Sex determination in Chlamydomonas

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Abstract

The sex-determination system of the unicellular green alga, Chlamydomonas reinhardtii, is governed by genes in the mating-type (MT) locus and entails additional genes located in autosomes. Gene expression is initiated by nitrogen starvation, and cells differentiate into plus or minus gametes within 6 h. Reviewed is our current understanding of gametic differentiation and fertilization, initiation of zygote development, and the uniparental inheritance of organelle genomes.

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1. Introduction

The mating system of Chlamydomonas reinhardtii has been the subject of several reviews that give detailed accounts of particular facets [1–5]. Offered here is an overview that focuses on the molecular genetics of the system and includes recent results from this laboratory.

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2. Evolutionary context

The origins of meiotic sex – the fusion of two haploid gametes of opposite sex to form a diploid zygote, followed by meiosis at some later stage in the life cycle to restore the haploid state – is a controversial topic [6,7]. However, the recent finding of meiosis-related genes in two so-called basal eukaryotes, Giardia [8] and Ostreococcus [9], suggests that meiosis was instantiated at least a billion years ago. Meiosis itself has been remarkably conserved, whereas countless modes of sex determination, fertilization, and patterns of zygote development have evolved in countless sexual lineages since meiotic sex established itself as a core eukaryotic property.
Chlamydomonas reinhardtii, a unicellular green soil alga [10], can be argued to resemble the ancient common ancestor to modern plants, animals, and fungi. It retains the flagellar apparatus subsequently lost by most plants and the fungi; its chloroplast is functionally equivalent to the chloroplasts of green plants; and many of its mating parameters – sexual differentiation in response to nitrogen depletion, formation of a stress-resistant zygote – are found in other lineages. This is not to say that C. reinhardtii mating strategies are the same as the original sexual eukaryotes – the strategies have emerged during evolutionary history – but to say that as we come to understand the molecular basis of C. reinhardtii sexuality, we are likely looking at modern strategies that derive quite directly from core ideas.

3. Life-cycle overview

As life cycles go, the life cycle of C. reinhardtii is exquisitely simple ([10] and Fig. 1). Two of the haploid products of each meiosis inherit a Linkage Group (LG) VI carrying the MT+ locus and two inherit an LG VI carrying the allelic MT− locus. Each product divides mitotically to produce clones of vegetative cells. When environmental nitrogen levels fall below threshold [11], vegetative cells carrying the MT+ locus express genes that allow them to mate as plus gametes, and cells carrying the MT− locus express a different set of genes that allow them to mate as minus gametes. Contact between a plus and a minus gamete initiates a rapid fertilization process that produces a binucleate zygote. During the next hour, the two nuclei fuse and a novel set of zygote-specific genes is expressed, many of which self-assemble as a zygote-specific cell wall that renders the zygote resistant to both freezing and desiccation, the key environmental challenges in its temperate-zone soil habitat. When conditions improve, the dormant zygote initiates meiosis and the four recombinant haploid products resume vegetative growth.

There is one additional option (Fig. 1). Occasionally, a young zygote fails to express the zygote-specific program and, in the presence of restored nitrogen levels, resumes vegetative growth as a stable MT+/MT− diploid. When such cells are starved for nitrogen, they differentiate as minus gametes—that is, minus is dominant to plus [12,13]. Since these rare diploids usually mate with haploid plus partners and the subsequent triploid meioses are unsuccessful, this option is presumably not perpetuated in nature, but it has provided important clues about the mating system in the laboratory.

It is important to note that while this life cycle is seemingly quite different from those of multicellular plants and animals, the commonalities are in fact striking. In all cases it is the diploid, whether multicellular or unicellular, that expresses the key environment-negotiating traits of the organism; the diploid forms only as a consequence of sexual fertilization; and the diploid alone undergoes the meiosis necessary to produce haploid products. Indeed, the vegetative mitotic phase of the C. reinhardtii life cycle can be thought of as functionally equivalent to the production of numerous gametes in the testis/anther/ovary. Hence mating in C. reinhardtii, like mating in multicellular organisms, is a required, and not an optional, feature of the life cycle. That the zygotes of plants and animals go on to divide into multicellular organisms rather than forming dormant spores is a detail with important evolutionary consequences, but a detail nonetheless.

