A Series of Fortunate Events: Introducing Chlamydomonas as a Reference Organism

Patrice A. Salomé and Sabeeha S. Merchant

Plant Cell. Advance Publication June 12, 2019; doi: 10.1105/tpc.18.00952

Corresponding author: Patrice A. Salomé salome@chem.ucla.edu

Review timeline:

TPC2018-00952-REV	Submission received:	Dec. 14, 2019
	1st Decision:	Feb. 14, 2019 *revision requested*
TPC2018-00952-REV1	1st Revision received:	May 20, 2019
	2nd Decision:	May 29, 2019 *acceptance pending, sent to science editor*
	Final acceptance:	June 8, 2019
	Advance publication:	June 12, 2019

REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2018-00952-REV 1st Editorial decision – *revision requested* Feb. 14, 2019

Thank you for your hard work on this review article, which we think will be a valuable addition to this series, with some revision as outlined below. We received four reviews and comments of a few members of the editorial board. All of the reviewers make a number of constructive comments. We ask that you please consider all of the review comments carefully in your revision. Additional editor comments:

Reviewer 1 makes some very interesting and valid observations. The article could be more balanced in its outlook on the value of Chlamy as a model system, as discussed by this reviewer. Reviewer 3 make comments in a similar vein, and suggests addition of an introductory section about "global relevance". Maybe the history section could be pared down somewhat (as intimated by reviewer 4) to keep it to a reasonable length and within the word limit.

Minor:
One point missing is the foundational role of Chlamy channel rhodopsins for opts-genetics (papers by Hegemann are cited, but not put into perspective). *nptII* is missing from Table 2 as a selectable marker gene (Barahimipour, et al. 2016. Efficient expression of nuclear transgenes in the green alga Chlamydomonas: synthesis of an HIV antigen and development of a new selectable marker. Plant Mol. Biol. 90, 403-418).

> **RESPONSE: We thank the reviewers for their time and thoughtful comments. We have gone about the revision in two steps: first, we included all comments in the text (insert/delete words, minor changes to the text, insertion of missing citations). After this first step, word count went up to 23,000, including citations. In the second step, we shortened the text when possible to bring the word count closer to 21,800 (15,300 without citations). Not all edits from step 1 survived step 2.**
>
> **We envision the target audience for this review as researchers not already familiar with microalgae in general, and Chlamydomonas specifically. This review's goal is to offer researchers the context necessary to understand how and when Chlamydomonas has emerged as a model organism, for which research topics it may be more suited than others, as well as some of the limitations intrinsically associated with the organism, for example its high propensity for transgene silencing and poor performance with Cas9/CRISPR gene editing. We have modified the end of the abstract to spell out the intended audience more clearly.**
>
> **We have also modified the "outlook" section at the end of the manuscript to address the future of Chlamydomonas research.**

---------- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2018-00952-REV1 1st Revision received May 20, 2019

Reviewer comments and **author responses**:

Reviewer #1:

This essay covers the history of Chlamydomonas research, the current state of technologies for working with it, and provides some snapshots of specific research areas. This is all very useful. The historical review is interesting and helpful, as is the global overview of the biology. The level of specificity is variable across different aspects of the biology, but this is natural. The essay clearly reflects deep knowledge about Chlamydomonas, and as well, communicates a very likeable affection for the organism. Into this pleasant domain, though, I feel obliged to inject some less agreeable considerations.

Point 1. I am thinking of a few different possible readers of this essay, in addition to the already-dedicated Chlamy researchers who will read and enjoy the essay, but without any major changes resulting in their outlook:

(1) A reasonable person, not already a Chlamy researcher, wondering whether to work with Chlamy to address some biological question. 'The little alga that could' is charming, but leads directly to the question: could what? And do we mean 'could in the past', 'could now', 'could into the future', or 'could in the future dependent on some technological advances that are not, so far, going so great'?

RESPONSE: The sentence was deleted in the new abstract.

(2) A reviewer of grant applications who starts out doubtful about the future prospects for Chlamydomonas. (That such reviewers exist is easily documentable).

(3) A land-plant scientist, who may start with the opinion that green algae are bizarre and forgettable creatures. (I'm sorry to say that I think this is far from an unusual opinion. Of course, land plants are in fact a specialized and bizarre version of green algae).

