing most likely effectively interfered expression of both LOX proteins in VS3 and led to reduction of the products of both pathways with different extent.

5.5 Inhibition of lipoxygenase pathway suppresses the growth of *P. arenysensis* cells

In many organisms LOXs are well characterized for both the pathway and the function. In diatoms, oxylipins are suggested to impair the reproductive biology of copepods, the principal plankton grazers, and to mediate intracellular communication (Ianora et al. 2004; Fink et al. 2011; Vardi et al. 2006; Fontana et al. 2007) but no diatoms with available genome sequence possess a known LOX pathway.

LOXs are involved in the cell growth both in human and in plants (HayashI et al. 2006; Kolomiets et al. 2001). In human, the 5-LOX pathway is important in regulating the growth of certain types of tumors. In particular, the use of a 5-LOX-specific inhibitor, AA861, induced strong growth inhibition of bladder cancer cells.

In plants the production of mutants by antisense coding specific tuber LOX sequence, *POTLX-1*, showed a significant reduction in LOX activity in potato tubers. The LOX reduction correlated with reduced tuber size and the production of malformed tubers. The results of this thesis show for the first time a correlation between the LOX reduction and the cell growth reduction in diatoms.

In order to study the possible phenotypical effects of the LOX silencing on the *P. arenysensis* cells we compared the growth of silenced cells and wild type cells. On

the strains with a strong reduction of the LOX protein levels and a marked loss of LOX- derived products, a growth curve was performed. The results obtained with this experiment were extremely interesting since the growth of the silenced strains was strongly impaired. The same strain showed a marked photoinhibition and a NPQ much lower than the wild type control. LOXs are involved in different stress response. In *Arabidopsis thaliana,* LOX activation is involved in the red light-induced defense response (Zhao et al. 2014). It seems that in *P.arenysensis*, LOX reduction correlated to rapid photoinhibition in comparison to wild type cells, thus suggesting that inhibition of LOX expression makes cells more sensible to stress (e.g. high light) compared to the control strain.

6. CONCLUSIONS AND FUTURE PERSPERCTIVES

The possibility of dissecting gene and function by reverse genetics represents a major advance to understand diatom biology and the mechanisms underlying the evolutionary success of these organisms in the oceans. Genetic manipulation tools, already developed for diatom models such as *P. tricornutum* and *T. pseudonana*, are now possible for the first time in the *Pseudo-nitzschia* genus. In the first part of this study I demonstrated the feasibility of biolistic transformation, overexpression and gene tagging in *P. multistriata* and *P. arenysensis*. Crosses experiments showed the possibility of transgene inheritance from parental cells to F1 cells progeny. This result has a crucial relevance as it allows to maintain the mutation for a long time in *Pseudo-nitzschia* cells that physiologically experience size reduction and die if they do not undergo sexual reproduction. The first part of this study has been included in a manuscript: “Establishment of Genetic Transformation in the Sexually Reproducing Diatoms *Pseudo-nitzschia multistriata* and *Pseudonitzschia arenysensis* and Inheritance of the Transgene” (2015) Valeria Sabatino, Monia Teresa Russo, Shrikant Patil, Giuliana d’Ippolito, Angelo Fontana & Maria Immacolata Ferrante.

A primary achievement of my thesis work is the RNAi-dependent silencing in species of the genus *Pseudo-nitzschia*. This is the first time that non-model diatom species are functionally interfered by this molecular approach that promises to be an important tool to shed light on ecological interactions in plankton communities, such as blooming and defense against grazers.

Interference of LOX pathways, that were the target of my thesis, is a clear demonstration of the effectiveness of the method and of the prominent information we can gather by this approach. Silencing of LOX pathways reduce synthesis of

LOX products and provide the first direct piece of evidence about the role of these pathways in diatom growth. Oxylipins production is also suggested to cause reproductive failure in copepods due to apoptosis and teratogenesis (Fontana et al. 2007), for this reason studies of the interaction between silenced cells and copepods will be performed with the aim of understanding the effect of oxylipins reduction in grazers.

Preliminary analyses correlate interference of LOX expression with photoinhibition. This may be due to a reduced ability of the silenced clones to respond to stress induced by light. On the other hand, LOX overexpression is expected to offer complementary results and increase strain resilience. Therefore, in addition to the ecological relevance, this study could pave the way to improve the growth of diatoms in closed photobioreactors or open ponds, thus opening to the biotechnological exploitation of these microalgae as feedstocks for functional products. Other experiments are already programmed to investigate in the deep the role of LOX in diatoms.

7. REFERENCES

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ORIGINAL ARTICLE

Establishment of Genetic Transformation in the Sexually Reproducing Diatoms *Pseudo-nitzschia multistriata* and *Pseudo-nitzschia arenysensis* and Inheritance of the Transgene

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Abstract We report the genetic transformation of the planktonic diatoms *Pseudo-nitzschia arenysensis* and *Pseudo-nitzschia multistriata*, members of the widely distributed and ecologically important genus *Pseudo-nitzschia. P. arenysensis* and *P. multistriata* present the classical size reduction/restitution life cycle and can reproduce sexually. Genetic transformation was achieved with the biolistic method, using the *H4* gene promoter from *P. multistriata* to drive expression of exogenous genes. The transformation was first optimized introducing the *Sh ble* gene to confer resistance to the antibiotic zeocin. Integration of the transgene was confirmed by PCR and Southern blot analyses. Subsequently, we simultaneously transformed in *P. arenysensis* two plasmids, one encoding the β-glucuronidase (*GUS*) gene together with the plasmid carrying the *Sh ble* resistance gene, demonstrating the possibility of co-transformation. By transforming a gene encoding a fusion between the histone H4 and the green fluorescent protein (GFP), we demonstrated that fluorescent tagging is possible and that studies for protein localization are feasible. Importantly, we crossed *P. arenysensis*- and *P. multistriata*-transformed strains with a wild-type strain of opposite mating type and demonstrated that the transgene can be inherited in the F1 generation. The possibility to transform two diatom species for which genetic crosses are possible opens the way to a number of new approaches, including classical loss of function screens and the possibility to obtain different combinations of double transformants.