4. Sex-related genes are both MT-linked and autosomal

Genes expressed in gametes fall into the following categories: (1) Some, such as the NSG (nitrogen-starved gametogenesis) genes [14,15] and the GAS (gamete-specific) genes [16,17] are up-regulated with ammonium depletion; they are expressed in both mating types and some are also expressed during vegetative growth; most that have been characterized prove to be involved in adapting the cells to nitrogen-starvation conditions rather than in generating mating phenotypes. (2) Some are expressed in only one mating type, contribute to mating-related traits, and are encoded either in the MT+ or the MT− locus (MT+-linked-sex-related). (3) Some are expressed in only one mating type,
contribute to mating-related traits, and are encoded elsewhere in the genome (autosomal-sex-related). As detailed below, mating type-specific expression of these genes is dependent on MT-encoded information. (4) Some are expressed in gametes of only one mating type but do not appear to function during the gametic stage. Instead, they are sequestered in the gametic cytoplasm and contribute to activation of the zygote-specific program following fertilization. In this sense they are analogous to the “maternal” proteins stored in the eggs of multicellular organisms.

5. The MT loci contain rearrangements and unique genes

The MT locus was mapped to the left arm of LG VI in early genetic studies, and it soon became apparent that the locus was distinctive in showing invariant linkage to a number of additional markers, the hallmark of recombinational repression [18]. Identification of a RFLP displaying such linkage permitted a chromosome walk through contiguous sequences in both plus and minus chromosomes [19].

Our current understanding of the MT+ and MT− loci is shown in Fig. 2. Several large inversions and translocations characterize the region, presumably contributing to recombinational suppression. This central rearranged (R) domain is flanked by centromere-proximal (C) and telomere-proximal (T) sequences that also fail to recombine, the extent of recombinational suppression extending ~1 Mb. Recombinational suppression proves to characterize mating-type and self-incompatibility loci in many diverse lineages (reviewed in [20]), in some cases due to chromosome rearrangement [21–23], in others to epigenetic mechanisms [24].

In addition to rearrangements, the MT+ R domain (Fig. 2) contains three DNA regions (a–c) not found in the MT− locus, as well as a block of two tandemly reiterated genes: Ezy2, whose expression is confined to the zygote [25], alternating with OTU2 that is expressed exclusively in plus gametes (Joo, Goodenough, and Lee, unpublished). Reciprocally, the MT− locus contains three regions (d–f) not found in the MT+ locus. Genes resident in regions c (FUS1), d (MTD1) and f (MID) have been assigned MT-specific functions in gametogenesis and mating, as detailed below. The presence of genes without homologues also characterizes MT loci in other lineages [22,26,27] and, of course, the sex chromosomes of vertebrates and some invertebrates (reviewed in [28,29]). The consensus view is that recombinational suppression serves to assure that MT-linked or sex-chromosome-linked genes dependent on one another for expression or for generating sexual phenotypes will be co-inherited [20].

6. The plus and minus sexual differentiation programs govern three traits

Multicellular organisms display a complex array of traits that distinguish male from female: somatic appearance, mating behavior, hormonal profiles, type of gonad and gamete, and so on. The list is far more modest in C. reinhardtii. (1) Fertilization competence: Gametes express sex-related genes (MT-linked and autosomal) that encode proteins required for recognizing and fusing with gametes of opposite type. (2) Initiation of zygote differentiation: Gametes harbor stored sex-specific transcription factors that form heterodimers when they encounter one another in the common zygotic cytoplasm; the heterodimers then move into the nuclei, initiate transcription of zygote-specific genes, and turn off expression of fertilization-related genes. (3) Uniparental inheritance of organelle genomes: Although C. reinhardtii gametes are of equal size and contribute equal numbers of mitochondrial and chloroplast chromosomes to the zygote, meiotic progeny overwhelmingly inherit mitochondrial genomes derived from the minus gamete and chloroplast genomes derived from the plus gamete.

Our current understanding of how each of these traits is instantiated is described in the following sections.

6.1. Fertilization competence: genetic regulation

In the laboratory, mating is initiated by mixing together cultures of mature plus and minus gametes. These immediately adhere to one another via plus and minus agglutinin glycopro-
teins, displayed on their flagellar surfaces, the result being the formation of large clumps of cells. Soon, however, plus/minus pairs within these clumps fuse together in a process mediated by differentiated regions of the apical plasma membrane known as mating structures. Molecular details of these events are presented in the following section.

Fig. 3 shows three gametes engaged in mating, tethered by flagellar adhesions (cf. Fig. 1). The two lower cells have just completed fusion and hence are formally a single zygote, at this stage called a quadriflagellated cell (QFC). The third cell displays an apical microvillus (arrow) filled with actin filaments, the activated configuration assumed by the plus mating structure in response to flagellar agglutination (see below). By unknown mechanisms, the agglutinins of newly formed zygotes lose their adhesivity – hence the zygote in Fig. 3 would soon have swum away from the third cell – whereas unmated gametes released from clumps remain adhesive and go on to find other partners. Hence a 50:50 mixture of plus and minus gametes achieves close to 100% zygote formation within ~15 min, allowing synchronous zygote differentiation to be monitored.