RESPONSE: We have modified the abstract to cast the intended audience for this review as non-Chlamy researchers who may consider switching or expanding to microalgae. As the reviewer points out, it is unlikely that Chlamydomonas researchers will learn anything novel from this manuscript. We bring this into a modified "OUTLOOK" section at the end of the manuscript as well.

Point 2. For me, the fundamental issue for all these readers is really what research in Chlamydomonas is, or could be, FOR - both in the past, and, critically, going into the future. I think the present MS offers an excellent opportunity to evaluate these issues in a balanced manner. Much of the material already in the MS is directly relevant to these issues; I think this should be made a more explicit aspect of the discussion - not a salesmanship job, obviously, but a rational appraisal.

Take cilia as an example. There is absolutely no question that Chlamy has been central to understanding cilia (it is the only really usable microbial-genetic model, since all usable fungal models lack cilia), and this is well covered in the overview here. HOWEVER, I do think that there are likely animal cell researchers who believe that the availability of genome editing and other methods sharply reduces or indeed entirely erases the need for any microbial model (for cilia, or really for anything else) in the future. (It may not be so easy to find anyone who will say this out loud, but I am sure this point of view would get a lot of votes in a secret ballot.) So when they say 'the little alga that could', are the authors referring only to the historical record, or to what can still be done better, faster, cheaper etc. in Chlamy than in other systems, now and for at least the next decade or so?

Similarly, photosynthesis has certainly been elucidated historically with tremendous contributions from Chlamy, as indicated in the review. But starting today, should a person new to the study of photosynthesis take up Chlamy, or should they suppose that efficient genome editing and other tools means that they can study it in land plants (where

applications to agriculture, for example, will surely be more direct). Or is the implication of the 'greener pastures' line in the Abstract that once (not to say 'if') genome editing works well in Chlamy, THEN (if not now), Chlamy will be the premier system - but even given equivalent genome editing, WHY would Chlamy be better? I think there's an argument to be made, but it's not a sure thing.

RESPONSE: We have articulated the place of Chlamydomonas as a great model for photosynthesis and cilia biology. However, we anticipate that Chlamydomonas could also provide the blueprint to discover a new algal model system that will be more amenable to genome editing and transgene expression, which would capitalize on the increasing numbers of algal genomes generated. We believe there is an argument to be made, both for Chlamydomonas and other classical systems, about the balance between the resources currently available and the accumulated history versus the technical limitations that are slowing down the progress of Chlamydomonas research.

Point 3. The phylogenetic discussion in the essay (pp 27 ff) is useful but brief. I think that independent of technological advances, these considerations indicate distinct ways in which Chlamy could be a useful model. Here are the main three:

Chlamydomonas as a microbial model:

1. Serve as a microbial model for biological features that fungi have evolved away (obvious example: cilia). I think this is definitely a real advantage and a niche for Chlamydomonas, though it requires some justification in terms of why it is good to have a microbial model (that is, if you're interested in some feature in animal cells, why not just study it there? There are reasons, but it's not a black-and-white case).

Chlamydomonas and absence of gene duplications:

2. Serve as a microbial model for land plants, especially for features not found in animals/fungi (obvious example: photosynthesis). Again, the reason for a microbial model requires justification. In the particular comparison of Chlamy to land plants, the rampant gene duplication in the latter provides a specific pro-Chlamy argument, at least for loss-of-function genetic studies.

RESPONSE: We had initially considered including a figure comparing gene family sizes in Chlamydomonas and Arabidopsis, but did not go through with it due to space limits. We have since generated the figure (Chlamy vs. Arabidopsis, as well as Arabidopsis vs. poplar for a comparison between two land plants), now included as the last panel in Figure 5. What we find is that, although Chlamy gene family sizes are indeed somewhat smaller compared to Arabidopsis, they are by no means equal to 1-2. In fact, the bulk of differences in gene number in Chlamydomonas appears to be explained by the large number of genes that are present in the alga and missing in Arabidopsis (and vice versa). There are some anecdotal examples of smaller gene family sizes in the alga, but they lack genetic data to confirm decreased redundancy. Looking at the metal homeostasis field, iron and zinc transporters are present in multiple copies, and no single loss of function in Chlamydomonas have a phenotype.

