

The *Chlamydomonas* Zygospore: Mutant Strains of *Chlamydomonas monoica* Blocked in Zygospore Morphogenesis Comprise 46 Complementation Groups

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ABSTRACT

Chlamydomonas monoica undergoes homothallic sexual reproduction in response to nitrogen starvation. Mating pairs are established in clonal culture via flagellar agglutination and fuse by way of activated mating structures to form the quadriflagellate zygote. The zygote further matures into a dormant diploid zygospore through a series of events that we collectively refer to as zygosporulation. Mutants that arrest development prior to the completion of zygosporulation have been obtained through the use of a variety of mutagens, including ultraviolet irradiation, 5-fluorodeoxyuridine, ethyl methanesulfonate, and methyl methanesulfonate. Complementation analysis indicates that the present mutant collection includes alleles affecting 46 distinct zygote-specific functions. The frequency with which alleles at previously defined loci have been recovered in the most recent mutant searches suggests that as many as 30 additional zygote-specific loci may still remain to be identified. Nevertheless, the present collection should provide a powerful base for ultrastructural, biochemical, and molecular analysis of zygospore morphogenesis and dormancy in *Chlamydomonas*.

THE zygospore of *Chlamydomonas* allows the species to enter dormancy and to survive, often for prolonged periods, in an environment hostile to vegetative growth (Bristol 1920; Coleman 1983; Trainor 1985; Harris 1989). Dormancy in this unicellular alga is also coupled to sexual reproduction. Gamete differentiation, mating between cells of opposite mating type, and maturation of the zygote into the zygospore (zygosporulation) occur sequentially in response to nitrogen depletion. The sequence of events leading to the formation of the zygospore in the homothallic species, *Chlamydomonas monoica*, are illustrated in Figure 1.

The *Chlamydomonas* zygospore differs from the vegetative cell in many ways (see Brown *et al.* 1968; Treimer and Brown 1975; Cavalier-Smith 1976; Van Winkle-Swift and Rickoll 1997): it is nondividing, houses a diploid nucleus committed to meiotic rather than mitotic division, is protected from environmental extremes by an elaborate and distinctive multilayered cell wall, and contains extensive food reserves in the form of starch granules and large lipid bodies (derived in part from membrane dedifferentiation) that can be mobilized at the time of spore germination. These morphological differences reflect unique processes that are

initiated upon gamete fusion and proceed sequentially for at least several hours (and perhaps days) after mating.

The unraveling of developmental pathways is often facilitated by the application of classical genetic approaches that focus on the isolation of particular mutant strains unable to carry out one or more steps in the sequence. Haploid clones recovered after mutagenesis of *C. monoica* will form zygotes in clonal culture; all zygotes derived from one clone are thus homozygous for any zygote-specific mutations that may have been induced. The presence of the mutations is easily identified by visual inspection of the zygotes or by assaying for the absence of zygotes showing the typical resistance (*e.g.*, resistance to chloroform vapors) of wild-type mated populations.

More than 10 years ago, we reported the first such zygote maturation (*zym*) mutant of *C. monoica* (VanWinkle-Swift and Bauer 1982) and were later able to demonstrate complementation among several different *zym* strains (VanWinkle-Swift and Burrascano 1983). Here we summarize the results of complementation testing of additional *zym* mutants obtained from many independent searches conducted over the past decade and involving use of a variety of different mutagens, including ultraviolet (UV) light, 5'-fluorodeoxyuridine (FdURD), ethyl methanesulfonate (EMS), and methyl methanesulfonate (MMS). Based on complementation testing among these mutants, at least 46 zygote-specific functions are essential for zygosporulation. Calculations based on the number of new *zym* loci identified in the

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most recent search suggest that additional *zym* loci remain to be identified. Nevertheless, the present collection should provide a starting point for ultrastructural, biochemical, and molecular analysis of dormancy in *Chlamydomonas*.

MATERIALS AND METHODS

Strains and culture conditions: The mutants described here were derived from the *C. monoica* wild-type strains WT15c (*cf.* VanWinkle-Swift and Burrascano 1983) and UTEX 220 (*cf.* Van den Ende and VanWinkle-Swift 1994) and from a spectinomycin-resistant mutant (*spr-fd1*) derived from the WT15c strain (VanWinkle-Swift 1980). Vegetative cells of wild-type and mutant strains were routinely maintained on agar-solidified HS medium (Sueoka 1960) under continuous illumination (3000–5000 lux) at 20–23°. Conditions for the induction of gamete differentiation, mating, zygote maturation, and zygospore germination were as described by VanWinkle-Swift and Bauer (1982).