Synchronous gametogenesis can also be studied by entraining the cell cycles of vegetative cells with an alternating 12-h light/12-h dark regime [30]; cells enter G1 at the onset of the light phase and, when washed into nitrogen-free medium, activate their gametogenesis program and achieve mating competency in 4–6 h [14]. Mature gametes enter G0 and remain viable and mating-competent for several weeks. Differentiation is also fully reversible: gametes washed into nitrogen-containing medium lose mating ability and resume vegetative mitotic growth within 18 h [11], a process that has not yet been investigated experimentally.

Studies of synchronous gametogenesis document that gamete-specific gene expression occurs in stages. Genes encoding catabolism-related proteins are expressed within the first 2 h after nitrogen depletion; their products participate in the massive protein and nucleic-acid breakdown involved in coping with nitrogen starvation and establishing a stable G0 state [3,14,31–33]. By contrast, genes encoding the flagellar agglutinins and a mating-structure-associated glycoprotein are not turned on until 4–6 h [14,33a], indicating that nitrogen depletion is a necessary but not the sole factor in activating the gametogenesis program. Two genes, MID and MT D1, have been shown to be directly involved in activating minus gametogenesis.

The MID gene, unique to region f of the MT locus, is so-named because it is responsible for the minus-dominance originally observed in diploids (see above). Cells expressing a MID gene differentiate as minus; loss-of-function mutation [13,34,35] or deletion [25] of MID prevents cells from differentiating as minus. The Mid protein [35] is a bZIP transcription factor in the RWP-RK family that also includes Nia2, a C. reinhardtii nitrate assimilation regulatory protein that activates genes involved in nitrate uptake and metabolism [36,37], as well as several higher-plant proteins involved in nitrogen-sensing programs [38–40]. The MID gene proves to be expressed at low (basal) levels in minus vegetative cells. A pulse of up-regulated expression (to an intermediate Level-1) occurs within 30 min of nitrogen depletion in concert with the catabolism-related genes, after which expression returns to basal levels. This is followed by a sustained up-regulation (to full Level-2) at 4–6 h in concert with the acquisition of mating competency [33a].

A key feature of mid mutants is that while they fail to express minus-specific genes, they instead express plus-specific genes, the exception being the FUS1 gene unique to region c of the MT locus and hence absent from minus cells. The lack of Fus1, a glycoprotein associated with the plus mating structure [41,42], prevents these mid mutants from fusing with minus gametes – hence their phenotype is designated pseudo-plus – but they agglutinate with minus gametes and form otherwise normal plus mating structures that erect actin-filled microvilli in response to adhesion [34]; moreover, transformation with an exogenous FUS1 gene allows them to fuse [41]. The pseudo-plus phenotype of mid knockouts indicates that MID is necessary both to activate minus gene expression and to prevent plus gene expression.

This conclusion is reinforced by the phenotype of RNAi knockdowns of a second gene unique to the MT locus, MTD1 (region d), hereafter referred to as mtd1 mutants. The mtd1 mutants express low levels of Mid, and they prove to express neither plus-specific nor minus-specific genes [33a]. It thus appears that Level-1 expression of MID is adequate to repress plus-specific genes whereas Level-2 expression is necessary to activate minus-specific genes. The mid mutants, unable to do either, express plus-specific genes.

Additional experiments indicate that Level-1 expression of MID is also necessary to induce strong expression of MTD1, with strong expression of MTD1 in turn necessary for Level-2 expression of MID [33a]. The "point" of this complexity, like the "point" of many complex feedback loops in genetic systems, is at present obscure, all the more so in that the Mtd1 protein gives no indication of being a transcription factor as one might infer; instead, it is predicted to form a membrane protein that crosses the membrane three times and carries putative N-glycosylation sites, with no homologues yet detected in the database that might give clues as to function.
Compounding the complexity is the following: When the MID gene is introduced into a MT* background lacking any MTD1 gene, it is able to direct an apparently normal minus gametogenesis program in response to nitrogen starvation [35]. This result generates the inference that plus gametes express a system, not repressible by Mid, that is functionally equivalent to the minus “MTD1 system” in its ability to activate MID expression to high levels, but that does not require the Mtd1 protein itself. Importantly, at least one essential gene in this posited plus “MTD1-equivalent” system must be resident in the MT* locus. If the system were fully encoded elsewhere in the genome and Mid-repressible, then the MT* cells carrying a MIND gene would fail to differentiate as minus. If it were fully encoded elsewhere in the genome and not Mid-repressible, then MTD1 knockdowns would presumably be complemented by this second system and would not have a mating-null phenotype.