This is mentioned briefly on pp 15-16; I think this discussion could use expansion. For example, cite cases (there must be many) where a loss-of-function mutant identified a critical gene in Chlamy, that is in a multigene family in Arabidopsis and hence buffered from direct functional identification (until a 7-way knockout plant is constructed, or something of the sort). So the point here would be that if it is convincing that the basic phenomenon is likely largely unchanged comparing Chlamy and land plants, then Chlamy is clearly the better place to study it - with classical genetics even if gene editing never works well in Chlamy; all the more if/when gene editing becomes efficient.

RESPONSE: We would argue here that the redundancy in land plants can be an advantage when working on genes that would be considered essential in Chlamydomonas. In Arabidopsis, very large insertion mutant libraries make it fairly easy to identify loss of function alleles in single genes, and crossing them to generate high-order mutants is also trivial, and will provide a graded series of loss of function as more genes are inactivated. This is not to say that Chlamydomonas cannot be more useful in some cases, but we have not gone through all gene families and their degree of genetic characterization.

Chlamydomonas as a model for unicellular green algae:

3. Serve as a model for unicellular green algae. It certainly is such a model; I personally am doubtful that this line has much general appeal. If the authors want to argue in favor of the study of green algae for their own sake, more power

to them; but I think it's swimming against the tide. Which means not that you don't do it, but you do have to swim harder.

RESPONSE: We now frame Chlamydomonas as a model for green algae due to their importance as primary producers.

Point 4. The authors cite biofuel applications in the Abstract - here it is sad but relevant that most of the companies that promised practical algal biofuels are out of business, or at least transitioning away from algal biofuel as fast as they can (https://www.greentechmedia.com/articles/read/lessons-from-the-great-algae-biofuel-bubble#gs.tCQR9zDX). It may yet turn into something useful, but it seems there was considerable overstatement of the benefits and/or neglect of the problems. At present, this is not, for me, much of an argument for Chlamy. (It may not appeal much to the authors as well, since there is practically no discussion of this aspect in the body of the review.)

RESPONSE: We agree that Chlamydomonas has not, thus far, lived up to its potential for cost-effective biofuel production. We have changed "biofuel" in the abstract to "production of bioproducts".

Point 5. In situ cryo-electron tomography is cited also as a positive for Chlamy research. It is true that the small size of Chlamy cells is a big advantage with present methods. How long this differential will last is a question related to technological advances, which it seems to me generally go much faster than anyone might expect. (In the present case, cryo-sectioning, or something called 'cryo-FIB', are proposed as ways to get around the size limitations even with big thick eukaryotic cells.)

RESPONSE: Chlamydomonas is much better suited than yeast cells for cryo-TEM, because the yeast cytosol is too dense and likely scatters electrons too much to provide a good contrast in images. Even so, observation of Chlamydomonas cells by this method does require thinning of samples by cryo-FIB, since effective cryo-TEM demands thin (<200 nm) sections. We suspect that the advantage provided by Chlamydomonas cells will remain for some time, even with technical improvements. We now mention cryo-FIB for sample preparation, to obtain the thin sections needed for cryo-EM in the text.

Reviewer #2:

Point 1. I'm sure Moewus was bogus, but for the record, here are the Levine-lab stats: "a genetics orgy, with technicians Elizabeth Levine and Peggy Olmstead at the fore. Some 250 crosses are performed and more than 20,000 tetrads analyzed".

RESPONSE: An earlier draft of this manuscript included the Sapp book from 1990, but a review of the book in Science did not speak highly of it. I found the earlier citation (Sapp, 1987) to be more informative, and downloadable too. From what we were able to gather from older articles is that all Chlamydomonas isolates, with the exception of those from Gilbert Smith, were *C. eugametos/moewus*, and thus strict photoautotrophs. Without Moewus, Smith would likely have not isolated the current *C. reinhardtii* strains, which would have precluded any work on photosynthesis. We were more interested in how scientists reacted to Moewus at the time than to what degree his claims were founded, since they undoubtedly influenced the thinking of Smith and contemporaries.

Point 2. It was my understanding that he made most of this stuff up, in which case his results didn't support anything. If he's been reinstated more recently, a ref would be good. Otherwise I'd leave out the last sentence of this paragraph, and also switch the order of the two preceding sentences so we get second and then first and move right in to Smith.

RESPONSE: We have switched the order of the sentences as suggested.

