Life Cycle Regulation

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Abstract

Diatom life cycles are unusual among microalgae by being diplontic with a long diploid vegetative phase and a short-lived haploid phase (gametes). Life cycle progression in diatoms is controlled by the cell size reduction-restitution cycle and is intimately linked to their peculiar mode of cell division and siliceous cell wall. Sexual reproduction is primarily cell-size dependent although environmental cues may be needed to trigger gametogenesis in centric diatoms. Although population genetic data suggest sexual reproduction to occur in most species and meiotic genes are widely conserved among diatoms, sexual events are seldom observed in nature. Recent laboratory studies have started to unveil complex pheromone signaling cascades during sexual reproduction in pennate diatoms. Likewise, significant progress has been made in the identification of mating type determination mechanisms in heterothallic species, where several conserved, but as yet functionally uncharacterized, genes involved in sexual reproduction have been identified. While many aspects of diatom life cycle regulation remain to be

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discovered, the recent development of new model species allowing genetic modification and the rapidly increasing genomic and transcriptomic resources hold much promise for advanced understanding of this key process.

Keywords

Life cycle · Diatoms · Sexual reproduction · Pheromone

Abbreviations

AFLP	Amplified fragment length polymorphism
BBS	Bardet-Biedl Syndrome
CDPS	Cyclodipeptide synthase
cGMP	Cyclic guanosine monophosphate
EGF	Epidermal growth factor
GC	Guanylate cyclase
GPCR	G-protein coupled receptor
IFT	Intraflagellar transport
m/z	Mass/charge ratio
MMETSP	Marine Microbial Eukaryote Transcriptome Sequencing Project
MR	Mating type related
MT-	Mating type minus
MT+	Mating type plus
NOX	NADPH oxidase
PDE	Phosphodiesterase
RP-UPLC	Reverse phase ultra-performance liquid chromatography
Sig	Sex induced gene
SIP	Sex inducing pheromone
SNP	Single-nucleotide polymorphism
SST	Sexual size threshold
TF	Transcription factor

1 Introduction: Basic Features of the Diatom Life History

Sexual reproduction can be traced back to the last eukaryotic common ancestor and is now widespread in eukaryote lineages, accompanied by the evolution of highly diverse life cycle strategies (Speijer et al. 2015). Unicellular eukaryotic organisms sometimes have complex heteromorphic life histories that include stages that differ in function, morphology and physiology. Unlike most other microalgae, diatoms have a diplontic life cycle. Their life history consists of a long vegetative phase in which the mitotically dividing cells are diploid, and a comparatively short sexual phase in which the short-lived gametes are haploid (Chepurnov et al. 2004). A

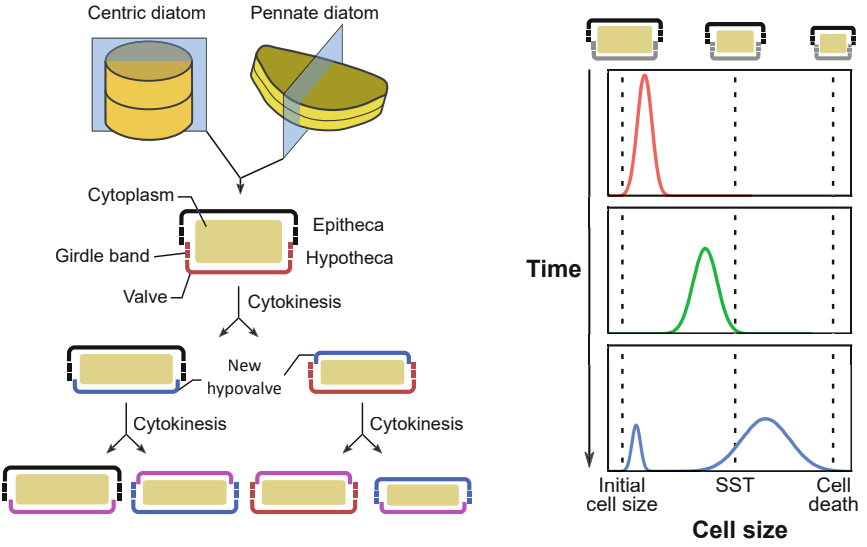

Fig. 1 The MacDonald-Pfitzer rule. Left: schematic depiction of cell size decline over successive mitotic cell divisions. Each daughter cells synthesizes a new hypovalve, resulting in population cell size decline over several generations. Right: evolution of cell size over time for a hypothetical diatom population. A population that has recently descended from initial cells contains only large cells, which gradually get smaller until the sexual size threshold (SST) is reached. In the bottom panel, some cells below the SST have reproduced sexually to avoid cell death by miniaturization. As a result, initial cells have been formed, originating a new population of large cells on the left. We assume that cell size distributions are Gaussian (Hense and Beckmann 2015)

unique feature of the life cycle of diatoms is the gradual reduction of the mean cell size of a clonal population as a consequence of repeated rounds of mitotic divisions, referred to as the MacDonald-Pfitzer rule (Fig. 1) (Macdonald 1869; Pfitzer 1869). Cell size decrease is linked to cell division, producing two slightly unequal daughter cells due to the peculiar architecture of the siliceous cell wall (frustule) and the formation of new valves inside the confines of the parent valves. Restoration of the initial cell size by the formation of an enlarged zygote called the auxospore is the most common mechanism to avoid critical miniaturization and eventually cell death (Mann 2011). A decrease of the cell size below a species-specific sexual size threshold (SST) is the primary requirement for sex to occur. Cell-size dependency of sexual reproduction is regarded as one of the main 'rules' to which the majority of diatom life cycles conform (Chepurnov et al. 2004). Spontaneous and experimentally induced abrupt cell size reduction demonstrates that sexualization is size-dependent rather than based on population age or the number of cell divisions (Chepurnov et al. 2004).

Sexual reproduction has beneficial consequences such as the generation of genetic variation by recombination and the prevention of accumulating disadvantageous mutations (Lewis 1984; Speijer et al. 2015). Meanwhile, sex is associated with

substantial short-term costs, one of them being the delay in mitotic growth during the period of mate finding and meiosis (Lehtonen et al. 2012). This trade-off explains the almost universal occurrence of intermittent sexuality in unicellular eukaryotes (Lewis 1984). In diatoms, this takes the form of an endogenous cell size clock that causes an alternation between sexual reproduction and long intervals of vegetative growth, which can span several years (Lewis 1984; Mann 2011). The large temporal separation between sexual events could explain the rarity of sexual stages observed in natural conditions (Montresor et al. 2016). Furthermore, several diatom species do not show a cell size decrease when dividing, and others are able to restore their cell size asexually (Mann 2011; Kaczmarska et al. 2013). Taken together, diatom cell size decrease might be an adaptation to balance the frequency of vegetative and sexual divisions to optimize the cost-benefits for sexual reproduction rather than an inherent consequence of the diatom cell wall architecture (Lewis 1984; Mann 2011).