While there is clearly much left to be learned, the understanding that MTD1 is involved in minus gametogenesis addresses a puzzle pertaining to the sex-determination system of C. reinhardtii. When it was assumed that MID was the sole determinant of mating type, it was not obvious why C. reinhardtii possesses complex MT loci under recombinational repression. Would it not be sufficient that cells carrying the MID gene differentiate as minus, and cells not carrying MID differentiate as plus? The finding that MID and MTD1 are mutually dependent on one another for bringing about minus gametogenesis, and that at least one component of the posited complementary system in plus is confined to the MT* locus, indicates that it may be essential that MID and MTD1 remain in genetic linkage. If so, the puzzle shifts to the question of how such a system evolved in the first place.

6.2. Fertilization competence: cell biology

Since C. reinhardtii is both a cell and an organism, analyses of its mating reaction using the approaches of molecular cell biology have provided extensive understandings of what its sex-determination program achieves.

The plus agglutinin is encoded by the autosomal (LGVIII) gene SAG1 [43,44]; the minus agglutinin is encoded by the SADI gene lying just outside the R domain of the MT locus [25,45]. Interestingly, a functional copy of SADI is found in the same location in both the MT* and MT− loci [25]; the MT* copy is not expressed in plus cells but is expressed in plus cells carrying a MID transgene [35], presumably because Mid is required for its transcription. Limited sequencing indicates that the MT* copy carries very few differences from the MT− copy, consistent with the possibility that it represents a recent transposition, but the fact that the gene has incurred no disabling frameshift/nonsense mutations suggests that its maintenance is under selection, for reasons as yet not understood.

The Sag1 and Sadi agglutinins are enormous fibrous proteins [46], ∼240 nm in length [47], and composed of 3349 and 3853 amino acids, respectively [48]. They are members of the hydroxyproline-rich glycoprotein (HRGP) family [49], a family that also includes cell-wall proteins in C. reinhardtii, in other Volvocales, and in higher plants. The two agglutinins share the same overall domain structure but are completely different in sequence except for two conserved hydrophobic α-helical sequences [48] thought to stabilize the structure of the globular C-terminal head domains. These heads rest on long central hydroxyproline-rich shafts (Sag1, 245 nm; Sad1, 225 nm), and globular N-terminal domains serve to associate the proteins with flagellar membranes [48]. The sequence divergence between SAG1 and SADI indicates that their common ancestry is deep; the conservation of domain structure suggests that these domains are important in achieving adhesion.

Cell-wall HRGPs self-assemble via interactions between heads, between heads and shafts, and between shafts [50–52] and presumably the agglutinins play this game as well, but no details are known: adherent flagella are interconnected by a vast network of fibers [53] that has defied analysis. That C. reinhardtii achieves species-specific and MT-specific adhesion by basically co-assembling an extracellular matrix between interacting gametes exemplifies the stunning ingenuity encountered in sexual recognition systems.

The agglutinins bring opposite-type gametes together. In addition, their interactions perform a second key function: adhesion induces a cascade of enzyme activity that results in a 10-fold increase in intracellular cAMP levels [54–56] accompanied by additional signal-transduction events [57–60]. The mating-related effects of cAMP elevation, which can be mimicked by presenting non-adherent gametes with the membrane-permeant dibutyryl cAMP, include the following: (1) Agglutinins stored in a cellular compartment are mobilized to the flagellar surface to further enhance flagellar adhesiveness [61–63], a process mediated by the kinesin/dynein-mediated intraflagellar transport system also important in flagellar assembly [60,65,66]; (2) gametic lytic enzyme, a metalloprotease [66] stored in its proform in the unnated gametes [67], is cleaved to its active form and released [68], allowing a rapid disassembly of gametic cell walls [50,69] so that the gametes are able to fuse; (3) mating structures, assembled during gametogenesis, are activated (see below) such that they are able to participate in cell fusion. Importantly, newly fused zygotes, like the one illustrated in Fig. 3, form as rapidly as 15 s after gametes are mixed; that is, mature gametes are fully “primed” to carry out these complex reactions.

The unactivated mating structure of a plus or minus gamete consists of a round (∼0.5 μm diameter) differentiated region of the plasma membrane [70] adjacent to the basal-body complex [71], underlain by electron-dense material (the “membrane zone”) and overlain by a fuzzy coat (“fringe”) [34,72]. The plus mating structure has a second double-layered structure (the “doublet zone”) beneath the membrane zone [72]. Elevation of intracellular cAMP causes the minus mating structure to bulge outward slightly, while the plus mating structure undergoes a dramatic transformation: actin filaments polymerize between the membrane and doublet zones, generating the microvillus (“fertilization tubule”) noted earlier in Fig. 3, with fringe at its tip [34,42,72–74]. Cell fusion initiates with an adhesive interaction between plus and minus fringe, followed by localized membrane fusion such that the gametes are initially conjoined via a “cytoplasmic bridge” the diameter of the microvillus. Cytoplasm then
flows through the basal aspect of this bridge until the two cells are fused as in Fig. 3 [72].