Keywords Diatoms · Transformation · *Pseudo-nitzschia* · Promoter · Genetic crosses

Introduction

Diatoms are unicellular organisms which constitute the main component of phytoplankton. Diatoms have important ecological, energetic, and biogeochemical roles. They are responsible for 20 % of global carbon fixation (Field et al. 1998), they play a key role in the biogeochemical cycles of carbon, nitrogen and silicon (Smetacek 1999), and the attention towards them increased as they are promising candidates for biofuel production (Hildebrand et al. 2012). Knowledge of diatom biology is crucial for understanding the basis of their ecological success, for predicting their capability to adapt to a rapidly changing environment, and for the exploration of their unique features. We know a great deal about ecology and physiology for many diatom species while extensive molecular and functional genomics studies have been conducted mainly on two representative species, *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*. Five diatom genomes are currently available, *T. pseudonana* (Armbrust et al. 2004), *P. tricornutum* (Bowler et al. 2008), *Thalassiosira oceanica* (Lommer et al. 2012), *Fragilariopsis cylindrus*, and *Pseudo-nitzschia multiseries* (unpublished but available

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at http://genome.jgi.doe.gov/bacillariophyta/bacillariophyta.info.html), and a recent initiative (http://www.marinemicroeukaryotes.org) has led to the generation of over 170 diatom transcriptomes (Keeling et al. 2014). Genomes and transcriptomes are providing us with a plethora of information, and complementary approaches are needed to decode and exploit this information. As expected, given that explorations of the field are relatively recent and that extrapolation of knowledge from related organisms is not always informative, a high percentage of diatom genes have unassigned function (Bowler et al. 2010). Molecular tools are desirable to assign function to genes as well as to manipulate strain properties. In addition to techniques that allow gene manipulation, controllable genetics of diatom species, and specifically experimental control of sexual crosses, would greatly enhance the opportunities for forward and reverse genetic studies. Diatoms with life cycles attributes that allow control of the genetics include the benthic species *Seminavis robusta* and several planktonic *Pseudo-nitzschia* (Chepurnov et al. 2008).

Pseudo-nitzschia are pennate diatoms of global importance, the genus comprises ca 37 species that have been recorded at different sites in the world oceans (Trainer et al. 2012). Some of these species are known to produce the neurotoxin domoic acid (DA), a molecule that can cause Amnesic Shellfish Poisoning (Lelong et al. 2012). Of the current model diatoms, both *P. tricornutum* and *T. pseudonana* do not reduce their size and do not reproduce sexually under laboratory conditions. *Pseudo-nitzschia* species on the other hand present the classical size reduction/restitution cycle and reproduce sexually when two strains of opposite mating type, "+" and "−", are mixed together, producing gametes, followed by zygotes and auxospores that form initial cells (Round et al. 1990). Among *Pseudo-nitzschia* species, *Pseudo-nitzschia arenysensis* and *Pseudo-nitzschia multistriata* are chain-forming species that can be responsible for blooms in coastal and oceanic waters (Trainer et al. 2012). *P. arenysensis* was known as *Pseudo-nitzschia delicatissima* and was subsequently named *P. arenysensis* in Quijano-Scheggia et al. (2009). For both species, the life cycle is well described (Amato et al. 2005; D'Alelio et al. 2009; Scalco et al. 2014). *P. multistriata* can be distinguished from other *Pseudo-nitzschia* due to its prominent sigmoid shape in girdle view, and it is moderately toxic (Orsini et al. 2002).

In order to study the molecular properties of diatoms, methods for stable genetic transformation have been developed in various species including *P. tricornutum* (Apt et al. 1996), *Cyclotella cryptica* and *Navicula saprophila* (Dunahay et al. 1995), *Cylindrotheca fusiformis* (Fischer et al. 1999), *T. pseudonana* (Poulsen and Kröger 2005; Poulsen et al. 2006), *Chaetoceros* sp. (Miyagawa-Yamaguchi et al. 2011), and *Fistulifera* sp. (Muto et al. 2013). Despite reports in which electroporation has been shown to be effective in transforming *P. tricornutum* (Niu et al. 2012; Miyahara et al. 2013), the method of choice to transform diatoms is particle bombardment. Genes encoding GFP and GUS have been used as reporter genes in *P. tricornutum* (Falciatore et al. 1999; Zaslavskaia et al. 2000). The GUS activity is easily detected and quantified in diatoms and particularly useful in plant molecular biology (Jefferson et al. 1987).

Our laboratory is using both *P. arenysensis* and *P. multistriata* as model systems to address questions related to domoic acid production and to signaling among cells during sexual reproduction. Molecular approaches, such as qPCR, are feasible in these two species (Adelfi et al. 2014), and we are developing additional resources, including the genome sequence for *P. multistriata* (Ferrante, unpublished) and several transcriptomic data sets for both species.

In this article, we report the genetic transformation of *P. arenysensis* and *P. multistriata,* achieved using the biolistic method.

To this aim, a *P. multistriata* promoter was cloned to drive exogenous gene expression.

We describe transformation with a plasmid containing the antibiotic resistance gene *Sh ble*, conferring resistance to the antibiotics zeocin and phleomycin, and demonstrate co-transformation using this plasmid together with either one encoding the *GUS* gene or one encoding the histone *H4* gene fused with GFP.

In addition, we show that genetics is controllable in *P. multistriata* as well as in *P. arenysensis* and that genetic crosses allow to transfer the transgene to the progeny.

Materials and Methods

Cell Culture

Strains of the marine planktonic diatoms *P. arenysensis* and *P. multistriata* were grown in F/2 medium (Guillard 1975) at 18 °C under white fluorescent lights at approximately 80 μmol photons m^{-2} s^{-1}, 12 h:12 h dark–light cycle. These are the standard growth conditions used for all the experiments mentioned herein. For transformation, we used the *P. arenysensis* strain B858 and the *P. multistriata* strain B856 (Adelfi et al. 2014). For crosses, the wild-type *P. multistriata* B936 strain was isolated from samples collected at the LTER station MareChiara in the Gulf of Naples (40° 48.5′ N, 14° 15′ E) on the 24 May 2012, while the B947 *P. arenysensis* strain was a sibling of two wild-type strains, B668 and B675 (Adelfi et al. 2014).

