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Review

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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Keywords: *Chlamydomonas*; Chloroplast genome; Fertilization; Homeoprotein; Mating type

Contents

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.

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.

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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,

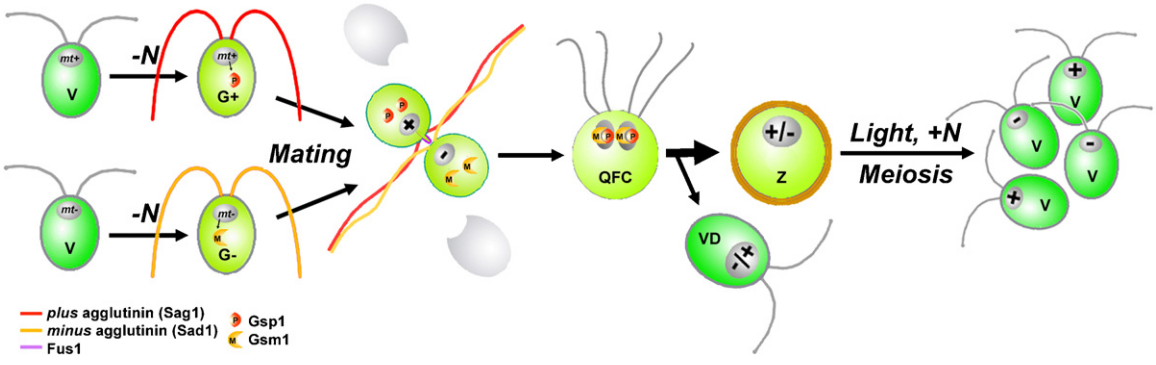

Fig. 1. Life cycle of *Chlamydomonas reinhardtii*. Haploid vegetative (V) cells of two mating types (mt^+ and mt^-) divide mitotically. When exogenous nitrogen becomes limiting, they differentiate into gametes (G+ and G−), expressing mating type-specific gametic traits. When gametes are mixed, the *plus* (Sag1) and *minus* (Sad1) agglutinins displayed on their flagellar surfaces mediate the initial adhesion reaction; adhesion generates a rise in intracellular cAMP which triggers gamete cell wall release and mating-structure activation; the Fus1 protein on the *plus* mating structure (cf. Fig. 3) interacts with partner protein(s) on the *minus* mating structure to trigger cell fusion and the formation of the binucleate quadriflagellated cell (QFC). Homeoproteins Gsp1 and Gsm1, pre-synthesized in *plus* and *minus* gametes, respectively, interact to activate transcription of zygote-specific genes. Nuclei fuse, flagella are resorbed, and a thick cell wall is assembled around the zygote (Z). In the laboratory, zygotes subjected to 5 days of dormancy in the dark and returned to light in N-containing media undergo meiosis to release four haploid meiotic products that resume vegetative growth. Occasional QFCs forego the meiotic pathway and instead resume vegetative growth as +/− vegetative diploids (VD).

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-incompatability 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-

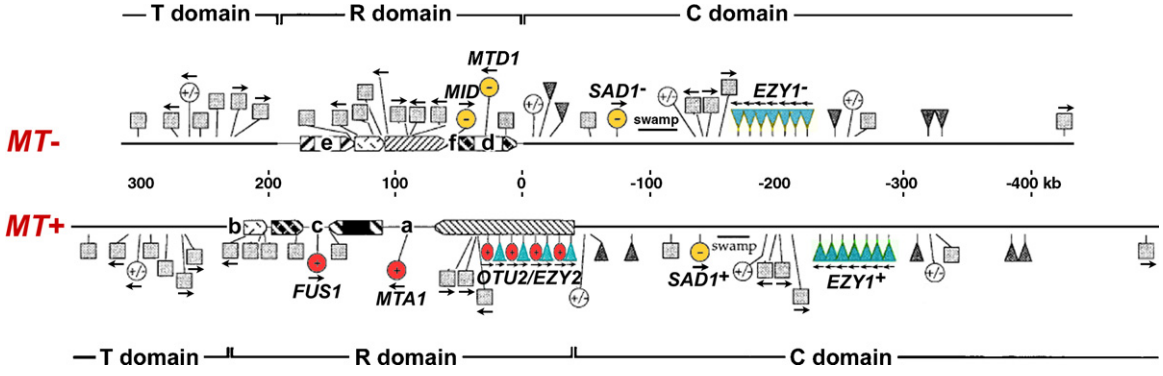

Fig. 2. Mating-type loci (MT^- and MT^+) of *Chlamydomonas reinhardtii* located in the left arm of LG VI (after [25]). The T (telomere-proximal), R (rearranged) and C (centromere-proximal) domains are indicated. The four segments of homology within the two R domains are drawn as shaded boxes, with the shapes indicating their relative orientations. Letters (a)–(f) indicate regions within the R domain that are unique to MT^- or MT^+. Orange circles identify *minus*-specific genes; red circles identify *plus*-specific genes. Squares indicate genes expressed in vegetative cells; +/− indicates genes expressed in gametes of both mating types; triangles indicate genes expressed exclusively in zygotes. Short arrows indicate direction of transcription where known.