The plus fringe is encoded by the FUS1 gene in region c of the MT locus [42,43]. Mutation of this gene generates a fringe-less mating structure which, while capable of forming an actin-filled fertilization tube in response to cAMP, is unable to fuse with minus mating structures [34]. When plus gametes are incubated in antibody directed against the Fus1 protein, this also blocks fusion [42].

An autosomal gene called GCS1 (GENERATIVE CELL SPECIFIC) has been identified in C. reinhardtii, as well as in other algae, protists, and higher plants, that encodes a transmembrane protein essential for cell fusion and that is expressed far more strongly in minus than in plus gametes [75]. Although GCS1 possibly encodes the minus fringe protein, its ubiquity in many lineages favors the possibility that it represents a second component in the minus mating structure that mediates the fusion that follows fringe-fringe adhesion. The temperature-sensitive gam1 mutant fails to fuse at restrictive temperature in a minus but not a plus background, but its phenotype is more consistent with its being defective in adhesion-induced signal transduction [76].

We can pause here to consider an interesting feature of the two gamete recognition systems: the MT locus encodes a gene (FUS1) essential for fringe-fringe recognition between plus and minus gametes, while the MT locus encodes a gene (SAD1) essential for agglutinin-agglutinin recognition (as noted earlier, the MT locus also carries a copy of SAD1 but it is not expressed in plus gametes). The gene encoding the plus agglutinin (SAG1) is autosomal, and the gene encoding minus fringe is presumably autosomal as well since MID-transformed mt+ gametes fuse as minus. Again we are confronted with the puzzle as to how such arrangements evolved in the first place.

6.3. Initiation of zygote differentiation

In multicellular lineages, large eggs contain “maternal” information, either as mRNA or as protein, that is activated by fertilization and initiates and/or directs early events in zygote development, with transcription of genes in the fused diploid nuclei commonly postponed until sometime later in development [77]. The plus and minus gametes of C. reinhardtii, by contrast, have the same size and contribute equivalent cytoplasmic volumes to the zygote (Fig. 3), and both gametes prove to contribute information required to initiate zygote development. Moreover, this information triggers the expression of novel genes within 10 min of gamete fusion [78–83], well before the two nuclei fuse together at 30 min to 1 h. At least one zygote-specific gene is not expressed until 1.5 h after cell fusion [84], and two genes encoding HRGPs utilized by the zygote are transcribed late in gametogenesis [85,86].

While future research may well reveal the existence of additional systems, available data indicate that the full program of zygote development, beginning with the immediate gene expression following cell fusion and continuing out to meiosis in the mature spore, is activated by the heterodimerization of two homeoproteins: Gsp1 contributed by plus gametes and Gsm1 contributed by minus gametes. Pioneering studies of Gsp1 from the Snell laboratory are reported in [55,87,88]; additional studies of Gsp1 and Gsm1 have taken place in our laboratory (Lee, Lin, and Goodenough, manuscript in preparation).

Both Gsp1 and Gsm1 are encoded by autosomal genes (GSP1 in LQII, GSM1 in LGVII), expressed at very low levels in vegetative cells, up-regulated towards the conclusion of gametogenesis, and expressed at maximum levels when cAMP is elevated in response to agglutination or artificially with dibutyryl-cAMP administration. The GSM1 gene requires MID for expression while GSP1 expression is Mid-inhibited, a pattern similar to the autosomal genes governing gametogenesis albeit no role for Gsp1 and Gsm1 in gametogenesis has yet been observed.

That these homeoproteins function in the zygote was first demonstrated by transforming minus cells with a GSP1 gene driven by a constitutive promoter. When the transformants were starved for nitrogen, they went on to assemble zygote cell walls (a phenotype readily monitored because walled zygotes stick together in liquid to form cellular sheets called pellicle), and were shown to express several zygote-specific genes identified earlier studies [88].

Given that homeoproteins often interact, a search was conducted for a homeoprotein, expressed only in nitrogen-starved minus gametes, that might form a heterodimer with the plus-specific Gsp1. Of the five homeoprotein-encoding genes in the C. reinhardtii genome, GSM1 alone was minus- and gamete-specific in expression, and when plus cells were transformed with a GSM1 protein driven by a constitutive promoter, cells formed pellicle and expressed zygote-specific genes when nitrogen-starved. Moreover, yeast-two-hybrid assays showed that the middle portion of the Gsm1 protein, containing its Knox 1 and 2 homology domains, interacts with the C-terminus of Gsp1 which contains the homeodomain (Lee, Lin, and Goodenough, ms in preparation).