Plasmids Construction

The plasmid vector pFCPBp-Sh ble (Falciatore et al. 1999) was used as the backbone transformation vector. The promoter

sequence of the *H4* gene, from base 663 to base 38 upstream of the *H4* ATG, was amplified from *P. multistriata* genomic DNA using the sense primer H4pKpnI for: 5′-GGCG GCCGGTACCGGCCATTTTGGAATTTGTCG-3′ and the antisense primer H4pKpnI rev: 5′- GGCGGCCGGTA CCGCCGAAGACTGAGGTAGTCG-3′ (*Kpn*I sites underlined). The resulting product was digested with *Kpn*I generating a 625-bp fragment that was cloned replacing the fucoxanthin chlorophyll *a*/*c*-binding protein (FcpB) promoter to obtain the PmH4pShble plasmid. The *H4* gene was amplified from *P. arenysensis* genomic DNA using the sense primer H4StuI for: 5′-GGCGGCCAGGCCTATGTCTGGAAGAGG AAAAGGAGG-3′ (*Stu*I site underlined) and the antisense primer H4NotI rev: 5′-GGCGGCCGCGGCCGCTTATCC ACCGAATCCGTAAAGG-3′ (*Not*I site underlined). To clone the *P. arenysensis* Histone *H4* gene, we designed primers on the MMETSP0329-20120918-8692 transcript described in Adelfi et al. (2014) (subsequently renamed MMETSP0329-20121206-8692 at http://camera.crbs.ucsd.edu/mmetsp/list.php, note that only the date in the ID has changed, not the initial and final identifiers for the sequence). The *P. arenysensis* Histone *H4* gene was cloned replacing the *Sh ble* fragment in the PmH4pShble plasmid. For the amino-terminal fusion construct, the *EGFP* gene was amplified from the plasmid pEGFP-N1 (Clontech) by PCR using the sense primer GFPKpnI for: 5′-GGCGGCCGGTA CCATGGTGAGCAAGGGCGAG-3′ (*Kpn*I site underlined) and the antisense primer GFPStuI rev: 5′-GGCGGCCAGGC CTCTTGTACAGCTCGTCCATGCC-3′ (*Stu*I site underlined). The product was cloned to generate the final PmH4pH4N-GFP plasmid. The PmH4pGus plasmid was obtained by replacing the FcpB promoter in the FcpBp-GUS-FcpA 3′ plasmid (De Riso et al. 2009) with the *P. multistriata H4* promoter amplified using the sense primer H4pKpnI for and the antisense primer H4pEcoRI rev: 5′-GGCG GCCGAATTCGCCGAAGACTGAGGTAGTCG-3′ (*Eco*RI site underlined).

The 625-bp *H4* promoter fragment sequence has been deposited at the European Nucleotide Archive and has been assigned accession number LN609578.

Growth on Solid Medium and Determination of Antibiotic Sensitivity

To test growth on solid medium, agar and agarose were used as gelling agents at the concentrations of 0.8, 0.6, and 0.4 % in F/2 medium. Approximately 10^3 and 10^4 *P. arenysensis* or *P. multistriata* cells were spread on the plates that were subsequently incubated at normal growth conditions. Colony formation was checked after 10 and 15 days.

In order to determine the lethal concentration of zeocin, wild-type *P. arenysensis* and *P. multistriata* cells were inoculated in liquid F/2 medium supplemented with 25, 50, and 75 μg ml^{-1} zeocin, kept at normal growth conditions and inspected after 12 days.

Transformation of *P. arenysensis* and *P. multistriata*

The transformation of the B858 strain was performed by microparticle bombardment using the Biolistic PDS-1000/HE Particle Delivery System (Bio-Rad). The day before bombarding, cells were harvested at the mid-logarithmic phase. Approximately 5×10^7 cells were spread onto 0.4 % agarose plates containing F/2 medium and allowed to dry under a sterile hood. Plasmid DNA (5 μg) was coated onto M10 (0.7 μm, Bio-Rad) or M17 (1.0 μm) tungsten particles using the $CaCl_2$-spermidine method according to the manufacturer's protocol. The plates were positioned at a distance of 6 cm from the stopping screens and a burst pressure of 1550 psi was used. After bombardment, the plates were incubated for 24 h at standard growth conditions. For each bombarded plate, the cells were then scraped from the agarose, resuspended in F/2 liquid medium (250 ml) containing 50 μg ml^{-1} zeocin (Invivogen) and incubated under standard growth conditions. Putative transformants were obtained after about 12 days of incubation. For co-transformation, cells were bombarded using conditions described above; particles for bombardment were coated with 3 μg of PmH4pShble plus 3 μg of PmH4pH4N-GFP or with 3 μg of PmH4pShble plus 3 μg of PmH4pGus. The optimized protocol, with M10 particles, was used to transform B856 *P. multistriata* cells.

PCR Analysis on Transformed Cells

PCR was performed on genomic DNA from transformants and wild-type cells, obtained by centrifugation of the culture followed by resuspension of the pellet in 500 μl of CTAB extraction buffer (Tesson et al. 2011). The thermal profile used was: 94 °C for 3 min (94 °C for 45 s, 55 °C for 45 s, 72 °C for 90 s)×30 cycles, 72 °C for 7 min. The primers used were: H4p 5′-GACTACTTCCGGGTCCATG-3′; Forble 5′-ACGACG TGACCCTGTTCATC-3′; Revble 5′-GTCGGTCAGTCCTG CTCCT-3′. The amplification with H4p and Revble produces an 889-bp fragment, the amplification with Forble and Revble produces a 250-bp fragment. Amplification fragments were analyzed on agarose gel.

Hoechst Staining and Fluorescence Microscopy

Five microliters of 0.1 mg ml^{-1} Hoechst (33342, Molecular Probes, Invitrogen) solution was added to each milliliter of live sample and incubated for 15 min in the dark before observation.

Live transformant cells were imaged using the Leica DMI 6000B inverted epifluorescence microscope equipped with a DFC360FX camera.