Several diatom species are known to form resting stages, including spores or resting cells. While the former are morphologically distinct from vegetative cells, resting stages have valves that are indistinguishable compared to their vegetative counterparts, but they are characterized by a condensed protoplast (Kaczmarska et al. 2013). Resting stages have a strongly reduced metabolism and were shown to successfully germinate even after >100 years of dormancy (Härnström et al. 2011; Kaczmarska et al. 2013). They may accumulate in the sediments, constituting a reservoir analogous to the seed banks of higher plants, and are implicated in the initiation of planktonic diatom blooms (McQuoid 2002).

Molecular and functional studies of the different life cycle phases are less advanced compared to other aspects of diatom biology. This is mainly due to the historical focus on the traditional model species *Phaeodactylum tricornutum* (pennate) and *Thalassiosira pseudonana* (centric). Both species lack a cell size reduction–restoration cycle, and there are no conclusive observations of sexual reproduction despite recent indirect indications from both species that sex might be possible (Moore et al. 2017; Koester et al. 2018; Mao et al. 2020). However, the advent of next-generation sequencing technologies has spurred research on the mechanisms regulating diatom life cycles also thanks to experimental protocols developed to control life cycle progression. The availability of de novo genome sequences, transcriptomic resources and an increasing number of genomic tools now allows to explore the mechanisms underpinning transitions between the various stages and to investigate the transmission and perception of biotic and abiotic signals that regulate the life cycle of diatoms. In this chapter we outline the main features of the diatom life cycle stages, focusing on recent contributions by molecular approaches. Readers interested in the general biology of diatom life cycles can refer to various review papers (Chepurnov et al. 2004; Davidovich et al. 2015; Mann 2011; Montresor et al. 2016; Poulíčková et al. 2019) and a recent work proposing a unified terminology for diatom life cycle stages (Kaczmarska et al. 2013).

2 Sexual Reproduction

2.1 Sexual Strategies and Genetic Model Organisms

While processes of cell size decrease and auxosporulation are conserved among most diatoms, their sexual behavior is highly diverse (Chepurnov et al. 2004). With some exceptions, mating strategies largely coincide with the main morphological groups of centric, araphid pennate and raphid pennate diatoms (Chepurnov et al. 2004) (Fig. 2). Most centric diatoms display oogamy, in which large egg cells are fertilized by small, flagellate spermatozoa. In general, centric reproduction is homothallic (self-fertile), i.e., there are no genetically defined mating types and clonal cells can differentiate into egg or sperm cells. The evolutionary younger group of pennate diatoms, on the other hand, are often heterothallic and sexual reproduction requires a partner of the compatible mating type. An exception to these "rules" is *Ardissonea crystallina*, a toxariid centric diatom that exhibits no oogamy and engages in both homothallic and heterothallic reproduction involving two mating types (Davidovich et al. 2017). A key difference of centric and araphid pennate diatoms with raphid pennates is that a transfer of function from gametes to the gametangia has occurred. In raphid diatoms, gametangia from opposite mating types interact and form a mating pair whose formation will initiate gametogenesis. The most studied raphid pennate species produce morphologically indistinguishable gametes (isogamy), while the phylogenetically older group of araphid pennates are characterized by non-flagellate motile male gametes and larger immobile female gametes (anisogamy). Depending on the species, one or two gametes are produced per gametangium, leading to the formation of one or two auxospores, respectively.

The centric *Thalassiosira weissflogii* is the oldest genetic model organism for sexual reproduction in diatoms (Armbrust 1999). It displays an archetypical centric life cycle characterized by homothallic and oogamous reproduction (Fig. 2). Spermatogenesis can be reliably induced in cultures below the SST by release from a dark period (Vaulot and Chisholm 1987). Although all sexual stages necessary for sexual size enlargement have been observed (Chepurnov 2019), the species can also increase its cell size by vegetative enlargement (Von Dassow et al. 2006).

The centric model diatom *Skeletonema marinoi* is a chain-forming diatom, playing an important role in phytoplankton blooms and forming benthic resting stages that stay viable for over 100 years (Härnström et al. 2011) (Fig. 2). Sperm cells and oogonia can be induced in cells below the SST by an increase in salinity, offering a high level of control into sexual reproduction in laboratory cultures. The sexual phase elapses according to the default pathway of centric diatoms and results in the formation of an auxospore, although evidence suggests that large cell size can also be restored by vegetative (clonal) auxospore formation (Godhe et al. 2014) (Fig. 2). A genetic transformation protocol has recently been developed, opening possibilities for further validation of putative life cycle and sexual genes using reverse genetic techniques (Johansson et al. 2019).

Model species of raphid pennate diatoms include two marine species with a different lifestyle: the planktonic *Pseudo-nitzschia multistriata* and the benthic