Two other observations from our lab document the centrality of Gsp1/Gsm1 to the C. reinhardtii life cycle. (1) Vegetative cells carrying constitutively expressed GSP1 and constitutively expressed GSM1 form pellicle and express zygote-specific genes without any nitrogen starvation or expression of any of the gamete-specific genes described in this review, indicating that Gsp1 and Gsm1 are the only gamete-specific (nitrogen-starvation-induced) proteins that are needed to switch on the zygotic differentiation program. (2) When MT+/MT− diploid strains are constructed to carry both constitutively expressed transgenes, they not only form zygote cell walls but, when subjected to laboratory protocols for zygote maturation and germination, proceed to undergo meiosis with 2:2 marker segregation patterns. Diploid strains are never observed to undergo meiosis on their own—as noted earlier, they differentiate into minus gametes when nitrogen-starved; hence the Gsp1/Gsm1 heterodimer is apparently sufficient not only to initiate sporogenesis but also to drive the entire sporulation program. There may be additional pathways downstream from Gsp1/Gsm1 that instantiate later events, but, if so, Gsp1/Gsm1 appears to be required to jump-start any such later cascades.
Not only do zygotes switch on zygote-specific genes; they also rapidly turn off expression of gamete-specific genes such that their transcripts are no longer detectable by 1 h after cell fusion [25,35,41]. It is not yet known whether this is a direct effect of the Gsp1/Gsm1 heterodimer or whether other zygote-specific genes are involved.

Immunolocalization studies performed by us show that Gsp1 is abundant in the cytoplasm, but absent from the nucleus, of plus gametes, and that Gsm1 is abundant in the cytoplasm, but absent from the nucleus, of minus gametes. By contrast, within 10 min after zygotic cell fusion, both proteins immunolocalize to both nuclei. Hence, heterodimer formation is apparently necessary for the proteins to traverse nuclear pores and, presumably, to initiate the transcription of zygote-specific genes once they enter the nucleoplasm.

The finding that Gsp1/Gsm1 heterodimerization is sufficient to drive the sporulation/meiotic phase of the Chlamydomonas life cycle raises an obvious question: Why is it that mating in Chlamydomonas is so complicated? Granted the importance of outbreeding, and hence of mating types to prevent mating between mitotic clones, why is it not the case that mitotic cells, sensing nitrogen deprivation, simply express either Gsp1 or Gsm1 and some straightforward heterologous fusion system and proceed to the diploid phase, rather than assembling two independent mate recognition systems – agglutinins and mating structures – with all their attendant molecular paraphernalia?

We suggest that the answer may be the same as the one on offer for multicellular lineages: sexual selection. When sex is complicated, only the “fit” can pull it off – be it peacock tails or agglutinin display – leaving behind the rest. Indeed, it is well known in the Chlamydomonas community that strains carrying mutations affecting biosynthetic or photosynthetic pathways are commonly compromised in mating efficiency even when supplemented with exogenous resources. The evolution of male–female dimorphisms has introduced countless opportunities for sexual selection to occur via male–female competition and choice, but this can be thought of as an add-on to a fundamental precept: mating represents a life-cycle juncture that demands the instantiation of complex phenotypes and hence distinguishes the healthy from the frail.

### 6.4. Uniparental inheritance of organelle genomes

In multicellular organisms, mitochondrial and chloroplast chromosomes are usually inherited from the female [89], and this was long thought to be the passive outcome of the fact that eggs contain copious numbers of organelles whereas male gametes contribute, at best, only a few. Recently, however, the process has been shown to be more dynamic [90,91]: shortly after a mouse egg is fertilized, male mitochondrial genomes can be PCR-amplified from egg-cytoplasm preparations whereas later they cannot—the male DNA has been destroyed. Even more interesting, if eggs are fertilized by sperm from a closely related mouse species, the male DNA persists. That is, destruction of male mitochondrial genomes is both an active and a species-specific process, suggesting the mediation of regulatory pathways. Subsequent studies with other mammals demonstrate that this phenomenon involves the ubiquitin-dependent proteolysis of a mitochondrial membrane protein [92,93].

These studies bestowed “relevance” to students of C. reinhardtii organelle genome inheritance who, for 50 years, have been engaged in documenting that meiotic progeny inherit chloroplast DNA contributed to the zygote by the plus gamete and mitochondrial DNA contributed to the zygote by the minus parent. While the molecular basis of the mitochondrial system is as yet not clear, and the system will therefore not be considered further, there is abundant evidence that in the early zygote, chloroplast (cp) DNA from the minus parent is actively destroyed. Hence the Chlamydomonas arrangement, once considered an aberration imposed by having isogamous gametes, turns out to be “mainstream”.

A number of theories have been offered as to why uniparental (UP) inheritance of organelle genomes is so ubiquitous, and so carefully regulated, throughout the sexual eukaryotes, but no consensus is apparent, possibly because some key feature of the situation is being overlooked. For purposes of this review, the regulation of UP cpDNA inheritance in C. reinhardtii provides an additional window on its sex-determination system.