GUS Assay

After about 10 days from the co-transformation, resistant *P. arenysensis* cells were isolated, expanded, and analyzed by histochemical GUS assay. The *P. tricornutum* Pt/GUS strain was grown and treated as described in De Riso et al. (2009). Histochemical assays were performed on cells plated on agarose in 5.5 cm Petri dishes containing 400 μl X-Gluc solution (0.1 % X-Gluc, Clontech); 100 mM Na-phosphate at pH 7.0; 0.5 mM $K_4Fe(CN)_6$; and 0.5 mM $K_3Fe(CN)_6$, placed in the dark and incubated at 37 °C overnight. For spectrophotometric assays, 50 ml of cells were collected in exponential phase and centrifuged at 3800 rpm for 15 min. The pellet was immediately frozen in liquid nitrogen and stored at −80 °C. Later, the cells were resuspended in 200 μl of fresh GUS extraction buffer (50 mM Na-phosphate pH 7.0; 10 mM β-mercaptoethanol; 0.1 % Triton X-100; 1 mM PMSF; 1× cocktail of protease inhibitors (Complete, Roche 11873580001, 1 tablet/2 ml)), frozen twice in liquid nitrogen and thawed at 37 °C. The samples were centrifuged at 12,000 rpm for 5 min at 4 °C. GUS assays were performed by incubating 20 μg of total protein extract with the substrate *p*-nitrophenyl glucuronide (PNPG) at 1 mM final concentration. After a 4-h incubation at 37 °C, the reaction was stopped by adding 0.4 ml of 2.5 M 2-amino-2methyl-1,3 propandiol and absorbance was measured at 415 nm. Enzymatic activity was calculated on the basis of the OD recorded and the molar extinction coefficient of the GUS substrate *p*-nitrophenol.

Crosses and F1 Cells Isolation

To demonstrate inheritance of exogenous DNA from transformed cells to the next generation, transformed cells were crossed with sexually compatible wild-type strains. Mating experiments were carried out by mixing a single resistant clone with a wild-type strain of opposite mating type and incubating them at standard growth conditions. F1 generation initial cells, easily distinguishable by size difference, were observed after 2 to 3 days. The day after initial cells appeared, the mixed cultures were placed in zeocin selection (50 μg ml^{-1} zeocin). After 8 days, the surviving initial cells were serially re-isolated (two times) from the mating culture by micropipette under inverted microscope, and each initial cell was placed into a separate well of a 24-well plate containing sterile seawater enriched with F/2 nutrients. The initial cells were subsequently grown under standard growth conditions and later genotyped for the incorporation of *Sh ble* gene by PCR analysis and Southern blot analyses.

Genomic DNA Preparation and Southern Blot Hybridization

For genomic DNA preparation *P. arenysensis* cultures with cell density of 1×10^5 cells ml^{-1} were harvested by filtration onto 1.2 μm pore size filters (RAWP04700 Millipore). Cells were disrupted by vortexing in the presence of 0.4 g glass beads 0.2–0.3 mm diameter (G1277, Sigma-Aldrich), 0.5 ml TE buffer, and 0.5 ml buffered phenol. Following steps of DNA extraction and purification were performed as reported in Vanstechelman et al. (2013). Digoxigenin (DIG)-based Southern blot was performed on 5 μg of genomic DNA digested with *Hin*dIII. Only one site for this restriction enzyme exists in the antibiotic resistance expression vector used for transformation, and no site was present in the hybridization probe. The digested DNA was separated on 0.9 % agarose gel, and then transferred to a positively charged nylon membrane (Roche) according to Sambrook and Russel (2001). The DIG-labeled *Sh ble* DNA probe was prepared by PCR DIG Probe synthesis kit (Roche), according to the manufacturer's instructions, using the primers Forble and Revble (see "PCR Analysis on Transformed Cells"). Hybridization and autoradiography were performed according to the DIG Application Manual for filter hybridization (Roche).

Results

Identification of the *P. multistriata H4* Gene Promoter

The choice of an active promoter is the first critical step to achieve efficient transformation. The *H4* promoter has been identified as a good candidate to drive constitutive expression of transgenes (De Riso et al. 2009).

Our laboratory is in the process of sequencing the genome of *P. multistriata* (Ferrante, unpublished).

A BlastN search against the draft genome sequence of *P. multistriata* with the nucleotide sequence of the *P. tricornutum* histone *H4-1a* retrieved two hits on scaffold 41 (at positions 47527–47838 and 151143–150832) with an identity of 87.50 and 87.18 %, respectively. These two sequences differ between them for two nucleotides only. At both these two loci, a homologue of the *H3* gene is present upstream of the *H4* gene, with a reverse orientation. The intervening sequences between the *H3* and *H4* copies and the sequences downstream of the genes at both loci are not conserved, indicating that the two loci represent a duplication and not an assembly error (data not shown).

We cloned a 625-bp sequence upstream of the *H4* gene present in the second locus on scaffold 41 (151143–150832) and placed it upstream of *Sh ble* (Falciatore et al. 1999) to obtain the PmH4pShble plasmid (Fig. 1). In order to verify whether the selected sequence could act as a promoter and