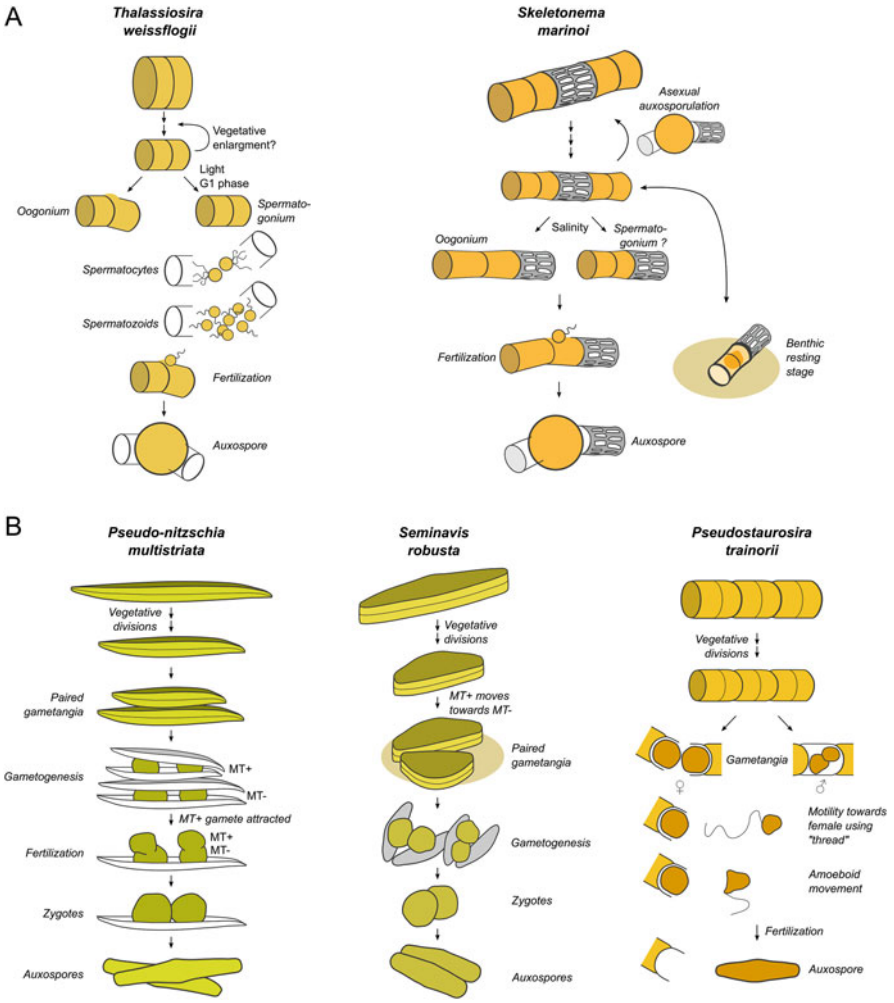

Fig. 2 Schematic life cycle of genetic model diatom species for sexual reproduction. (**a**) Centric diatoms. Left: *Thalassiosira weissflogii,* right: *Skeletonema marinoi*. *T. weissflogii* presumably uses vegetative enlargement to partially restore cell size, while the chain-forming *S. marinoi* can form asexual auxospores to restore cell size. *S. marinoi* additionally forms long-lived resting stages in benthic sediments, which are characterized by a thick frustule and condensed cytoplasm. Environmental cues activating sexualization in each species are indicated next to the arrow pointing towards gametangia. *T. weissflogii* drawings are based on microscopic pictures from Von Dassow et al. (2006) and Chepurnov (2019), while for *S. marinoi* they are based upon Godhe et al. (2014). (**b**) Pennate diatoms: Left: the planktonic raphid *Pseudo-nitzschia multistriata* in oblique girdle view, center: the benthic raphid *Seminavis robusta* and right: the araphid *Pseudostaurosira trainorii*. Thin lines represent the border between thecae. Drawings are based on microscopic pictures from D'Alelio et al. (2009b), Scalco et al. (2016), Chepurnov et al. (2002, 2008), Sato et al. (2011), respectively

Seminavis robusta. Their sexual cycles are largely analogous, consisting of heterothallic gametangia that form mating pairs and each gametangium releasing two gametes that fuse to form zygotes (Fig. 2) (Chepurnov et al. 2002; D'Alelio et al. 2009b). The zygotes expand to form elongated auxospores from which initial cells will hatch. While in *S. robusta* the MT+ gametangium is attracted towards the MT- gametangium, behavioral differentiation in *P. multistriata* takes place at the level of gametes, with MT+ gametes moving towards their MT- counterparts confined to the gametangial frustule (Fig. 3) (Gillard et al. 2013; Scalco et al. 2016). Gliding of *P. multistriata* gametangia to form mating pairs has been observed in petri dishes, but it is uncertain how cells pair in planktonic conditions, although a model has recently been proposed where collective sinking promotes passive cell pairing of planktonic diatoms (Scalco et al. 2016; Font-Muñoz et al. 2019). In both pennate species, the sexual process can be easily followed by mixing sexually compatible strains below the SST. Additionally, genome and transcriptomic datasets are available for both species (Basu et al. 2017; Osuna-Cruz et al. 2020), and *P. multistriata* can be transfected through biolistic transformation (Sabatino et al. 2015). Meanwhile, the raphid pennate species *Cylindrotheca closterium* meets the requirements to become an additional model species to study life cycle regulation, including a high growth rate, the possibility of experimental cell size reduction by cell cutting to shorten generation time, frequent crossing in culture conditions, pheromone attraction assays and the availability of a draft reference genome and efficient genetic transformation protocol (Vanormelingen et al. 2013; Klapper et al. 2021; Belišová and Audoor et al. pers. comm.).

The genetic regulation of the life cycle in araphid pennate diatoms is less studied, despite their interesting intermediate position between the oogamous centrics and the isogamous raphid pennate diatoms. A promising candidate model organism is the chain-forming araphid diatom *Pseudostaurosira trainorii,* the first diatom for which the existence of sex pheromones was demonstrated in a laboratory setting (Sato et al. 2011)**.** *P. trainorii* shows anisogamous heterothallic reproduction, with each mating type producing morphologically different gametes: motile male gametes that move towards the immotile female gametes using unique "threads" and amoeboid gliding (Sato et al. 2011) (Fig. 2).

2.2 Sex Determination in Diatoms

In centric diatoms, genetic sex determination is absent as is evident from their homothallic mating system, with male and female gametangia originating from the same clonal population (Davidovich et al. 2015). Since environmental cues are major triggers for the induction of sexual reproduction in centrics, environmental sex-determining systems are possibly at play to determine the male or female development of gametangia. Interestingly, in numerous centric species the ratio of male and female gametangia is dependent on cell size, with larger mother cells differentiating into eggs more frequently (Chepurnov et al. 2004). The size dependence of sex determination is likely related to eggs being much larger than sperms