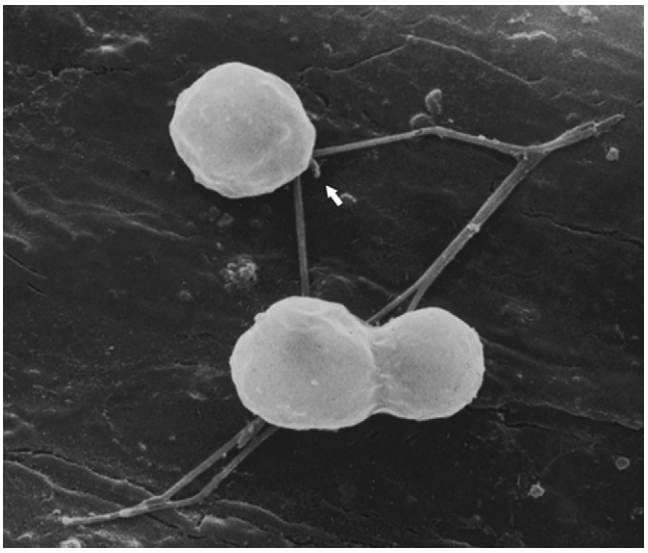

Fig. 3. Three *Chlamydomonas* cells, adhered by flagella, the lower two just fused to form a zygote, the upper displaying an activated mating structure (arrow).

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 G_1 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 G_0 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 G_0 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 *MTD1*, 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 *Nit2*, 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 *MID* 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 *SAD1* gene lying just outside the R domain of the *MT* locus [25,45]. Interestingly, a functional copy of *SAD1* 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 Sad1 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 *SAD1* 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 pro-form in the unmated 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 tubule 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* gemetes [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, are 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 LGII, *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 in 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 paraphenalia?

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 FUdR 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 repeatedly turn up new *mat3* alleles. A possible reason for such failures is that the genes controlling protection and destruction 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 deubiquintion (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 Ezy2 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 Ezy1 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 Ezy1 and Ezy2 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

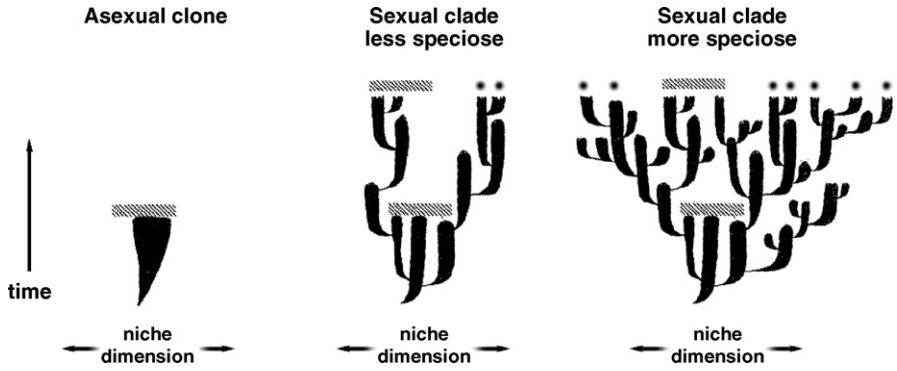

Fig. 4. Cartoon of evolutionary patterns. Gray bars denote events that wipe out major niches; circles indicate extant members of a clade. The speciosity of a clade influences its niche dimensions and hence its representation in present-day ecosystems (after Stanley [109]).

ligand is recognized by a variant egg receptor. Presumably the first novel dyad interactions are relatively crude and imprecise, but by the time speciation is complete – that is, by the time divergent populations are identified by investigators as distinct species and their sex-related genes are analyzed – there would have occurred selection on these dyads to render their interactions more effective and hence the mating process more reliable. Evolvable sex-related genes would more rapidly generate fodder for the co-evolution and fine-tuning of these new, and eventually species-specific, dyads that reinforce species isolation.

The second possibility can be presented in the context of Stanley's [111] concept of speciation, akin to Wright's Shifting Balance Theory [112] (critiqued in [113]) and summarized in Fig. 4. The obligate genomic deck-shuffling (panmyxia) of, and natural selection on, Mendelian populations at each generation generates a narrow range of phenotypes, buffered to generate organisms that are adapted to a particular niche dimension but thereby vulnerable to extinction when that dimension is compromised. In a clade endowed with mutation-prone genes and/or variation-tolerant proteins that mediate sex-determination and pre-zygotic/post-zygotic specificities, the potential for generating new dyads – however crude and imprecise – keeps arising. Should such a dyad arise in a subset of individuals that also carry novel and putatively adaptive trait(s) that would otherwise be swamped out by deck-shuffling (outbreeding depression; reviewed in [114]), this would allow for an inbreeding of such trait(s) and hence transitions into novel niches. That is, evolvable sex-related genes, by continuously offering up variant mating 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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