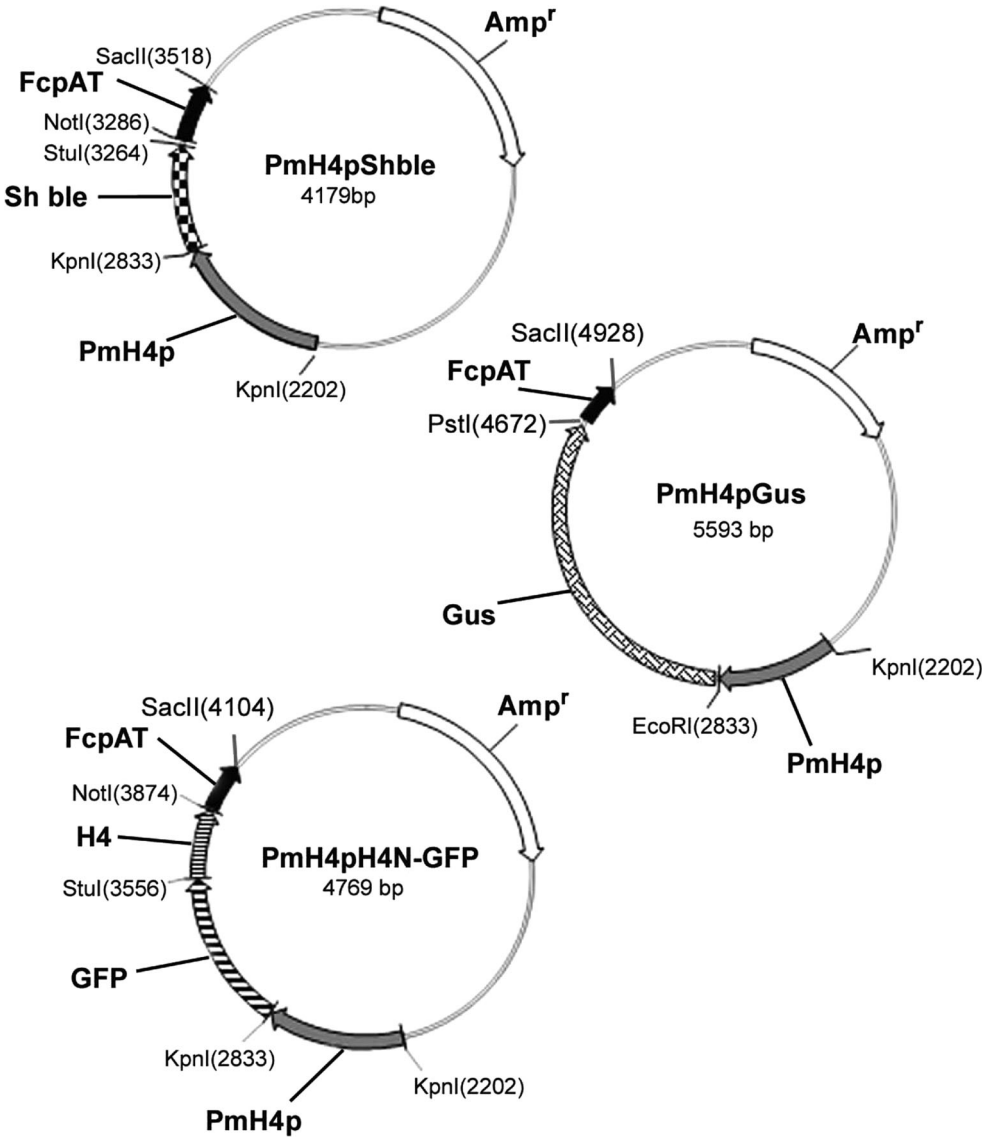

Fig. 1 Vector maps of the plasmids PmH4pShble, PmH4pGus, and PmH4pH4N-GFP used to transform *Pseudo-nitzschia arenysensis* and *Pseudo-nitzschia multistriata*. *FcpAT* fucoxanthin chlorophyll *a*/*c*-binding protein terminator; see text for other abbreviations

could drive expression of the *Sh ble* gene, we first used this construct to transform *P. tricornutum*. We obtained resistant clones and were able to confirm the presence of the transgene by PCR (Online resource 1).

Genetic Transformation of *P. arenysensis* and *P. multistriata*

In order to find an efficient transformation method for *Pseudo-nitzschia,* we modified the transformation protocol commonly used for *P. tricornutum* (Apt et al. 1996; Falciatore et al. 1999). To drive expression of the *Sh ble* gene in *Pseudo-nitzschia* species, we used the *H4* promoter from the Histone *H4* gene. We tested growth on solid plates: we used agar and agarose as gelling agents in different concentrations and observed that *Pseudo-nitzschia* cells grew on 0.4 % agarose better than on agar plates (data not shown), however in general cell growth on solid plates was not robust and cells were routinely maintained in liquid. We then tested zeocin antibiotic sensitivity. We established the antibiotic concentration to use for selection in liquid medium, testing doses of 25, 50, and 75 μg ml^{-1}. We observed that concentrations of 50 and 75 μg ml^{-1} were efficient in suppressing growth (Table 1), therefore we chose 50 μg ml^{-1} for subsequent experiments.

P. arenysensis cells were plated the day before the transformation. We used M10 and M17 tungsten particles for bombardment, according to the manufacturer's protocol. The day after transformation, for each bombarded or control plate, the cells were transferred from the plate to selective liquid medium and kept at normal growth conditions. After about 12 days, all cells were dead in the control flasks containing the non-transformed strain. We did not find live cells in the experiments in which M17 particles were used, whereas we could

Table 1 Antibiotic sensitivity of *Pseudo-nitzschia arenysensis* and *Pseudo-nitzschia multistriata*

Cells	Zeocin (μg/ml)		
	25	50	75
10^5	+	–	–
10^6	+	–	–

The signs "+" and "–" indicate presence or absence, respectively, of viable cells in flasks after 10 days in selection

observe live cells in flasks containing cells transformed using M10 tungsten particles.

Zeocin-resistant cells collected from independent flasks were analyzed by PCR screening. PCR analysis showed the presence of *Sh ble* as well as of the *PmH4* promoter in transformant cells, whereas no amplified product was obtained from wild-type cells (Fig. 2).

We obtained transformed cells with variable efficiency in the experiments performed (Table 2).

The protocol described above was tested in *P. multistriata* cells (strain B856) using M10 tungsten particles and the PmH4pShble plasmid. As for *P. arenysensis*, resistant *P. multistriata* cells in zeocin selection appeared 10–12 days after transformation. We verified the presence of the *Sh ble* transgene in transformant cells by PCR analysis (Online resource 2). The success of transformation was variable (Table 3), most likely due to the variable state of the starting culture: the highest number of positive flasks was obtained when using a culture collected exactly in the mid-exponential phase of growth, whereas cells collected later along the growth curve led to a lower number of positive flasks.

We also tested selection of resistant strains on solid plates, re-plating on selective plates 24 h after bombardment. Although a few transformant colonies could be observed 3 weeks after re-plating, cells survival was generally low on solid plates and different experiments did not give consistent results (data not shown). Liquid selection appeared faster and allowed a better recovery of cells after transformation; therefore, this method has been used for all subsequent experiments.