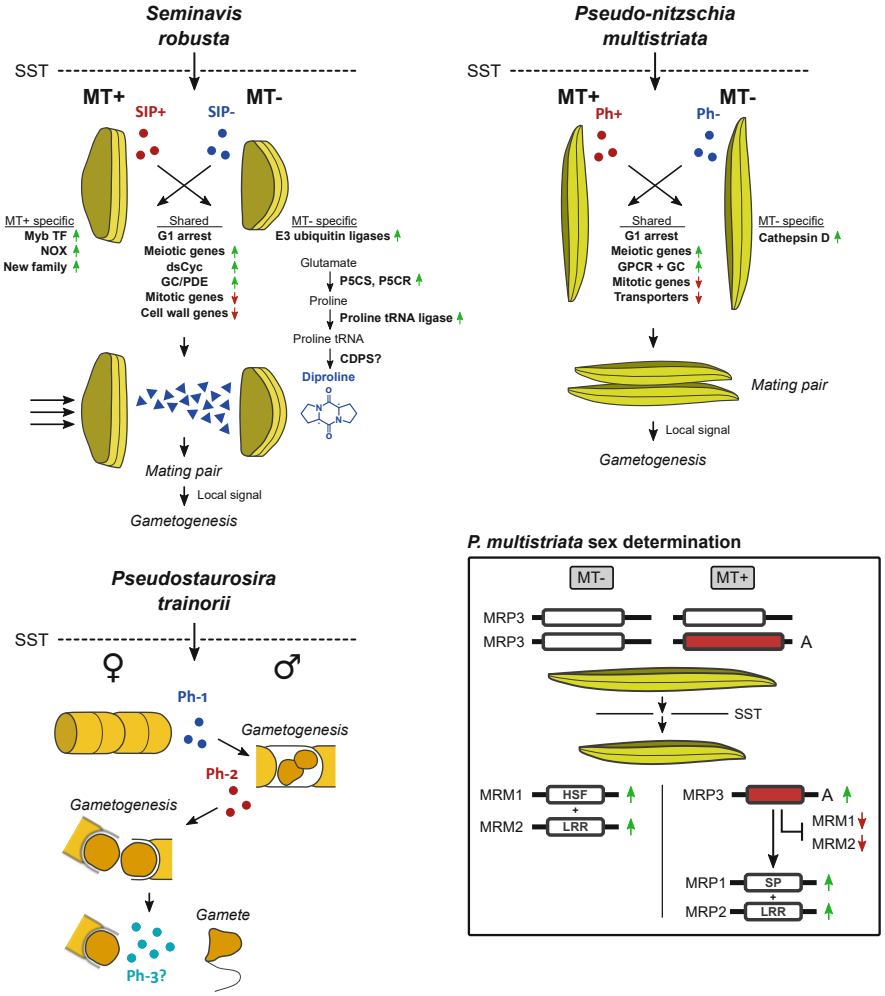

Fig. 3 Pheromone signaling and sex determination in pennate diatoms. Known and putative sex pheromones are shown in red (from mating type + or male) and blue (from mating type – or female). Transcriptomic responses to the presence of sex pheromones is shown with arrows (green = upregulated, red = downregulated). Ph+/− = putative pheromone from mating type + and – respectively, SIP = sex inducing pheromone, GPCR = G-protein coupled receptor, GC = guanylate cyclase, GC/PDE = guanylate cyclase/phosphodiesterase fusion gene, TF = transcription factor, NOX = NADPH oxidase, P5CS = Δ1-pyrroline-5-carboxylate synthetase, P5CR = Δ1-pyrroline-5-carboxylate reductase and CDPS = cyclodipeptide synthase. *Inset:* sex determination in *P. multistriata*. A = alto ("high") allele of the sex determining gene MRP3. SST = sexual size threshold. Gene with active expression are indicated with a green arrow, while a red downwards arrow signifies downregulation and a flat-ended line indicates inhibition. Predicted protein domains are mentioned within boxes, with HSF = heat shock factor DNA-binding domain, LRR = leucin-rich repeat receptor-like domain, and SP = endoplasmic reticulum signal peptide

and the observation that initial cell size is often correlated with the size of the parental oogonium (Chepurnov et al. 2004; Davidovich 1994; Shirokawa and Shimada 2013).

The transition to heterothally has been accompanied by the development of genetic sex determination systems in pennate diatoms. Sex-determination in pennate diatoms seems to be driven by a single sex locus for which one mating type is generally heterozygous (Davidovich et al. 2015; Russo et al. 2018; Vanstechelman et al. 2013). The existence of a sex locus is corroborated by an MT+:MT- ratio of about 50:50 in experimental crosses (Amato et al. 2007; Vanstechelman et al. 2013; De Decker et al. 2018; Russo et al. 2018). The first efforts towards identifying a genetic sex-determining locus have been made in *Seminavis robusta* (Vanstechelman et al. 2013). Amplified Fragment Length Polymorphism (AFLP, a restriction digest-based DNA fingerprinting technique) linkage mapping showed that mating type phenotype cosegregated with one MT+ specific linkage group, identifying the sex determining region as a single locus and pinpointing MT+ as the heterogametic sex (Vanstechelman et al. 2013). Recent comparative transcriptomic evidence identified several *S. robusta* genes exhibiting a mating type-specific expression in vegetative conditions, including an MT+ specific uncharacterized gene family and a Myb transcription factor (Bilcke et al. 2021a), although their expression pattern will need to be investigated in multiple other strains to confirm a role in mating type determination.

In the pennate diatom *Pseudo-nitzschia multistriata,* a simple sex determining mechanism has been elucidated (Fig. 3) (Russo et al. 2018). Using comparative transcriptomics, Russo and colleagues identified five mating type-related (MR) genes that show mating type-specific expression (three MT+ and two MT- specific genes). One of these genes, *MRP3* (MR Plus 3), was always expressed in MT+, with the longest ("A") MRP3 allele always segregating with this mating type. Below the SST, the A allele of the *MRP3* gene was expressed exclusively in MT+ in a monoallelic manner. When transformed into an MT- strain, expression of *MRP3* induced sex reversal, evident from the production of sexual stages when crossed with MT- strains as well as from the transcriptional inhibition of MT- specific genes *MRM1* and *MRM2* and activation of MT+ genes *MRP1* and *MRP2*. Thus, the mating type identity in *P. multistriata* is determined by the presence of the A allele of *MRP3* in the heterogametic mating type MT+, which is expressed below the SST and acts as a master regulator that activates downstream MT+ genes while inhibiting MT- determinants (Fig. 3). Homologs of *MRP3* and the other sex-specific genes are restricted to the genomes of the phylogenetically related genera *Fragilariopsis* and *Pseudo-nitzschia,* suggesting that multiple independent sex-determining systems exist in pennate diatoms (Russo et al. 2018).

2.3 Induction of the Sexual Phase

2.3.1 Induction by Environmental Factors

Available experimental evidence suggests that chemical communication between gametangial cells is not necessary to induce meiosis and gamete formation in centric diatoms, considering the fact that a clonal cell line is able to subsequently produce eggs and sperm cells as vegetative cell size decreases (Shirokawa and Shimada 2013). Additionally, the vegetative fraction of cells in the centric diatom *S. marinoi* does not exhibit a growth arrest under sex-inducing conditions, which may point towards the absence of sex inducing pheromones (Ferrante et al. 2019). Rather, spermatogenesis and/or oogenesis in centrics with a cell size below the SST can be induced by a shift in species-specific environmental conditions such as salinity, temperature, irradiance, photoperiod and ammonium concentration (Amato 2010; Godhe et al. 2014; Moore et al. 2017). Molecular studies on environmental induction of sexual reproduction have focused on the centric *S. marinoi.* A shift in salinity induces sexual reproduction in this species, which was confirmed using microsatellite analysis of the parental and the offspring strains (Godhe et al. 2014). Additionally, Ferrante and colleagues performed transcriptomic analysis on salinity-treated cultures of this species and observed an upregulation of meiotic and other conserved sexual genes (Ferrante et al. 2019). It is of note that background expression of flagella synthesis genes was observed in non-induced cultures of several centric diatom species, suggesting that gametogenesis might continuously occur at a low frequency in vegetative conditions in centric diatoms (Nanjappa et al. 2017). It is imperative to consider that environmental requirements need to be fulfilled in pennate diatoms as well before sexual reproduction can occur. For example, sexual reproduction of the pennate *Pseudo-nitzschia multistriata* was most successful when cultures were in the exponential growth phase (Scalco et al. 2014). Sexual reproduction does not occur in the dark in *Haslea ostrearia* and *S. robusta*, in the latter species potentially through inhibition of sex pheromone biosynthesis (Mouget et al. 2009; Gillard et al. 2013). Gametogenesis was maximal at a photon flux density of 96 μE m^{-2} s^{-1} in *Nitzschia lanceolata*, but the highest level of auxosporulation in *H. ostrearia* and *S. robusta* was observed at intensities below 40 and 27 μE m^{-2} s^{-1} respectively (Davidovich 1998; Mouget et al. 2009; Bilcke et al. 2021b). In addition, sexual reproduction was highly dependent on the presence of red light in *H. ostrearia* and blue light in *S. robusta* (Mouget et al. 2009; Bilcke et al. 2021b). Finally, the physiological state of many pennate diatoms needs to be excellent before cells can become sexualized (Amato 2010).