Mutagenesis: Procedures for UV mutagenesis were as described by VanWinkle-Swift and Burrascano (1983). FdURD mutagenesis was carried out as described by VanWinkle-Swift and Hahn (1986). Conditions for MMS mutagenesis were similar to those for EMS, as described by VanWinkle-Swift and Bauer (1982).

Identification of *zym* mutants: We suspended 3000–10,000 individual clones obtained from postmutagenesis populations separately in 0.5 ml of low phosphate/low nitrate (LPN) medium (VanWinkle-Swift and Bauer 1982) and after a 7-day mating/maturation period, a 20 μ l aliquot from each mating was plated on Bold's basal medium (BM; see VanWinkle-Swift and Bauer 1982), stored in darkness for 1 wk, and then exposed to chloroform vapors to kill unmated cells. Clones yielding no growth after exposure to chloroform were retested and observed by light microscopy. These mutants fell into several classes: flagella-less strains (unable to agglutinate); mutants motile but unable to mate (including homothallism-defective strains); mutants producing morphologically normal zygospores (presumed to be germination defective, but which may include mutants with defects in the zygospore wall not detected by light microscopy); and mutants that undergo mating and cell fusion but that fail to complete the zygosporulation process (*zym* mutants). This report focuses exclusively on the latter class of mutants.

Complementation testing: Mutants obtained in a particular search were first crossed to each other in all pairwise combinations. Approximately equal numbers of cells of each strain were suspended together in 0.5 ml of LPN. Gamete differentiation thus occurred concurrently for both strains in the mixed population and a significant number of mating events were between strains ("crossing") rather than within strains ("selfing"). At the end of the 7-day period, an aliquot was examined by light microscopy for the presence of wild-type zygospores or was plated and exposed to chloroform vapors as a test for wild-type zygospore resistance. Any combinations yielding negative results were retested along with selfing controls (to verify that both strains produced gametes and mated). Pairs of mutants failing to yield wild-type zygospores were classified as noncomplementing and were assumed to carry mutations in genes affecting the same essential zygote-specific function.

Mutants chosen to represent each unique gene locus from a particular search were then crossed in all pairwise combinations to mutants obtained in earlier searches. To begin to estimate the number of *zym* loci in *C. monoica*, it is necessary to

know the minimum number of independent times a particular locus has been mutagenized. For UV mutagenesis, because separate aliquots were irradiated on each plate and because several doses were used, mutants obtained from different aliquots or different doses could be assumed to be independent. With FdURD mutagenesis, multiple flasks were sometimes used as well as different mutagen concentrations; these also represented independent populations. Following EMS or MMS mutagenesis, cells were plated as separate aliquots, and because incubation times in the mutagen were short, cell division was unlikely to have occurred during mutagenesis in the majority of cells. Therefore, these separate aliquots from a single population, as well as the use of separate populations exposed to different concentrations of mutagen, provide sources of independent mutants. In all experiments, mutants obtained from the same aliquot and the same dose/concentration cannot be considered independent because of the period (2–3 days) of postmutagenesis recovery and growth that was allowed before subcloning and screening for mutants.

RESULTS

Isolation of zygosporulation mutants: Mutant strains of *C. monoica* blocked at each of the stages of sexual reproduction depicted in Figure 1 have been obtained: strains (*mtl-2*, *mtl-3*, *mtl-4*, *mtl-5*, and *mtl-6*) that fail to agglutinate or establish mating pairs in clonal culture because of mating-type specific defects (VanWinkle-Swift and Hahn 1986; VanWinkle-Swift and Theurauf 1991; K. VanWinkle-Swift, unpublished data); a strain (*mtl-7*) that agglutinates and activates the mating structures in clonal culture but is unable to form the cytoplasmic bridge (Shi 1995); a strain (*cf-1*) that establishes the cytoplasmic bridge via mating structure adhesion and fusion but fails to complete plasmogamy (VanWinkle-Swift *et al.* 1987); and numerous strains (*zym*) that fail to complete morphogenesis from quadri-flagellate zygote to zygospore (VanWinkle-Swift and Burrascano 1983). The latter zygote maturation mutants are the subject of this report.

zym mutants have been obtained following treatment of wild-type strains with a variety of mutagens (see materials and methods). Postmutagenesis subclones were individually self-mated and screened for the presence of morphologically normal zygospores either by visual inspection of mated cultures and/or by the recovery of zygote progeny after exposure of the mated population to chloroform vapors (see materials and methods). Table 1 summarizes the results of numerous mutagenesis experiments aimed at the isolation of a variety of sexual cycle mutants. Data regarding the recovery of *zym* mutants only are included here.