Point 3. Lines 252-254 (Word) 255-257 (PDF): Unless I've missed something, GSP1 isn't involved in gametic differentiation; knockdowns only have zygotic phenotypes. SAG1 expression is transcriptionally restricted to mt+ gametes because its expression is repressed in mt- gametes that express the MINUS-DOMINANCE (Mid) gene. You don't mention this gene until p. 21, but I think it belongs here as well. You cite Ferris et al. 1996, but that paper is about *fus1*, and Ferris et al. is about the agglutinin genes. The relevant Mid paper is Ferris, P.J., and Goodenough, U.W. (1997). Mating type in Chlamydomonas is specified by *mid*, the minus-dominance gene. Genetics 146, 859-869.

RESPONSE: We have re-written the section and added a panel in Figure 3B to add the regulatory cascade leading from MID1 induction by nitrogen starvation to zygote development with the heterodimerization of GSP1 and GSM1.

Point 4. Lines 567-568 (Word) 555 (PDF): Bacterial chromosomes attach to the inner cell membrane. Is there any evidence that nucleoids attach to the homologous inner chloroplast envelope membrane? "Intraplastidal" is vague.

RESPONSE: Such evidence does exist in pea and spinach, but so far not in Chlamydomonas. The protein PEND, shown in pea to bind to the plastid genome and to the thylakoid membrane, has no homolog in Chlamydomonas. We have deleted the second half of the sentence to remove our comment about nucleoid attachment to the chloroplast envelope.

Point 5. Line 683 (Word) 664 (PDF): Why is it a problem in haploids to distinguish between gain- and loss-of-function mutants?

RESPONSE: The function of a protein is typically derived from the analysis of a strain lacking it. The interpretation of a phenotype will therefore depend on whether the mutated allele is a gain- or a loss-of function. In the nematode *C. elegans*, this distinction is important enough to have warranted the development of the lf / gf nomenclature to report the state of an allele, although this has not expanded beyond the worm community.

Point 6. Line 857 (Word) 785-786 (PDF): Can't amiRNAs be synthesized with correct GC?

RESPONSE: A functional amiRNA needs to match its target, so the sequence of the amiRNA cannot be corrected. The high GC content of the Chlamydomonas genome means that most candidate amiRNAs are poor (their hybridization energy with their target is low, thereby decreasing their efficiency). Natural plant amiRNAs contain an adenine at position 10, which will not be as easy to find in Chlamydomonas. We have added this last statement to the text

Point 7. Lines 1140 (Word) 1044-1045 (PDF): change "mapping populations … in *pf* mutants" to "mutated populations … in *pf* strains"

RESPONSE: We have modified the text to make our intent clearer: by mapping populations we meant progeny of a cross derived from a wild-type strain and a *pf* mutant, using the absence of phototaxis as a way to enrich for them in the mixed population, for later identification of the causal mutation by bulk sequencing. Sentence later deleted during final editing.

Point 8. Line 1149 (Word) 1081 (PDF): You're of course free to leave it out, but TEM of live Chlamy cells using quick-freeze TEM technology has been utilized for decades in the Goodenough/Heuser lab, including highly cited studies of the axoneme that might be mentioned in the previous section.

RESPONSE: We have added a description of the axoneme work in the section on cilia biogenesis as suggested, and mention them again at the beginning of the cryo-EM work. 3D reconstruction is now possible from the integration of multiple cryo-EM images, which was not possible in the 1980s.

Reviewer #3:

The current review focuses on the green alga *Chlamydomonas reinhardtii* as model organism. It describes comprehensively the historical background, the algal life cycle and its morphology, its genetics and genetic approaches, as well as selected research highlights. The review would profit from a few changes, to make it even more attractive to a broad readership:

Point 1. The authors should present a short chapter right at the beginning of the review about the global relevance of algae e.g. with regard to photosynthetic capacity and CO_2 fixation on Earth and explain to a broad readership why we do need algal model systems. The *C. reinhardtii* example fits here especially well due to the long-standing and extended studies on photosynthesis in this organism. Key biological questions that can be especially well addressed with Chlamydomonas should be also highlighted here at the beginning within a Table. For example, the fact that *C. reinhardtii* research can result in curing diseases due to the studies on its flagella or due to the development of optogenetics (e.g. light switches used in de/activating neurons; see also later) is worthwhile to bring up. By making such changes, the general Plant Cell reader would be attracted right away to the review. .

RESPONSE: We have added a section at the beginning of the review to frame the importance of algae in research.