A C. reinhardtii gamete possesses a single cup-shaped chloroplast containing ∼80 copies of a 200-kb cpDNA genome; the zygote possesses two such chloroplasts. Studies from the Kuroiwa laboratory [94,95] have documented that within the first 30 min after zygote formation, virtually all the cpDNA genomes in the minus chloroplast are destroyed while virtually all the genomes in the plus chloroplast survive; indeed, they are also preferentially replicated late in zygote development [96]. Therefore, most of the meiotic progeny inherit the plus-derived genomes and not the minus-derived genomes, whether they are MT + or MT −, which is to say that the cpDNA itself has no MT specificity or preference; what is at stake is whether the chromosome comes to reside in the chloroplast of a plus or a minus cell.

It was originally proposed that restriction endonucleases were involved in destroying the minus-derived genomes [97], but there is no evidence of restriction-fragment intermediates in the digestion process [96]. Recently, a Ca-dependent nuclease activity has been detected in cytoplasmic extracts of plus but not minus gametes, and it is proposed that plus-derived genomes are somehow protected from this nuclease whereas unprotected minus-derived genomes are not [98]. The gene(s) encoding this nuclease activity have not yet been identified.

The system is not perfect. Some minus-derived genomes escape destruction, allowing them to recombine with plus-derived genomes and generate recombinant progeny (the source of data for early mapping studies of the cp genome [10]). Moreover, certain mutations and treatments increase the representation of minus-derived genomes such that cpDNA inheritance becomes biparental (BP), and these have proved informative.

The mat3 mutation [99–101] and FdR treatments [102] generate plus gametes with a drastically reduced number of cp genomes, in which case a zygote-based system somehow “counts” total cpDNA input and spares minus-derived genomes, permitting biparental (BP) cpDNA inheritance such that progeny
inherit an adequate overall number of cpDNA copies. Nothing is known about how this counting mechanism works, but it documents that the need for progeny survival is able to trump the selective MT-controlled system.

A second observation is that UV-irradiation of plus gametes just prior to mating results in a UP → BP switch; irradiation of minus gametes, by contrast, has no effect [103]. This suggests that a plus gamete contributes (components of) a “destroyer” system to the zygote that ordinarily targets minus-derived genomes for destruction and is UV-sensitive.

Since both plus-derived and minus-derived cp genomes reside in a single zygotic cell, most models of UP inheritance also posit, as above, that plus-derived cpDNA is “protected” from nuclease exposure and/or degradation, whereas unprotected minus-derived cpDNA is vulnerable.

Given the protector–destroyer model, one would expect it possible to mutationally disable components of the protector system, leading to zygotes that destroy all their cpDNA and are inviable, and to mutationally disable the destroyer system such that inheritance is uniformly BP. Intensive screens in a number of laboratories have failed to come up with such mutants, albeit they might be multi-copy, in which case single-gene knockouts would fail to generate mutant phenotypes. The multi-copy concept is given credence by the finding of three blocks of multi-copy genes in the MT loci, the only examples of such a genetic configuration (other than rDNA) in the C. reinhardtii genome.

One of the gene blocks carries six to eight copies of a gene, OTU2, that is unique to the R domain of the MT* locus, expressed only in plus gametes, non-repressible by Mid, carries a predicted chloroplast transit sequence at its N-terminus, and encodes a divergent version of otubain (Joo, Goodenough, and Lee, unpublished), a cysteine protease that functions in the deubiquitination (DUB) pathway [104].

The OTU2 gene resides in what was previously considered “spacer” DNA between the six and eight repeating modules of a second gene unique to the MT* locus called EZY2, a sequence with no homologues in the database [25]. The EZY2 genes are not expressed in plus gametes; instead, they are expressed almost immediately after zygote formation, peaking at 30 min, and greatly reduced by 2 h—the time frame during which minus cpDNA is destroyed. Moreover, the Ez2 protein carries a predicted chloroplast transit sequence at its N-terminus. It has not yet been immunolocalized nor tested for UV-sensitivity.

The third block of repetitive genes is found in equivalent locations in the C domains of both the MT* and the MT− loci, and carries 14–15 copies of the gene EZY1 [105]. EZY1 genes from both MT loci are expressed in the early zygote, slightly later than the onset of EZY2 expression. The Ez1 protein also carries a predicted chloroplast transit sequence, and it has been immunolocalized to the cpDNA containing regions (nucleoids) of both the plus and the minus chloroplasts in the zygote. Moreover, EZY1 expression is inhibited when plus but not minus gametes are UV-irradiated.

Models can be constructed that entail involvement of Otu2 in protection and Ez1 and Ez2 in destruction, and these will be tested in the future with a combination of immunolocalization, RNAi technology, UV-sensitivity studies, and analysis of minus cells transformed with the plus-restricted genes.