2.3.2 Sex Inducing Pheromones

While sexual propagation of centric diatoms appears to depend on environmental stimuli, the heterothallic pennate diatoms must find a partner of the opposite mating type. In recent years, it has become clear that pennate diatoms use sex pheromones to recognize potential mating partners. Once the SST is reached, each mating type of the raphid pennate diatoms *S. robusta* and *P. multistriata* start secreting unique sex inducing pheromones (SIPs) that signal the presence of a suitable partner (Fig. 3)

(Moeys et al. 2016; Basu et al. 2017). In *S. robusta*, an active fraction containing SIP secreted by MT- was purified using RP-UPLC, followed by mass spectrometric analyses that resolved some of its features, including the presence of at least one sulfur atom and a relatively large molecular weight compound with a mass charge ratio (m/z) of 842 (Moeys et al. 2016). In *P. multistriata,* metabolomics identified several mating type-specific metabolites, but since the experiment consisted of only one strain per mating type, additional evidence is necessary to identify pheromones (Fiorini et al. 2020).

Sex inducing pheromones trigger a number of responses that are conserved between *S. robusta* and *P. multistriata* (Fig. 3). Flow cytometry showed an induction of a temporary arrest in the G1 phase of the cell cycle, which delays commitment to the meiotic or mitotic cell cycle until successful mating pair formation or a failure thereof, respectively (Moeys et al. 2016; Basu et al. 2017; Bilcke et al. 2021a). *C. closterium* also employs a cytostatic sex pheromone, although pheromone production appears to be restricted to MT-, suggesting that cell cycle arresting pheromones are widespread among raphid pennate diatoms (Klapper et al. 2021). On a molecular level, the cell cycle arrest is exemplified by a downregulation of the expression of mitotic cell cycle markers and, in *S. robusta,* genes for silica cell wall synthesis that are associated with cytokinesis (Moeys et al. 2016; Basu et al. 2017; Bilcke et al. 2021a). Endometabolomics in *P. multistriata* demonstrated that the concentration of the chlorophyll precursor phytol strongly decreased during sexual reproduction, suggesting a more general metabolic arrest in addition to the growth arrest (Fiorini et al. 2020). On the other hand, meiotic genes such as RAD51 and MRE11 are induced by SIPs, which is intriguing given that meiosis and gametogenesis do not take place until a mating pair is formed, indicating that mate finding and meiotic genetic programs are not strictly temporarily separated (Basu et al. 2017; Bilcke et al. 2021a; Moeys et al. 2016). Strikingly, a diatom-specific cyclin was found upregulated rather than downregulated in *S. robusta*, suggesting a specialized role for this cyclin in the response to sex pheromones (Bilcke et al. 2021a). Another conserved genetic response to SIPs in both *P. multistriata* and *S. robusta* is the induction of genes containing a guanylate cyclase domain that are involved in the production of cyclic GMP (cGMP), suggesting a general role for signalling through this secondary messenger during sexual reproduction of pennate diatoms. In *P. multistriata* a sexual soluble (non-membrane bound) guanylate cyclase was identified, while in *S. robusta* several membrane bound proteins were upregulated that are also carrying a cGPM breakdown phosphodiesterase domain (Moeys et al. 2016; Basu et al. 2017; Bilcke et al. 2021a). In both species, transcriptomic studies have dissected asymmetric signalling pathways associated with mating type-specific responses to the pheromones. In *P. multistriata,* a cathepsin D protease showed a seven-fold increase in MT- cells, which may play a role in pheromone degradation (Basu et al. 2017). In *S. robusta*, on the other hand, MT+ showed a unique response of superoxide producing NADPH oxidase, as well as a Myb transcription factor that may regulate downstream MT+ specific responses (Bilcke et al. 2021a). Meanwhile, MT- showed specific upregulation of several genes in the biosynthetic pathway of the sex pheromone diproline (see Sect. 2.4).

Also for the araphid pennate *P. trainorii*, sex inducing pheromones have been identified (Sato et al. 2011). In contrast to the raphid pennates, there is a sequential sexualization, with the MT- producing a pheromone called ph−1 when cell size drops below the SST, while the MT+ only produces its pheromone ph−2 after perceiving ph−1 (Fig. 3). In contrast to the raphid pennates studied so far, sex inducing pheromones in *P. trainorii* directly induce meiosis and gametogenesis, followed by a motile MT+ gamete moving towards the female gametes (Fig. 3). In raphid species, gamete formation only takes place following pairing of gametangial cells.

2.3.3 Cell Cycle Dependency of Sexual Induction

The exit from the mitotic cell cycle and entry into meiosis in diatoms appears to be restricted to the G1 phase of the cell cycle (see Chap. "Cellular Hallmarks and Regulation of the Diatom Cell Cycle"), before meiosis is induced. Early studies showed that the transition to gamete formation is confined to the G1 phase in the centric *T. weissflogii* (Armbrust et al. 1990) as well as in the raphid pennate *N. lanceolata* (Davidovich 1998). Similarly, a G1 phase arrest induced by sex inducing pheromones in *S. robusta* and *P. multistriata* supports its role as a decision point for mitotic versus meiotic cell division (Moeys et al. 2016; Basu et al. 2017). The positioning of this sexual checkpoint in the G1 phase is analogous to many eukaryotic species, and it allows the cell to decide whether to proceed through a mitotic or meiotic S-phase (replication).

2.4 Mate Finding

Before fertilization can occur, cells must be in close proximity to each other. For each of the diatom morphological types, specialized mechanisms are likely present that allow compatible cells to get into close proximity (Mann 2011).