Point 2. Some parts of the History chapter can be shortened; a few examples are mentioned below. The development of optogenetics, which is an emerging field nowadays and was greatly enhanced by the studies on the Chlamydomonas channelrhodopsin photoreceptors (Deisseroth and Hegemann, 2017, Science, doi: 10.1126/science.aan5544), should be integrated into the review.

RESPONSE: We have added a section in "selected highlights" that covers optogenetics and the role channelrhodopsins have played.

Point 3. Line 46/47: The authors write: "His drawings of Chlamydomonas ... are rather underwhelming, but Volvox is unmistakable...". Is it attractive for the reader to find the reference organism labeled with the statement "underwhelming"? The authors should either omit the Figure or emphasize that the drawings of Ehrenberg were made in the early days of light microscopy and that the relation of Chlamydomonas to the colony forming genera such as Gonium, Eudorina or Volvox that exist purely or mainly of Chlamydomonas-like cells was obviously already recognized at that time. It may be also worthwhile stating in this context that Volvox is nowadays a simple model for developmental biology due to the existence of only two cell types (Chlamydomonas-like cells and gonidia).

RESPONSE: We have removed the word "underwhelming". We now mention the use of Volvox and other multicellular algae in the study of the switch from isogamy to oogamy in the Sexual Reproduction section of the manuscript.

Point 4. Lines 110-120: Please shorten, there are too many details.

RESPONSE: This is an important section, as it leads into the early genetic work performed in *Chlamydomonas reinhardtii*, and forms much of the basis for using the alga as a model system for cilia and photosynthesis biology. The names also come back later when we talk about the relationship between laboratory strains. We have deleted the Moewus sentence.

Point 5. Lines 213-215: The channelrhodopsin photoreceptors are generally believed to be located in a specialized area of the plasma membrane right above the chloroplast membranes and the carotenoid rich globules. Please correct the statement accordingly.

RESPONSE: We now give the correct localization for channelrhodopsins.

Point 6. Lines 237-240: Please note that there is some indication about a circadian clock in yeast (Eelderink-Chen Z, Mazzotta G, Sturre M, Bosman J, Roenneberg T, Merrow M., Proc Natl Acad Sci U S A. 2010 doi: 10.1073/pnas.0907902107. A circadian clock in *Saccharomyces cerevisiae*.). Please update the information on the circadian clock of *C. reinhardtii* here with the more recent review (Noordally ZB and Millar AJ., 2015) that is mentioned later.

RESPONSE: We meant "strong" circadian rhythms. The circadian oscillations detected in yeast are weak and dampen rapidly in constant conditions, which is obviously not the case in algae and land plants. We will omit the Eelderink-Chen reference due to space limitations, but qualified the types of circadian rhythms as "strong". We have added the Noordally reference.

Point 7. Lines 242-299 along with Figure 3: A role of nitrogen and light in gamete formation as well as in germination is known and should be indicated in Figure 3; further relevant photoreceptors (plant cryptochrome, animal-like cryptochrome) beside phototropin were recently described (Zou Y, Wenzel S, Müller N, Prager K, Jung EM, Kothe E, Kottke T, Mittag M., Plant Physiol. 2017 doi: 10.1104/pp.17.00493. An Animal-Like Cryptochrome Controls the Chlamydomonas Sexual Cycle.) and the information should be added.

RESPONSE: We have added boxes in Figure 3 to illustrate the roles of light/darkness and nitrogen status in gamete formation and zygote germination. The Zou reference has been added, as well as the Huang reference and Muller reference.

Point 8. Lines 1764/Table 2 - Reporters: Under Luciferases: the firefly luciferase should be added; it was used as chloroplast reporter (Matsuo T, Onai K, Okamoto K, Minagawa J, Ishiura M., Mol Cell Biol. 2006 Feb;26(3):863-70, Real-time monitoring of chloroplast gene expression by a luciferase reporter: evidence for nuclear regulation of chloroplast circadian period.) Several selection markers and reporters for the chloroplast are presented in a recent review that should be mentioned at this position again (Esland L et al., 2018).

RESPONSE: We have added firefly luciferase to Table 2 with its associated reference. We now also mention the Esland review article in the main text, but would like to omit some of the lesser-used selectable markers for the chloroplast due to space limits.