Co-transformation of *P. arenysensis*

To demonstrate that overexpression and fluorescent tagging of proteins is feasible in our system, we tried co-transformation

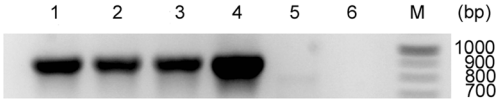

Fig. 2 PCR analysis of *P. arenysensis* transformant clones. *Lanes 1–3*, three independent zeocin-resistant strains (B858R1, B858R2, B858R3); *lane 4*, PmH4pShble plasmid; *lane 5*, wild-type strain B858; *lane 6*, blank; *M* 100 bp ladder

in *P. arenysensis* cells with PmH4pShble and PmH4pH4N-GFP, a plasmid with GFP fused with the *P. arenysensis* Histone *H4* gene *PaH4* under the control of the *P. multistriata H4* promoter (Fig. 1). From three independent experiments, we obtained seven zeocin-resistant clones of which five were also positive for green fluorescence in microscopy (Table 2). A live nuclear stain with Hoechst confirmed that the GFP signal was localized to the nuclei (Fig. 3).

As a further confirmation, we made a co-transformation with the *GUS* reporter gene. The plasmid PmH4pGus used had the *GUS* gene under the control of the *P. multistriata H4* promoter (Fig. 1). Resistant cells obtained after co-transformation were analyzed by histochemical GUS assay and showed an intense blue color (Fig. 4a–d). For quantification of the GUS activity, we performed a spectrophotometric GUS assay: we measured the enzymatic activity of GUS for two clones reported as percent of the *P. tricornutum* Pt/GUS strain (De Riso et al. 2009), used as reference value. The clones showed a level of GUS activity ranging from 92 to 70 % with respect to the reference strain (Fig. 4e).

Inheritance of the Transgene

To demonstrate that the exogenous DNA integrated in the transformed cells can be transmitted to the progeny, we crossed both *P. multistriata*- and *P. arenysensis*- resistant strains with wild-type strains of opposite mating type. The success rate in sexual reproduction of resistant strains derived from the same experiment was variable: some resistant strains could reproduce as efficiently as the non-transformed strains; others produced a lower number of F1 initial cells. To isolate F1-resistant cells, we placed the mixed cultures containing the two parental strains and the F1 cells in zeocin selection. After 10 days in selection, the wild-type parental and most of the F1 cells died; however, a number of F1 cells could still be found (Fig. 5a). A subset of these F1-resistant cells was manually isolated and grown as independent cultures, and PCR analysis demonstrated that they had inherited the *Sh ble* resistance gene (Fig. 5b).

Southern blot analyses were performed to assess the number of integration events in transformed strains and to confirm inheritance of the resistance gene in the F1 cells. Genomic DNA was digested with *Hin*dIII, a unique site in the PmH4pShble plasmid cutting in the terminator (Fig. 6a), and a 250-bp probe was designed against the *Sh ble* sequence (Fig. 6a). Three independent *P. arenysensis* resistant strains showed different patterns of hybridization (Fig. 6b, lanes 1, 5, and 8), confirming independent events of integrations. A culture established after manual isolation of a single cell from the B858GUS12 culture was also included (Fig. 6b, lane 6) to verify whether multiple bands derived from independent integrations in the same genome or from the presence of different transformed genotypes in the original resistant culture. As the

Table 2 Summary of the transformation experiments for *P. arenysensis*

Experiment	Plasmid	Number of bombardments	Flasks[a] positive for antibiotic resistance	Flasks[a] positive for second transgene
1	PmH4pShBle	2	2	
2	PmH4pShBle	3	1	
3	PmH4pShBle	6	5	
4	PmH4pShBle	10	2	
5	PmH4pShBle	10	2	
6	PmH4pShBle	10	3	
7	PmH4pH4N-GFP+PmH4pShBle	8	3	1
8	PmH4pH4N-GFP+PmH4pShBle	5	1	1
9	PmH4GUS+PmH4pShBle	12	3	2
10	PmH4pH4N-GFP+PmH4pShBle	10	3	3

[a] One flask was established from each bombardment

same bands appear in lanes 5 and 6, it appears that a single genotype with multiple integration events for the resistance cassette was present in the original B858GUS12 culture. Siblings from B858GUS2 and B858GUS12 were also analyzed: the same pattern of the parental strain was observed in two siblings of B858GUS2 (Fig. 6b, compare lanes 3 and 4 with lane 1), whereas two bands were absent for the third sibling of B858GUS2 (Fig. 6b, compare lane 2 with lane 1) and one band was absent for the only sibling of B858GUS12 analyzed in this study (Fig. 6b, compare lane 7 with lane 5), suggesting in the latter two cases that some of the *Sh ble* copies from the parental strain had not been inherited.

Discussion

Genetic transformation was established in the two pennate diatoms *P. arenysensis* and *P. multistriata* using particle bombardment.

Table 3 Summary of the transformation experiments for *P. multistriata*

Experiment	Plasmid	Number of bombardments	Flasks[a] positive for antibiotic resistance
1	PmH4pShBle	14	12
2	PmH4pShBle	6	3
3	PmH4pShBle	6	3
4	PmH4pShBle	7	3
5	PmH4pShBle	5	4

[a] One flask was established from each bombardment

Our initial attempts to transform *P. arenysensis* using the *P. tricornutum Fcp*B promoter to drive the resistance gene expression had failed (data not shown), indicating that either the biolistic method was not effective in *P. arenysensis* or that the exogenous *P. tricornutum* promoter was not efficient in promoting transcription in a different species. The availability of the genome sequence of *P. multistriata* allowed us to clone a *Pseudo-nitzschia* promoter. Two histone *H4* genes exist in *P. tricornutum*, *H4-1a* (protein ID 34971) and *H4-1b* (protein ID 26896). The *P. tricornutum H4* promoter, from the *H4-1a* gene, has been used to drive constitutive gene expression (De Riso et al. 2009), and histone genes are highly conserved. We identified two *H4* homologues in the *P. multistriata* genome presenting 87.50 and 87.18 % identity at the nucleotide level with the *P. tricornutum H4-1a* gene. Our results show that a 625-bp sequence upstream of one of these two homologues is effective in driving expression of endogenous and exogenous genes in *P. arenysensis* and in *P. multistriata* as well. This result also implies that an exogenous promoter is functional in *P. arenysensis*.