Egg cells or oogonia of centric diatoms are thought to produce attraction pheromones to allow sperm cells to move towards egg cells (Chepurnov et al. 2004). The existence of such pheromones has not been experimentally demonstrated, although the centric *Skeletonema costatum* is known to produce the brown algal pheromone ectocarpene when placed in gamete inducing conditions (Derenbach and Pesando 1986). In the araphid pennate diatom *P. trainorii,* male gametes become amoeboid when in the vicinity of the female gametangium and actively move towards it, suggesting an additional female pheromone that was provisionally called ph−3 (Fig. 3) (Sato et al. 2011). In the araphid *Tabularia fasciculata*, however, movement of male gametes in the proximity of female gametangia is non-directional, so a chemical cue for attraction seems absent (Edgar et al. 2014). SIP-treated MT+ gametangia of the pennate *S. robusta* actively move towards MT−, suggesting that MT− acts as an attractor (Fig. 3). Mass-spectrometry of the exometabolome identified the attraction pheromone as L-diproline, a diketopiperazine consisting of two proline moieties (Gillard et al. 2013). Diproline attraction bioassays were subsequently used to investigate

back-and-forth motility of MT+ towards diproline, and showed that very small cells can be attracted towards diproline regardless of previous conditioning by SIP- (Bondoc et al. 2016, 2019). Comparative transcriptomic analysis between the mating types largely elucidated the biosynthetic pathway of diproline. Enzymes catalysing the interconversion of glutamate to proline (Δ1-pyrroline-5-carboxylate synthetase and Δ1-pyrroline-5-carboxylate reductase) are upregulated uniquely by SIP+ in MT-, satisfying the increased need for proline in this mating type (Moeys et al. 2016; Bilcke et al. 2021a). A similar MT- restricted response of a proline-tRNA ligase pinpoints tRNA-attached proline as the substrate for diproline synthesis, suggesting that the final reaction is carried out by a cyclodipeptide synthase (CDPS) (Fig. 3) (Bilcke et al. 2021a). No attraction pheromone has been described for the planktonic *P. multistriata* and it is unclear how compatible cells encounter each other in the water column to form mating pairs, although observations show that *P. multistriata* undergoes sex more vigorously in non-turbulent conditions (Scalco et al. 2014).

In both *P. multistriata* and *S. robusta*, meiosis and gametogenesis only take place after successful pairing, indicating that an unknown local signal is interchanged between paired cells (Fig. 3) (Moeys et al. 2016; Scalco et al. 2016). Additionally, *P. multistriata* gametes belonging to MT+ move locally within the mating pair and merge with MT- gametes inside their maternal frustule, suggesting local attraction or recognition (Scalco et al. 2016).

2.5 Gametes, Zygote Formation and Auxospores

2.5.1 Meiosis, Gametogenesis and Gamete Fusion

Diatom gametangia undergo meiosis in order to form haploid gametes (Amato 2010). Meiosis is an ancestral mechanism in eukaryotes, and a conserved set of meiotic genes can be traced back to the last eukaryotic common ancestor (Ramesh et al. 2005; Goodenough and Heitman 2014; Speijer et al. 2015). Homologs of 42 eukaryotic genes involved in meiotic DNA replication, chromosome maintenance and recombination were identified across six diatom species, one centric (*T. pseudonana*) and five pennate diatoms (*P. tricornutum, F. cylindrus, P. multistriata, P. multiseries* and *S. robusta*) (Patil et al. 2015). Expression profiling in the sexual species *S. robusta* confirmed an increase in transcription during gametogenesis for most genes (Patil et al. 2015). Five of these genes play a role exclusively in meiosis, among which is the double-strand break inducing SPO11-2 that plays a central role in meiotic recombination. SPO11-2 was strongly upregulated in sexualized *S. marinoi, P. multistriata* and *S. robusta,* making it a valuable marker gene to detect sexual reproduction in culture (Patil et al. 2015; Moeys et al. 2016; Ferrante et al. 2019). Strikingly, SPO11-3/TOP VIA, a paralog of SPO11-2 that is involved in vegetative growth in plants, was upregulated (although to a lesser extent than SPO11-2) during mating in both *S. robusta* and *S. marinoi* suggesting a sexual function in diatoms, although no such response was visible in *P. multistriata* (Patil et al. 2015; Ferrante et al. 2019; Bilcke et al. 2021a).

Interestingly, most of the meiotic toolbox (Patil et al. 2015) has been retained in the genomes of *P. tricornutum* and *T. pseudonana*, suggesting that these presumed asexual species do have the potential for sexual reproduction or that these genes have become neo-functionalized. Some genes that are widely involved in meiosis in other eukaryotic groups could not be identified in diatom genomes, including the meiotic recombination proteins SPO11-1 and Hop2, although a putative Hop2 protein domain was recently predicted in a sexually induced gene in *S. robusta*, *P. multistriata* and *S. marinoi* (Patil et al. 2015; Ferrante et al. 2019; Bilcke et al. 2021a). Notably, the cyclin A/B gene family was enriched in genes upregulated during three stages of sexual reproduction in *S. robusta*, suggesting that cyclins play a regulatory role during meiosis (Osuna-Cruz et al. 2020).

Transcriptomic studies of gametogenesis have mainly focused on sperm production in centric diatoms. The first effort was made in *T. weissflogii* cultures where about 40% of cells differentiated into male gametes after release from a prolonged dark arrest (Armbrust 1999). The set of genes upregulated under these conditions contain a gene family of polypeptide sexually induced genes holding three members (Sig1-3) that are characterized by epidermal growth factor (EGF)-like repeats (Armbrust 1999). Later research showed that homologs of Sig proteins are present in the mastigoneme hairs on the flagella of stramenopiles (Honda et al. 2007). Thus, the upregulation of Sig genes during *T. weissflogii* spermatogenesis can be linked to flagella biosynthesis. The presence of cysteine-rich EGF-like repeat motifs in the Sigs may suggest that mastigonemes in centric diatom sperm have a function in partner adhesion or recognition (Fig. 4) (Honda et al. 2007). Sequencing of Sig1 homologs across the genus *Thalassiosira* showed high sequence divergence, which may explain reproductive isolation if these proteins are indeed involved in cell–cell recognition (Armbrust and Galindo 2001). A more recent effort described a reference set of 22 genes involved in flagella formation in the transcriptome of the centric *Leptocylindrus danicus* (Nanjappa et al. 2017). Distribution of these genes in the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) dataset indicated that centric diatoms have retained both IFT-A and IFT-B (intraflagellar transport) complexes but lost all BBSome subunits (a protein complex present in the basal body, involved in primary cilium biogenesis, defective in the Bardet-Biedl Syndrome, BBS) of the flagella basal body, which are involved in vesicular trafficking of proteins (Nanjappa et al. 2017). Expression profiling of a selection of six flagellar genes in *L. danicus* during different stages of sexual reproduction showed that high expression is confined to the stage of gametogenesis as sperm cells are the only cell type to carry flagella (Nanjappa et al. 2017). While several IFT-B genes were observed in the *S. marinoi* MMETSP transcriptome (Nanjappa et al. 2017), none of the reference set of 22 flagellar genes was upregulated in this species during salinity shift induced gametogenesis (Ferrante et al. 2019). However, several other flagellar genes with homology to cytoskeleton factors in *Emiliania huxleyi* were upregulated (Fig. 4) (Ferrante et al. 2019). Investigations on the ultrastructure of the flagellum coupled with identification of genes involved in flagellar assembly and intraflagellar transport are needed to shed light on the evolutionary pathways of these