Reviewer #4:

Point 1. It is always great to see a review of one's favorite experimental system. In this case, the target audience is not very well defined. The paper often seems to focus on details that are not important (like the long-ago history) while at the same time leaving out the details that would help the reader to understand the organism and how to work with it. Overall, the paper lacks up-do-date references. With some serious editing, the paper would be a better contribution to the Chlamydomonas literature. Here are just a few suggestions. More editing is strongly recommended to help the paper reach its potential.

RESPONSE: We have added many references, suggested by all reviewers, and hope they make this review more up-to date. We also qualify our intentions with this review more clearly at the beginning: it should be seen as a primer for non-Chlamydomonas scientists who may be considering expending to this unicellular alga. Seasoned Chlamydomonas researchers will unlikely find much new in this review, but we have attempted to gather enough background and details about the use of this alga as a research subject to get them started.

Point 2. The transmission EM in Fig. 2 is a poor representation of the apical end of the cell. It would be good to include an image such as the rare image (made by Bill Dentler) showing both basal bodies. It was published in a paper by Johnson and Rosenbaum Trends in Cell Bio. 1:145. (1991). Dentler has the full cell image as well but it does not include the pyrenoid.

RESPONSE: We now show both the original TEM image and one unpublished image from Bill Dentler that includes basal bodies and pyrenoid.

Point 3. The "Morphology" section beginning in line 195 fails to mention anything about the cell wall, which is certainly an important component of cell morphology.

RESPONSE: We have now added a description of the cell wall to the Morphology section. We also point out that many laboratory strains carry *cw* mutations but are viable, although perhaps more fragile.

Point 4. Lines 213-16: The eyespot involves a close association of specialized regions of the plasma membrane (containing the photoreceptors) and the chloroplast envelope (where the pigment granules are organized. The position of the eyespot on one side of the cell is dependent on the cytoskeleton (see for example Boyd et al, BioArchitecture 1:196, 2011).

RESPONSE: We have corrected the localization of channelrhodopsins and now mention the role of the cytoskeleton in the positioning of the eyespot.

Point 5. Line 264: It is important to point out that the increase in intracellular cAMP induced by adhesion of the flagella also triggers the release of the gamete cell wall, allowing fusion of the gametes.

RESPONSE: We have added a description of the triggering of the release of the cell wall after the formation of the fertilization tubule and dome.

Point 6. Line 272: Gametes fuse to form a temporary dikaryon with four cilia and a common cytoplasm. The four cilia are not quickly resorbed but are present for a few hours, providing a useful tool for addressing numerous questions concerning ciliary protein function and cilia assembly. See the review by Dutcher in Cytoskeleton 71:79, 2014.

RESPONSE: This fact is mentioned later in the review, as well as the Dutcher review from 2014. This section is arguably about sexual reproduction, not flagella biology, so we will not add the reference here so as not to repeat the later statements.

Point 7. Lines 280 - 287. This section is confusing, possibly because it does not follow the chronological order of biological events. The information is not entirely correct or complete. For example, line 284-285 states that germinated colonies carry one or the other mating type. If the colony is growing up from a zygote, then both mating types would be present. If the tetrad of cells are separated (not mentioned here) and the four cells grow up to colonies, then of course each colony would carry a single mating type, segregating 2:2. Nuclear fusion discussed in

lines 287-288 seems out of order because it would have occurred prior to zygote germination. Line 284 mentions putting zygotes into rich media, but it is important to note that meiosis and zygote germination are light dependent. Huang and Beck (PNAS 100:6269) showed the important role of blue and red light for this stage of the life cycle. More recently, the roles of aCRY, pCRY and PHOT genes have been revealed (e.g., Müller et al., Plant Physiol. 174:185, 2017; Zou et al., Plant Physiol. 174:1334, 2017). It should also be noted that the zygote cell wall begins to form from hydroxyproline-rich glycoproteins soon after gamete fusion (e.g. Suzuki et al., J. Phycol., 36:571, 2000) but that the desiccation-resistant zygote wall (line 281) is dependent on the activity of a polyketide synthase gene *PKS1* expressed later (Heimerl et al., The Plant Journal, 95:268, 2018).

RESPONSE: We have now added the listed references, modified the order of the paragraph, and mentioned the role of light in zygote germination. We did not say specifically that the germinated products of the zygote would be a tetrad where all four cells should be separated, but we are now.

Point 8. Line 361: Spray-plating is a very efficient method for single cell isolation and should be mentioned here. It was utilized to isolate the S1-D2 polymorphic strain (Gross et al. Curr Genet 13:503).