7. Evolutionary perspectives

The articles in this volume bear witness to the stunning variety found in eukaryotic sex-determination and mating strategies. A given lineage tends to employ the same overall strategy—for example, C. reinhardtii and C. eugametos are estimated to have last shared a common ancestor hundreds of millions of years ago [106], but they both still agglutinate via flagellar-displayed HRGP agglutinins and fuse at their apices [107]. By contrast, different lineages mate in very different ways.

While within-lineage strategies persist over time, it is also the case, by definition, that each species in a given lineage has evolved sufficient levels of discrimination to maintain species identity. This takes us to the central, and as yet deeply unresolved [109], topic of the relationship of sex to speciation.

We have explored this relationship using the species in culture that is most closely related to C. reinhardtii – C. incerta – where the two are estimated to have last shared a common ancestor <10 million years ago. A comparison of their sex-related genes has revealed striking amino-acid-sequence differences in their plus and minus agglutinins, Gsp1, and Gsm1 compared with control housekeeping genes (Lee, Waffenschmidt, and Goodenough, manuscript submitted).

Rapid evolution of many sex-related genes has been documented in numerous between-species comparisons in numerous phyla [108], raising the largely unaddressed question as to how and why a particular subset of genes might evolve more rapidly than most of the rest of the genome. In this same study (Lee, Waffenschmidt, and Goodenough, manuscript submitted) we found that this property may reside, in part, in the genes and/or the proteins themselves. Many sex-related genes, including those rapidly evolving in Chlamydomonas, prove to carry an abundance of regions low in amino-acid complexity (low-complexity regions or LCRs) that are vulnerable to slipped-strand mispairing that generates insertions/deletions (indels). Other sex-related proteins adopt protein folds that persist despite the accumulation of numerous amino-acid substitutions. A third category displays both abundant LCRs and substitution-resilient secondary structure. That is, many sex-related genes are inherently “evolvable.”

Given that successful mating is essential for the genomes of sexual organisms to continue through time, why might some of their sex-related genes/proteins be endowed with enhanced capacities to undergo variation given that most such variants will likely generate sterility? We can suggest two possibilities.

The first pertains to selection events that presumably occur during the course of speciation. Speciation takes place in countless contexts and rates, and may initiate at pre-zygotic and/or post-zygotic interfaces [109], but at some point in each process, a subset of individuals engages in preferential mating as a consequence of heritable sexual traits that differ from the parental population [110]. These novel interactions are by definition dyadic—e.g. a variant male behavior or coloration is recognized by a variant female preference, or a variant sperm
influences its niche dimensions and hence its representation in present-day ecosystems (after Stanley [109]).

Stanley's[111] concept of speciation, akin to Wright's Shifting species-specific, dyads that reinforce species isolation.

for the co-evolution and fine-tuning of these new, and eventually Evolvable sex-related genes would more rapidly generate fodder tions more effective and hence the mating process more reliable.

species and their sex-related genes are analyzed – there would divergent populations are identified by investigators as distinct but by the time speciation is complete – that is, by the time ligand is recognized by a variant egg receptor. Presumably the possibilities that the more speciose lineages have more evolvable sex-related systems is therefore a testable proposition. The highly speciose Chlamydomonas clade [115] offers an attractive system for future study along these lines: (1) it possesses identi
dyads, would be expected to promote the speciosity of a lineage and hence the likelihood that representatives of the clade, if not individual species, will move through time.

We suggest, then, that the very low proximate cost incurred by generating the occasional unsuccessful gamete, particularly given the large number of gametes produced per clone (sexual unicells) or per multicellular organism, may be overridden by the long-term cladal benefit of possessing evolvable sexual systems that are poised to abet either the initiation of a speciation event and/or subsequent species isolation and “fine-tuning.” We recognize that such arguments can be labeled as group-selection arguments and that group-selection arguments are controversial, but alternative explanations for the ubiquity of rapidly evolving sex-related genes are not, to our knowledge, currently on offer.

That certain clades are more speciose than others – fruit flies and beetles are more speciose than dragonflies, bats are more speciose than bears – has long been recognized. The possibility that the more speciose lineages have more evolvable sex-related systems is therefore a testable proposition. The highly speciose Chlamydomonas clade [115] offers an attractive system for future study along these lines: (1) it possesses identified, rapidly evolving dyads at both the pre-zygotic (agglutinins) and post-zygotic (Gsp1/Gsm1) interfaces of the life cycle; (2) many geographic isolates of C. reinhardtii from the northeast United States and Canada are in culture and can be probed for levels of dyadic sequence variation vs. mating and germination efficiency with laboratory strains and with one another; (3) many additional Chlamydomonas-related genera and related families (Gonium, Volvox) in the order Volvocales are also available for study.

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