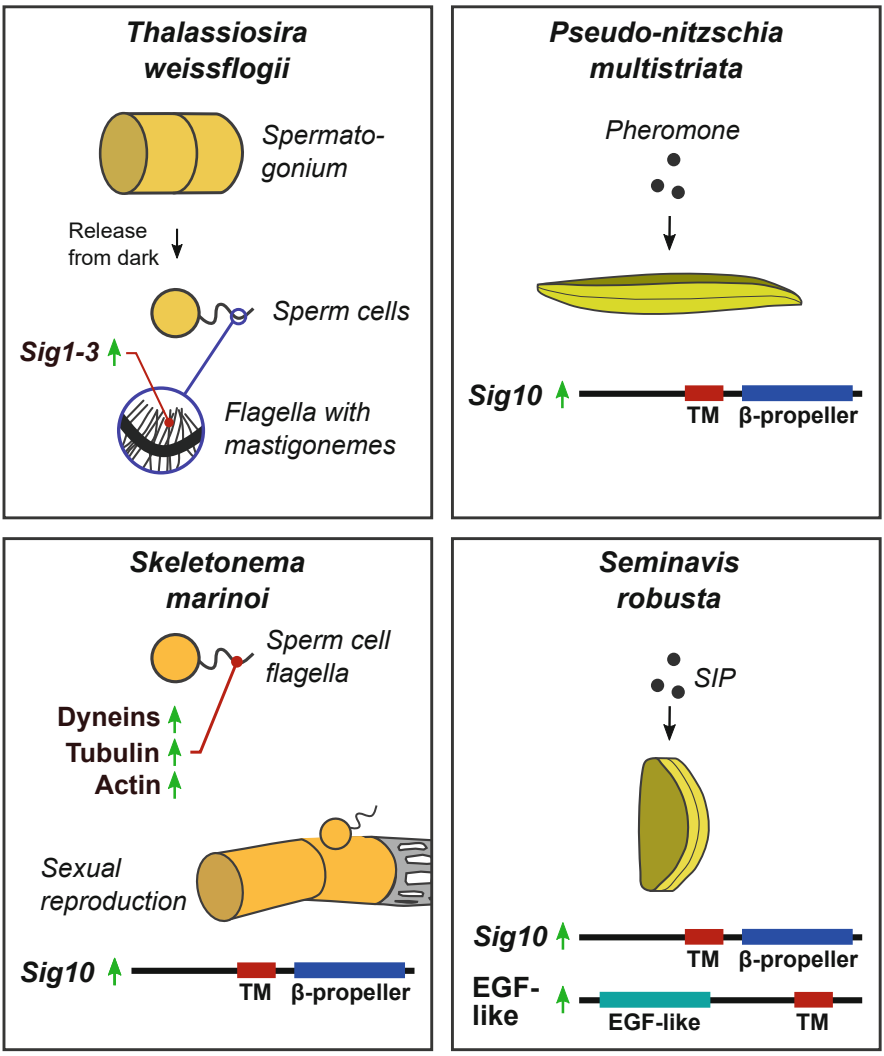

Fig. 4 Cartoon depicting gene expression changes related to spermatogenesis and cell–cell communication in two centric (*T. weissflogii, S. marinoi*) and two pennate diatom species (*P. multistriata, S. robusta*). Green arrows indicate an upregulation of a gene during sexualization. Predicted protein domains are indicated as boxes on the gene backbone (black line). TM = transmembrane domain, EGF = epidermal growth factor

genes in centric diatoms. Pennate diatoms have lost all IFT proteins, consistent with the absence of flagellated stages during their life cycle.

It is hypothesized that pennate diatoms employ local signals to verify the configuration into mating pairs or recognize gametes of the opposite mating type. One candidate for cell–cell recognition is a conserved gene called Sig10 that is induced

after treatment with sex pheromones in *P. multistriata* and *S. robusta* and also during sexualization in the centric *S. marinoi* (Fig. 4) (Basu et al. 2017; Ferrante et al. 2019; Bilcke et al. 2021a). The protein resembles an integrin-like receptor, which typically mediates attachment and signal transduction over the plasma membrane (Bilcke et al. 2021a). Another potential recognition gene that encodes a transmembrane EGF-like protein is strongly transcribed in response to sex pheromones in both mating types of *S. robusta* (Fig. 4) (Bilcke et al. 2021a). Ongoing research shows that four classes of adhesive genes are upregulated during mating in both *P. multistriata* and *S. robusta*, including lectins, EGF-like, fasciclin-like and integrin-like proteins (Annunziata & Bilcke, pers. comm.). Localization and knock-out studies will be necessary to further elucidate their role during gametangia recognition and gamete fusion. Successful gamete fusion in most eukaryotic lineages is mediated by the plasma membrane-associated Hap2 fusogen protein (Wong and Johnson 2010) while the Gex1/Kar5/Brambleberry nuclear membrane protein is involved in nuclear fusion (karyogamy) (Nishikawa et al. 2020). Strikingly, homologs for both genes are missing from the gene repertoire of sexual diatoms *P. multistriata* and *S. marinoi* (Ferrante et al. 2019), suggesting the presence of alternative fusogens or of highly diverged Gex1 and Hap2 genes.

2.5.2 Conserved but as yet Uncharacterized Sexual Genes

A comparison of the set of genes showing upregulation during pheromone signaling in *P. multistriata* and sexual reproduction in *S. marinoi* revealed a set of eight conserved genes with unknown function that were called Sex Induced Genes (Sig4-11), of which six have homologs in the *S. robusta* genome (Ferrante et al. 2019). While most of these genes remain uncharacterized, two have recently been annotated with a putative function: Sig7 is a potential homolog of the meiotic Hop2 gene and Sig10 encodes an integrin-like transmembrane protein (Bilcke et al. 2021a). Additional searches for conserved diatom genes that are uniquely expressed during reproduction will complement this set of genes as general markers for the detection of sexual events in natural conditions. High-resolution sampling combined with (meta)transcriptomic approaches could aid the detection of rare, localized and/or short-lived in situ sexual events. Combined with measurements of environmental parameters, this has the potential to greatly expand our understanding of the frequency of sexual reproduction in natural diatom populations and the environmental conditions favoring it.

2.5.3 Sexual Auxosporulation

Sexual reproduction and cell size restoration involves the formation of a zygote formed by the fusion of two haploid gametes. The zygote then expands into an enlarged auxospore to host the formation of the large-sized initial cell. Although typically auxosporulation follows from a sexual reproduction event, in rare cases asexual auxospores can be produced (Chepurnov et al. 2004). Two specific types of cell wall elements cover the auxospore: the outer organic or silica-containing incunabula elements, and the inner siliceous transverse and longitudinal perizonial bands (Kaczmarska et al. 2013). Despite the auxospore being a unique cell type for

diatoms that likely requires the action of specialized cytoskeletal and cell wall factors, the molecular pathways underlying the differentiation of zygotes into auxospores as well as the formation of initial cells within them remain completely unexplored.

2.6 Molecular Evidence for Sexual Reproduction in Natural Populations

Reports of sexual stages in natural diatom samples are scarce (Assmy et al. 2006; D'Alelio et al. 2010; Holtermann et al. 2010; Sarno et al. 2010), in part due to the relatively short duration of sexual events and the long timespan between them. Genetic and genomic evidence has been used to investigate the occurrence and frequency of sexual reproduction in natural populations of several diatom species.