RESPONSE: DONE. The Gross reference works well as evidence of phototaxis for the isolation of algal cells, and we have added the original Wiedeman reference that described the spraying method.

Point 9. Line 601: The description of the collections in the Chlamydomonas Resource Center should be clarified. There is a core collection (CC designators) of about 3635 strains contributed by numerous labs since the beginning of the Center in 1978. In addition, there are three collections of mutant strains generated more recently in specific large-scale projects. These include the CLiP strains (65,706 strains) and the Niyogi CAL strains (461 strains) generated by insertional mutagenesis. The Tulin and Cross TS-lethal and Null Mutant collection generated by UV mutagenesis (908 strains).

RESPONSE: We have moved the introduction of CRC earlier in the section, and moved the CLiP libraries and other collections down. We have also corrected which sets are part of the core collection, and which ones are not, and included additional links in Table 1.

Point 10. Line 679: The definition of "tightly linked" should be more specific. If only ten tetrads are examined and no recombination is found, then linkage would be within ~10 cM.

RESPONSE: We have removed “ten tetrads” and instead state that the extent of linkage will depend on the number of tetrads analyzed. I (PAS) come from the plant field, where it is very easy to genotype hundreds of plants at once derived from a single cross. Chlamydomonas is not quite as easy, although it is certainly possible to set up large number of crosses.

Point 11. Lines 686-694: If the audience of the paper is novice Chlamydomonas users, it would be advisable to first demonstrate and publish the proposed method for mating type switching prior to suggesting it.

RESPONSE: We have changed the wording of this section. mt+ transformants expressing MID1 were shown to be competent for crosses with other mt+ strains, so the proof of concept has already been demonstrated by Ursula Goodenough in 1997 when her laboratory cloned *MID1*.

Point 12. Lines 696 - 711: The recommended protocol for transformation rescue of mutations is puzzling because it does not mention widely-used methods such as co-transformation with dominant selectable markers for drug resistance. The reference to second-site suppressors should be clarified (or deleted). The last sentence of the paragraph should be clarified.

RESPONSE: DONE. The last sentence was modified earlier, and we hope it is now clearer.

Reviewer #5:

Point 1. Lots of work and real update on Chlamy resources but.....to be honest I am a bit embarrassed with its content.

RESPONSE: We are sorry to read that. We have put a lot of time and effort into this review, and hoped it would be received more positively.

Point 2. Of course I understand there is a strong bias towards the research interest of the Merchant lab and towards the resources available for present days Chlamy research. But the part devoted to the genealogy of lab strains of *C. reinhardtii* is in my opinion....of little interest for the broad readership of TPC and therefore by far too detailed (whereas other things are really missing as discussed below). Then what really puzzles me is that for whatever deals with chloroplast or photosynthesis, either major aspects of Chlamy innovative research over the years are missing or the references are inappropriate and sometimes what is written is simply wrong. Examples below.

RESPONSE: We think the genealogy is important to understand the genomic relationships that exist between commonly used strains by the community. Too many times we see articles citing "WT" are their laboratory strain, which is not informative. In the early days, "137c" was used interchangeably to describe what we now call CC-124 and CC-125. Yet these two strains are not identical (obviously outside the mating locus). However, we understand the point made here and have attempted to shorten the sections pertaining to genome sequencing.

Point 3. A major contribution of Chlamy research to photosynthesis in the early days has been the fluorescence screen on colonies. This started in Paul Levine's lab, first with a screen on fluorescence yield for photosynthesis mutants (Bennoun and Levine, 1967, Plant Physiol) then on fluo kinetics (for instance Bennoun et al. 1980, Genetics). Here the remarkable contribution of Pierre Bennoun should not be overlooked. The two references presently used p.5 (Girard et al., 1980; Xie et al., 1998) fall short in that respect.

RESPONSE: The two Bennoun references have been added. We have added one section in "highlights" on chloroplast biogenesis and function that provides more focus on chlorophyll fluorescence as a tool for the isolation of photosynthesis mutants.

Point 4. Another major contribution in that field, regarding chloroplast gene expression and the biogenesis of photosynthetic apparatus, is fully ignored. That is the nuclear control of chloroplast gene expression. Chlamy has been really unique in that respect (like *Saccharomyces cerevisiae* for mitochondria) and there are numerous papers from Geneva and Paris (plus a few from David Stern at Boyce Thompson) that document these remarkable insights into how the organelle manages in the cross-talk with the nucleo-cytosol.