Although many of the *zym* mutants were phenotypically indistinguishable from one another, they can be generally classified as "early" or "late" mutants depending upon whether maturation has progressed to the point of primary wall release (see Figure 1). All of the previously published *zym* mutants are of the "early" class in which shed primary zygote walls are not found in clonal culture (VanWinkle-Swift and Burrascano

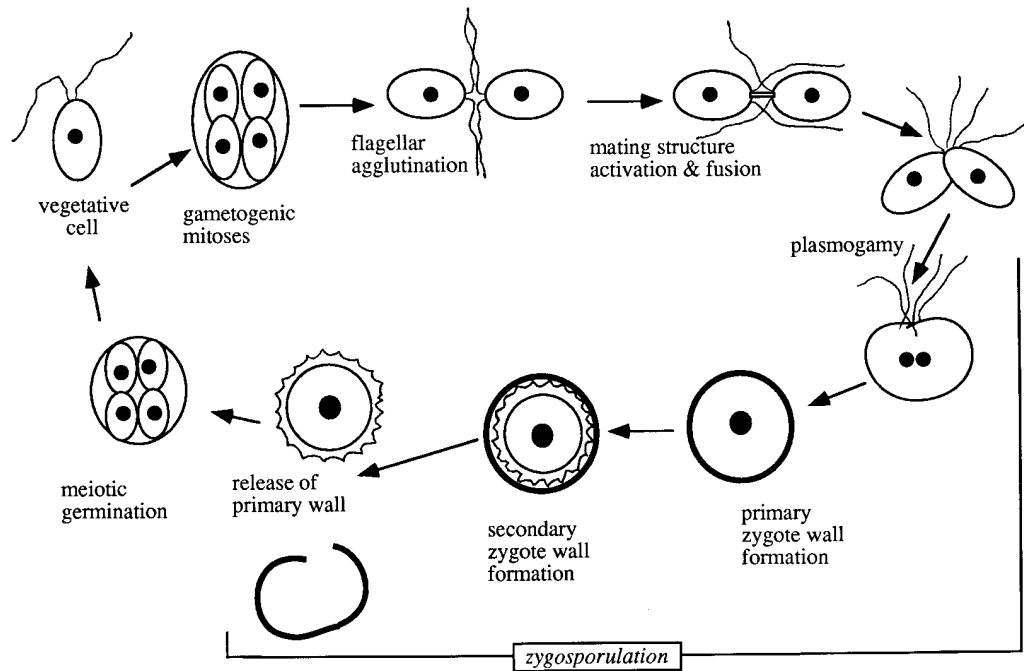


Figure 1.—Homothallic sexual reproductive cycle of *C. monoica*. Sexually compatible gametes are produced within a clonal population by mitotic divisions occurring under conditions of nitrogen limitation. Compatible gametes establish pairs via flagellar agglutination, activate mating structures that promote cell fusion, and fuse to produce the quadriflagellate zygote. The zygote matures into a heavily walled zygospore, discarding a transient primary zygote wall as secondary wall formation is completed.

1983). Many of the additional *zym* mutants reported here are “late” mutants. Ultrastructural characterization of these mutants will be presented elsewhere.