The *P. multistrata H4* promoter has been used to overexpress *Sh ble* in both species, and we have produced zeocin-resistant strains. Liquid selection appeared faster and more suitable than solid selection to allow recovery of cells after transformation and therefore was chosen for our experiments. One potential drawback of liquid selection is that, if multiple independent transformation events happen in one shot, the resulting resistant culture will be a mixture of different transformed strains with different genotypes. This issue can be overcome by aliquoting cells recovered after each bombardment in small vessels (small flasks or multiwell plates) and then manually isolating a single cell from each of the vessels

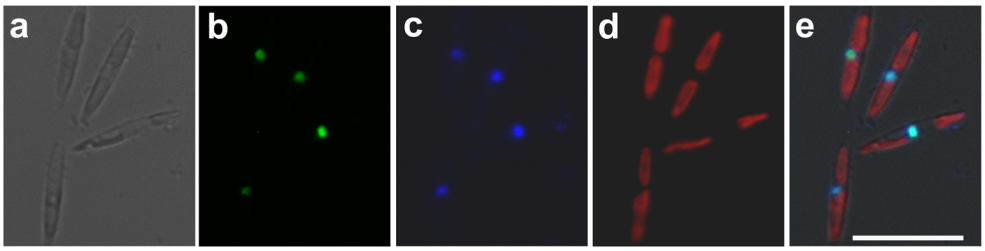

Fig. 3 Four *P. arenysensis* cells expressing the fusion protein H4-GFP. **a** Bright field. **b** GFP fluorescence. **c** Hoechst staining. **d** Chlorophyll auto-fluorescence. **e** Merge of the previous four images. *Scale bar*, 20 μm

in which resistant cells appear. In this way, independent transformed clones can be obtained.

From what we just discussed, liquid selection does not allow comparison of the transformation efficiencies with those estimated in other systems in which solid selection is used, because of a potential underestimation of the number of different transformants in a liquid culture established after bombardment.

The *P. multistrata H4* promoter was also used to overexpress a fusion protein, Histone H4-GFP, to obtain *P. arenysensis* cells with fluorescent nuclei. This experiment provided a proof of principle for the feasibility of protein tagging in *Pseudo-nitzschia* species and represents a new tool for the study of nucleus dynamics in live cells, for example, during mitosis or meiosis.

The production of GUS-overexpressing strains represented an independent confirmation of the feasibility of co-transformation.

P. arenysensis GUS-overexpressing strains were compared with the *P. tricornutum* Pt/GUS strain (De Riso et al. 2009), and their GUS activity ranged between 92 and 70 % with respect to the Pt/GUS strain. Similarly to what has been shown for *P. tricornutum* (De Riso et al. 2009), we plan to use the *P. arenysensis* GUS-overexpressing strain with the highest level of activity to verify whether gene silencing mechanisms operate in *Pseudo-nitzschia*. Preliminary searches in the *P. multistriata* genome retrieved proteins with two RNase III domains, typical of the Dicer family, indicating that the mechanism for gene silencing could be present in these species (Russo, unpublished). The possibility to downregulate genes in *Pseudo-nitzschia* species would greatly enhance the range of opportunities for functional studies.

Overall, our results indicate that the *P. multistriata* 625 bp *H4* promoter sequence can drive robust expression of downstream genes. This fragment is active not only in *Pseudo-nitzschia* species but also in *P. tricornutum* and represents a new entry in the repertoire of diatom promoters available.

The most important result here reported is the possibility to transfer the transgene from a transformed parental strain to the progeny by sexual reproduction. This is not currently possible in any other model diatom. We routinely maintain hundreds of *Pseudo-nitzschia* strains that are easily isolated from plankton samples. By repetitive crossing, we have produced a pedigree of four generations in *P. multistriata* and of three generations in *P. arenysensis*, observing that genetics in these species is controllable (data not shown). With the availability of transformation, and with sequence information (genome and transcriptomes), it will be possible to design approaches in *Pseudo-nitzschia* that are traditionally used in other genetic model systems.

Importantly, the possibility to transmit a given character to the progeny will allow to preserve interesting properties over

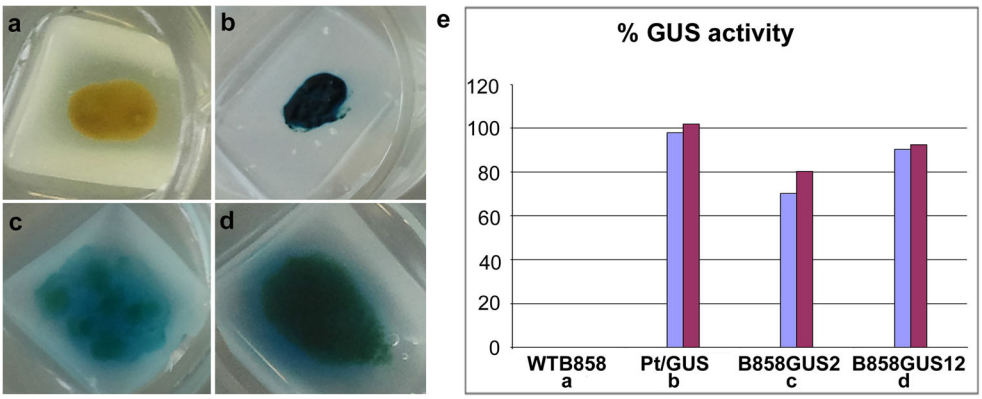

Fig. 4 GUS assay on *P. arenysensis* cells. **a**–**d** Images of cells plated on agarose stained for GUS activity. **a** *P. arenysensis* wild-type B858. **b** *Phaeodactylum tricornutum* Pt/GUS. **c** *P. arenysensis* B858GUS2. **d** *P. arenysensis* B858GUS12. **e** GUS activity of the clones shown in the previous panels, normalized to the GUS activity of the Pt/GUS strain (100 %). *Violet and purple bars* represent two independent measurements made for each clone