Genome-wide neutral microsatellite markers have been employed to assess the frequency of sexual recombination in *Ditylum brightwellii* (Rynearson and Armbrust 2004, 2005), *S. marinoi* (Godhe and Härnström 2010; Godhe et al. 2013) and several species of the genus *Pseudo-nitzschia* (Casteleyn et al. 2009b, 2010; Tesson et al. 2014; Ruggiero et al. 2018). More recently, whole genome resequencing has allowed for the detection of gene flow and recombination based on SNP patterns. Indications for ongoing sexual reproduction were found for specific subtypes of *T. pseudonana* (Koester et al. 2018), while high linkage disequilibrium between SNPs across ten accessions of the pennate *P. tricornutum* was interpreted as indicative of prolonged asexual reproduction (Rastogi et al. 2020). Interestingly, there is also evidence for hybridization between distinct diatom lineages as was demonstrated in the genus *Pseudo-nitzschia* by analyzing the internal transcribed spacer region of the ribosomal DNA (ITS-rDNA) and the plastid marker rbcL combined with knowledge of their plastid inheritance (Casteleyn et al. 2009a; D'Alelio et al. 2009a; D'Alelio and Ruggiero 2015). Although the identification of natural hybrids by rbcL genotyping is not possible in *S. robusta* because its uniparental chloroplast transmission (Chepurnov et al. 2002; De Decker et al. 2018), whole genome resequencing of co-existing natural isolates demonstrated repeated and ongoing hybridization throughout the diversification of the *S. robusta* species complex (De Decker, Bilcke et al. pers. comm., Osuna-Cruz et al. 2020). In contrast, analysis of genomic SNPs resulting from ddRAD genotyping of 45 *Fragilariopsis kerguelensis* strains revealed three genotypic variants that lacked signs of ongoing hybridization or introgression, despite the capability of two geographically separated variants to interbreed in laboratory crosses (Postel et al. 2020).

3 Other Life Cycle Stages

3.1 Resting Stages

The formation of resting stages (spores or resting cells) is reported for several diatom species and can be seen as an important process contributing germplasm to the benthic seed bank at the end of a bloom. Nutrient limitation is considered the main factor inducing the transition from vegetative cells to resting stages (McQuoid and Hobson 1996). Recent work on the centric diatom *Chaetoceros socialis* confirmed nitrogen starvation as an effective trigger but showed that spores were also produced at high cell density when nitrogen was not limiting (Pelusi et al. 2020). Moreover, the formation of resting spores can be induced in culture media obtained from healthy as well as from lysed cells, suggesting that a chemical cue is secreted by this species to communicate cell density status (Pelusi et al. 2020). In the same species, infection with the single-stranded RNA virus CsfrRNAV induced massive spore formation (Pelusi et al. 2021). Although qPCR identified viral genetic material in spores, no transmission was apparent after germination (Pelusi et al. 2021). Thus, sporulation appears to be a strategy to evade an infected population and serve as a non-infectious seed bank for the future. To investigate the benthic-planktonic coupling between resting and vegetative cells, resting cells of *S. marinoi* were revived from discrete sediment layers in a fjord spanning over 100 years and were analyzed with eight polymorphic microsatellite loci (Härnström et al. 2011). While populations originating from the fjord were clearly distinct from those of the coastal area, hardly any divergence between older and more recent populations was observed, suggesting that the resting cells provide a stable and locally adapted seedbank to the planktonic vegetative population (Härnström et al. 2011). However, little is known about the molecular mechanisms underlying resting stage formation. At the metabolic level, a sudden shift to anoxic and dark conditions was shown to induce a rapid depletion of nitrate through anaerobic nitrate respiration in *Amphora coffeaeformis*, likely providing energy and building blocks required for the transition to a long term dormant resting stage (Kamp et al. 2011). Mass spectrometric analysis of *S. marinoi* resting cells supplied with isotope-labelled nutrients showed that dormant cultures continuously assimilate sources of nitrogen, which might contribute to their long-term survival and viability (Stenow et al. 2020).

3.2 Chain Formation

Many diatom species can form chains or colonies during the vegetative phase of their life cycle, with implications e.g., nutrient uptake and sinking rates (Pahlow et al. 1997), carbon export (Tréguer et al. 2018) and prey selectivity by grazers. It has been shown that chemical compounds excreted by copepod grazers can induce a significative reduction in chain length in *S. marinoi*, interpreted as a threat evasion trait (Bergkvist et al. 2012; Amato et al. 2018; Selander et al. 2019). Moreover, both chain length and cellular gene expression are affected by oceanic turbulence (Amato

et al. 2017, 2018). A disintegration of chains into freely moving single cells or shorter chains are the first signs of sexualization after mixing strains of compatible mating types in the pennate diatoms *F. kerguelensis* and *Pseudo-nitzschia ssp.* (Chepurnov et al. 2005; Davidovich and Bates 1998; Fuchs et al. 2013).

4 Future Prospects

In recent years, life cycle regulation of diatoms has been receiving increasing interest from a molecular viewpoint, thanks to the introduction of new model species that complement the predominantly asexual model diatoms *P. tricornutum* and *T. pseudonana*. To date most insights stem from transcriptomic and genomics analyses, whereas functional confirmation of the regulatory mechanisms underlying most life cycle traits remain largely lacking. Progress in experimental tools including genetic transformation and genome editing will allow in-depth studies to dissect the function of some of the uncharacterized sexual genes mentioned before. Outstanding questions include the biochemical structure and diversity of sex pheromones, as well as how environmental cues are perceived and integrated to trigger the sexual phase. Another unresolved topic concerns the genetic mechanisms that drive the differences in mating behaviour between the centric, raphid and araphid pennate diatoms, as well as pathways behind resting stage induction and metabolic arrest. Finally, auxospore formation and the size-dependent sex clock of diatoms, two of the major hallmarks of the diatom life cycle, have received little attention from a genetic perspective.

In the meantime, comparative genomics can be applied to scan the available diatom genomes for sex-related genes, while comparative transcriptomic analyses are underway to discover diatom-wide marker genes for sexual reproduction. Such analyses will provide an extensive resource of candidate genes that can be evaluated in metagenomic and metatranscriptomic datasets, allowing to document and quantify the occurrence of sexual reproduction in the natural environment. Especially when combined with in situ environmental data, these studies could provide valuable insights into how sexual processes are constrained by external cues like seasonality, light, temperature, salinity or population density. Finally, the decreasing cost of next-generation sequencing improves the accessibility of extensive whole-genome resequencing and population genomic studies, allowing fine-scale analysis of genotypic population diversity and revealing genome-wide patterns of sexual reproduction and genomic regions under selection. Ultimately, a better understanding of life cycle regulation will provide insight into the importance of different speciation mechanisms in diatoms and help to understand the origins of their astonishing species diversity.

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