Summary of complementation analyses: In each mu-

tant search, new *zym* mutants were first crossed to one another in all pairwise combinations. The formation of chloroform-resistant zygospores in these crosses confirmed that the *zym* mutations affected unique func-

TABLE 1
Summary of recovery of *zym* following induced mutagenesis

Search ID no.	Strain	Mutagen	No. clones analyzed	No. <i>zym</i> mutants	No. gene loci ^a	Mutation frequency ^b
1-80 ^c	spr-fd1	EMS	2500	5	1	2×10^{-3}
2-82 ^d	WT15c	UV	1200	14	7	8.3×10^{-3}
3-83	UTEX220	UV	878	8	9 ^e	1×10^{-2}
4-84	UTEX220	UV	872	6	6	6.9×10^{-3}
5-84	spf-fd1	FdURD	NA	2	2	NA
6-85	WT15c	FdURD	415	5	4	1×10^{-2}
7-86	UTEX220	UV	168	2	1	5.9×10^{-3}
8-87	WT15c	FdURD	600	3	2	3.3×10^{-3}
9-87	UTEX220	FdURD	500	3	3	6×10^{-3}
10-88	UTEX220	UV	1219	8	7	6.6×10^{-3}
11-92	WT15c	EMS	2297	11	8	3×10^{-3}
12-92	WT15c	MMS	2223	18	9	6.3×10^{-3}
13-95	UTEX220	MMS	3224	14	11	3.7×10^{-3}

NA, data not available.

^a The number of gene loci is assumed to be equal to the number of complementation groups.

^b Mutation frequencies are based on the number of *zym* mutants recovered that complement one another and any noncomplementing mutants that are known to have arisen independently of one another. Noncomplementing mutants that cannot be assumed to have arisen independently are assumed to be mitotic daughters produced during postmutagenesis growth (see materials and methods).

^c VanWinkle-Swift and Bauer (1982).

^d VanWinkle-Swift and Burrascano (1983).

^e One mutant derived from this search was later found to be carrying two unlinked *zym* mutations (Parmelee 1983).

TABLE 2
Recovery of alleles at the same locus: estimation of the minimum number of *zym* gene loci

Search ID No.	Mutagen	New loci ^a /total loci	Estimated no. <i>zym</i> loci ^b
1-80	EMS	1/1	
2-82	UV	6/7	4
3-83	UV	9/9	
4-84	UV	6/6	
5-84	FdURD	2/2	
6-85	FdURD	2/4	38
7-86	UV	0/1	19
8-87, 9-87	FdURD	2/5	47
10-88	UV	4/7	49
11-92; 12-92	EMS, MMS	15/22	88
13-95	MMS	5/11	79

^a Although the sum of the new loci identified is 52, mutants representing only 46 of these loci have been retained. Mutants showing extremely low mating efficiencies or an excessively leaky phenotype have been discarded.

^b The number of *zym* loci is calculated from the formula $n = t/a$ where t is the number of test crosses performed (number of newly isolated complementing mutants \times number of mutants presently in the collection) and a is the number of cases of noncomplementation in the test crosses.

tions, *i.e.*, that complementation had occurred in the hybrid zygotes. (Because the *zym* strains remain homothallic, maturation-defective zygotes of each parental type were also found in the mixed cultures.) Complementation analysis provided the information needed to determine how many gene loci were represented within the population of mutants recovered in a particular search (see Table 1). When two or more mutants failed to complement, one of these was chosen to represent the locus and was used as the "tester" in subsequent crosses to *zym* mutants recovered in earlier searches (see materials and methods).

In summary, complementation analysis of all *zym* mutants recovered to date has identified 46 complementation groups. Each complementation group has now been assigned a *zym* locus number from 1 to 46. The *zym-1*, *zym-6*, *zym-7*, *zym-8*, *zym-13*, and *zym-20* loci correspond to mutants with the same designations in VanWinkle-Swift and Burrascano (1983). The strains designated *zym-2*, *zym-3*, *zym-4*, *zym-5*, *zym-10*, *zym-14*, *zym-15*, and *zym-21* by VanWinkle-Swift and Burrascano (1983) are alleles of *zym-1* and will henceforth be referred to as *zym-1-2* through *zym-1-9*, respectively. If non-complementing mutants could not be assumed to be independently induced, only one strain has been maintained in the collection. Thus, the *zym-9*, *zym-11*, *zym-15*, and *zym-18* strains of VanWinkle-Swift and Burrascano (1983) have been discarded and these numbers now refer to newly identified distinct gene loci. In addition, the *zym-16* and *zym-17* strains (VanWinkle-Swift and Burrascano 1983) have been discarded because

of excessive leakiness and very low mating efficiency, respectively. These numbers have also been reassigned.