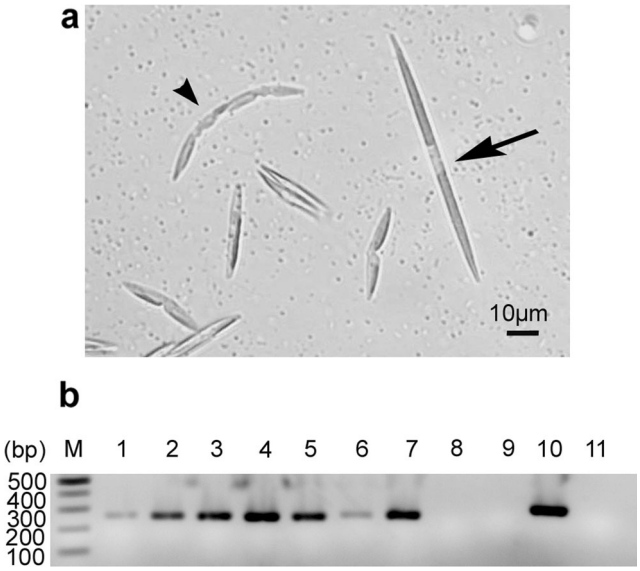

Fig. 5 Resistant *P. multistriata* F1 cells. **a** Light micrograph of live *P. multistriata* cells, the longest cell indicated by the *arrow* is an F1 initial cell, the other cells are the small parental cells, present as single cells or in a small two-cell chain (*arrowhead*). **b** PCR analysis of resistant *P. multistriata* F1 cells; *M* 100 bp marker; *lanes 1–3*, three independent F1 cells deriving from the cross B856T9×B936; *lanes 4 and 5*, two independent F1 cells deriving from the cross B856T10×B936; *lane 6*, parental strain B856T9; *lane 7*, parental strain B856T10; *lane 8*, wild-type parental strain B936; *lane 9*, wild-type non-transformed B856 strain; *lane 10*, plasmid; *lane 11*, blank

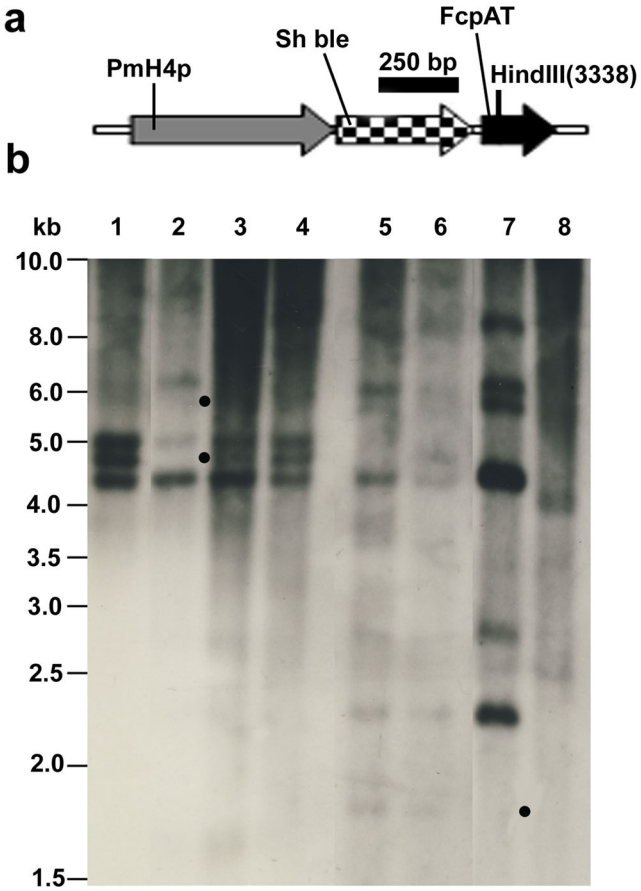

Fig. 6 Southern blot analysis of *P. arenysensis* transformants and F1 siblings. **a** Schematic representation of the expression cassette for the antibiotic resistance. The *black bar* represents the probe used for hybridization; the position of the *Hin*dIII restriction site used to digest DNA is indicated. **b** Southern blot autoradiography. *Lane 1*, strain B858GUS2; *lanes 2–4*, three independent F1 clones, C2, C3, and C5, deriving from the cross B858GUS2×B947; *lane 5*, strain B858GUS12; *lane 6*, clonal B858GUS12 (established from a manually isolated single cell); *lane 7*, one F1 clone deriving from the cross B858GUS12×B947; *lane 8*, strain B858T2A1. *Black dots on the right side of a lane* indicate bands that are missing in some of the samples. Molecular weights are indicated on the *left*

time. Moreover, different combinations of double transgenes could be created by crossing parental strains obtained from different experiments. This could be useful to test multiple combinations of tagged proteins or could be exploited with more sophisticated approaches such as Cre-LoxP systems (Chakraborti et al. 2008) or CRISPR (Burgess 2013; Cong et al. 2013) to obtain double mutants.

Crosses were incubated with zeocin to select the F1 cells that had inherited resistance and Southern blot analyses demonstrated that the resistant F1 had indeed inherited the transgene copies from the parental strain. We noticed that sexual reproduction in some transformed strains was less efficient than in other transformed strains; this could depend on the number of insertions of exogenous DNA that could lead to a disturbance in the meiosis process or to intrinsic variability in the system.

Modulating or changing the type of selection or modulating the amount of DNA inserted in the cell could further improve the yield of transgenic F1 strains. Lowering the antibiotic concentration could help in selecting transformants and F1 clones that have a single integration rather than multiple integrations.

Diatom research is increasingly making use of molecular tools to answer to longstanding questions with new methods. A wide fraction of diatom genes cannot be assigned a function with homology-based methods (Armbrust et al. 2004; Bowler et al. 2008) and more sequences are being discovered with the use of deep sequencing (Keeling et al. 2014). Traditional loss of function and gain of function approaches are sought to explore this mine of novel sequences. Gene silencing, overexpression, and protein tagging are routinely used for the model diatom *P. tricornutum* (Huysman et al. 2013). Species of the genus *Pseudo-nitzschia* can represent additional models to study the molecular properties of diatoms; indeed, the study of this specific group is particularly relevant for the understanding of ecosystem dynamics and of toxic blooms. Furthermore, specific secondary metabolisms characteristic of *Pseudo-nitzschia* species, such as those involved in DA production (Boissonneault et al. 2013) or in oxylipin production (Lamari et al. 2013), could be targeted. The study of specific metabolic features will benefit from the accessibility

of gene manipulation techniques in a range of diatom species, allowing comparative studies.

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