RESPONSE: We now mention nuclear control of plastid gene expression, RNA half-life and translation before state transitions in the new section mentioned above. Because of space limitation, we can only scrape the surface when it comes to the contribution of Chlamydomonas to chloroplast research, and think this should be the topic of another review article.

Point 5. Genuine mistakes: p.17, to my knowledge Chlamy as well as other microalgae do not have NEP, only PEP. This is in contrast to the plant chloroplast situation.

RESPONSE: Thank you for noting our mistake. We have now corrected the text accordingly.

Point 6. Next, p.26, the CES study is referred to as "control of epistasy of synthesis (CES), whereby the stability of individual components of a protein complex depends on the presence of the other constituents (Choquet and Wollman, 2002) ". In fact, CES is exactly the opposite: it shows that it is not the stability but the translation of some chloroplast-encoded subunits which depends of the other constituents. What Choquet and Wollman (and others) have documented is that the "accumulation" of the various subunits of the same photosynthesis protein is a concerted process, some behaving as CES (see above) other being degraded when unassembled (too many references available to list them here, but several reviews cover this aspect).

RESPONSE: We have replaced "stability" with "translation potential", which we think is what the reviewer meant by "accumulation".

Point 7. Then p.29: "These (photosynthesis) complexes each contain subunits that are encoded by the chloroplast genome." No, what matters here is that each complex contains a mix of chloroplast- and nucleus-encoded subunits: they are genetic mosaics.

RESPONSE: This appears to be the consequence of excessive editing on our part. The end of the sentence was intended for cytochrome b_6f. Technically, the sentence is however correct: the complexes do contain subunits that are encoded by the chloroplast genome – just not ALL subunits. We have modified the text accordingly.

Point 8. Regarding synthesis of subunits of photosynthetic proteins, pulse labeling with S35 is mentioned p.26 but

here the actual breakthrough was from Nam Hai Chua's lab at Rockefeller using C14 -acetate and translation inhibitors (Chua and Gillham 1977 J. Cell Biol) which has been extended in a seminal study by Delepelaire (Delepelaire 1983, Photobiochem. Photobiophys.) who delivered the most complete map (on gels) of which thylakoid membrane proteins were translated on cytosolic or chloroplast ribosomes.

RESPONSE: The Delepelaire article is not indexed at NCBI PubMed, nor is the article available online (possibly in part because the journal stopped publishing new articles after 1986), but we were sent an electronic copy by FA Wollman. This reference is now cited.

Point 9. That cytochrome b6f control activation of the LHCII-kinase is not Fleischmann et al. 1999 but is in Wollman and Lemaire (1988 BBA), further substantiated by the identification of the Qo site of the complex as the key activation site for the kinase (Zito et al. 1999 EMBO J.)

RESPONSE: We used the Fleischmann reference because it described the isolation of the *stt7* mutant. We now cite the Wollman and Lemaire 1988 reference at this location, and moved Fleischmann two sentences later. We note that many of the early work on photosynthesis in Chlamydomonas is not directly accessible via NCBI PubMed, but several articles can be accessed from the website of the photosynthesis research unit in Paris. The web link has been added to Table 1.

Point 10. I also would have highlighted the identification of the two pathways for heme biogenesis (CCS and CCB) because these studies very nicely illustrated the power of forward and reverse genetics with Chlamy, which is not that straightforward in plants or other algae.

RESPONSE: While we would be happy to cite many articles, we are running into space limitations and will therefore omit references covering the CCS and CCB systems. Studies of essential genes are not as difficult as this reviewer thinks, and this is due in large part to the very large collections of T-DNA insertions generated by the Arabidopsis community.

Point 11.The review is not optimized for chloroplast research.

RESPONSE: It was not our intention for this review to be "optimized" for chloroplast research, or for flagellar biology or any specific topic in Chlamydomonas research. We attempted to cover many topics and lines of inquiry followed by the Chlamydomonas community at large to give a flavor of what has been accomplished to new scientists eager to start working on microalgae, and what could be done better in other systems.

TPC2018-00952-REV1 2nd Editorial decision – *acceptance pending* **May 29, 2019**

We are pleased to inform you that your review article entitled "A Series of Fortunate Events: The Introduction of Chlamydomonas as a Reference Organism" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor **June 8, 2019**