Tables 2 and 3 summarize the data from *zym* mutant searches in terms of the identification of new loci *vs.* recovery of a new allele at a previously identified locus. The mutagenesis experiments are listed chronologically beginning with the first *zym* isolation in 1982 and progressing through the most recent search in 1995. The number of *zym* loci was estimated on the basis of the frequency with which new alleles at previously identified loci were recovered, using the equation $n = t/a$ where t is the number of test crosses performed (number of complementing *zym* strains in the present search \times number of *zym* mutants in the pre-existing collection) and a is the number of cases of noncomplementation found in the test crosses (Esposito *et al.* 1972). Estimates of the number of *zym* loci determined in this way are given in Table 2.

Within any mutant search, multiple hits at a single locus are possible. However, only if the mutagenized population has been subdivided in some way before postmutagenesis growth has occurred is it possible to distinguish between truly independent alleles and duplicates produced by mitotic division prior to the isolation of clones for phenotypic analysis. Table 3 summarizes the results of complementation analysis in terms of the minimum number of independently induced mutant alleles now represented in our collection for each *zym* locus. Inspection of the data regarding the sources of these alleles indicates no obvious locus specificity for the mutagens used.

DISCUSSION

Homothallic sexual reproduction in *C. monoica* has simplified the recovery of numerous mutant strains showing altered zygote development. The frequency at which new loci were identified in the most recent search (Table 2) indicates that the collection is not yet complete and that only about half of the essential zygote specific loci may have been identified to date.

Many of the *zym* strains have been used in standard crosses to wild-type or to each other and tetrad analysis verifies that they carry mutations in single genes showing typical Mendelian segregation ratios (VanWinkle-Swift and Burrascano 1983; K. VanWinkle-Swift, C. Burrascano, K. Linder and J. Maddock, unpublished data). Few cases of linkage have been detected (see VanWinkle-Swift and Burrascano 1983).

If we assume an average *zym* mutation frequency of 6×10^{-3} , the expected frequency of double mutants is about one per 25,000–30,000 clones analyzed. The total number of clones analyzed in all of our searches to date is about 15,000, and one double mutant has been confirmed (see footnote to Table 1). Thus, we expect that few if any additional double mutants will be found in the present collection as we continue our genetic analy-

TABLE 3
Recovery of alleles at the same locus: summary of loci with multiple alleles

zym locus ^a	No. independent alleles	Source(s) of alleles	Genetic background(s)
<i>zym-1</i>	12	EMS (6); MMS (2); UV (3); FdURD (1)	<i>spr-fd1</i> ; UTEX220; WT15c
<i>zym-3</i>	2	MMS (1); UV (1)	UTEX220; WT15c
<i>zym-5</i>	3	MMS (1); UV (2)	UTEX220
<i>zym-6</i>	2	UV (1); MMS (1)	WT15c
<i>zym-12</i>	2	UV (1); MMS (1)	UTEX220
<i>zym-17</i>	2	MMS	UTEX220; WT15c
<i>zym-22</i>	6	MMS (3); UV (2); FdURD (1)	UTEX220; WT15c
<i>zym-23</i>	2	UV	UTEX220
<i>zym-25</i>	2	UV (1); MMS (1)	UTEX220
<i>zym-27</i>	3	UV (2); MMS (1)	UTEX220' WT15c
<i>zym-28</i>	3	UV (2); MMS (1)	UTEX220; WT15c
<i>zym-31</i>	2	UV (1); MMS (1)	UTEX220; WT15c
<i>zym-35</i>	3	UV (2); MMS (1)	UTEX220; WT15c
<i>zym-40</i>	2	FdURD	<i>spr-fdl</i>

Any *zym* mutants that have been used in work previously published have retained their original numbers. Thus, for example, the *zym-6* mutant assigned to complementation group B by VanWinkle-Swift and Burrascano (1983) will continue to be referred to as *zym-6* (or, more accurately, as *zym-6-1*, since a second allele at that locus has since been obtained).

^a The gene loci are assumed to be equivalent to complementation groups. Locus numbers do not necessarily reflect the chronological order in which mutant alleles at these loci were obtained.

ses. Furthermore, as the collection continues to increase in size, the probability of identifying double mutants simply by complementation testing also increases.

Intragenic complementation, if it occurs, would lead to an overestimation of the number of *zym* gene loci. In contrast, noncomplementation between alleles of different genes can also occur if the gene products from two loci function as a complex and the presence of both mutant and wild-type subunits in a common cytoplasm interferes with function (Atkinson 1985; Klein and Deppe 1985; Stearns and Botstein 1988; Varkey *et al.* 1993; Rancourt *et al.* 1995). In contrast to intragenic complementation, intergenic noncomplementation will lead to an underestimation of the number of *zym* loci.

VanWinkle-Swift and Burrascano (1983) discussed the difficulty in interpreting complementation data if certain *zym* loci are expressed exclusively in one mating type, *i.e.*, if the gene product is produced in the gamete and contributed to the zygote from one mating type only. Mutations in different genes whose expressions are restricted to the same mating type would fail to complement because the wild-type gene product could not be provided by the partner of opposite expressed mating type. The isolation of mating-type-limited nonselfing mutants of *C. monoica* (VanWinkle-Swift and Hahn 1986; VanWinkle-Swift and Theurauf 1991) makes it possible to easily determine whether any of the *zym* alleles are themselves mating-type limited in expression. Each *zym* mutant was crossed to *mtl-2* (a strain that can mate only as *mt*⁻; VanWinkle-Swift and Hahn 1986) and to *mtl-5* (a strain that can mate only as

mt⁺; A. McNamara and K. VanWinkle-Swift, unpublished data). In all cases, viable zygotes were obtained from both crosses. Thus, the wild-type *zym* gene products must have been provided by both mating types and were not mating-type limited (data not shown).

VanWinkle-Swift and Burrascano (1983) argued that zygote maturation might be activated only when coregulators derived from each mating type (and perhaps products of the mating-type alleles *per se*) could interact in a common cytoplasm, *i.e.*, immediately after cell fusion. Ferris and Goodenough (1987) have proposed a similar model for *C. reinhardtii*. The apparent absence of mating-type-limited *zym* alleles in our collection may simply reflect the fact that the collection is incomplete. Alternatively, if the hypothesized regulators of zygote maturation are also regulators of events in the sexual cycle that precede cell fusion, the relevant mutants might have a nonmating and/or fusion defective phenotype.

However, it is also possible that no such mating-type-limited regulatory products are directly required for activation of the zygosporulation pathway. Flagellar agglutination in *Chlamydomonas* activates a cAMP-dependent signal transduction pathway (Pasquale and Goodenough 1987; Kooijman *et al.* 1990; Sato *et al.* 1993) and evidence for IP₃/DAG signal transduction during the sexual cycle is also accumulating (Musgrave *et al.* 1993; Quarmbay 1994). Activation of the zygote-specific *zym* loci could be accomplished through action of a zygote-specific kinase whose presence/activity is simply the end product of a traditional signaling pathway. A flagellar protein kinase whose activity is reg-

ulated during the early stages of flagellar sexual signal transduction has been described (Kurvari *et al.* 1996; Kurvari and Snell 1996).

The flagellar agglutinins (or mating-structure adhesion molecules) might be the only mating-type-specific gene products required for the entire progression of the sexual cycle. Although this appears not to be the case in *C. reinhardtii* (Goodenough *et al.* 1982, 1995), *C. monoica* is more closely related to *C. eugametos*, a species believed to have diverged from *C. reinhardtii* 300 million years ago (Larson *et al.* 1992). *Chlamydomonas* is an exceptionally diverse genus and species may differ in the precise mechanisms regulating their sexual development.

Using a molecular approach, several investigators have identified genes showing zygote-specific transcription in *C. reinhardtii*. Ferris and Goodenough (1987) identified eight such transcripts that accumulated relatively early in zygote development, Wegener and Beck (1991) identified three additional transcripts in older zygotes, and Uchida *et al.* (1993) obtained five cDNA clones showing expression early in zygote development that are apparently different from those described by Ferris and Goodenough (1987). Such molecular approaches are likely to reveal those genes whose transcripts are most abundant.

The large number of *zym* loci already identified in *C. monoica* underscores the complexity of the maturation process. Much of our present effort is being directed toward ultrastructural analysis of the *zym* strains to begin to clarify the particular aspect of zygote maturation being affected in each and to identify strains that may provide insights into the structure, assembly, and function of the various zygospore wall layers (see discussion by VanWinkle-Swift and Rickoll 1997). It is our hope that these mutants will help identify some of the critical and unique processes that allow the zygospore to survive environmental extremes and that define dormancy in *Chlamydomonas*.

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