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Chlorophyll fluorescence of *Chlamydomonas reinhardtii*: insights into the complexities

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Manuscript submitted 2022-07-15, 2022-09-19 (revision)

Manuscript accepted 2022-09-19
https://doi.org/10.26124/bec:2022-0010

Reviewer 2

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Manuscript reviewed 2022-08-28
https://doi.org/10.26124/bec:2022-0010.r2

*Only major points from review and responses included.

Reviewer 2

What do semi-quantitative analyses imply, why is it not quantitative?

Author

I do not think any protocol with chlorophyll fluorescence can be 100% accurate at quantifying a specific mechanism of NPQ, hence the use of the term 'semi', but since the protocol does accurately quantify the rate of change of chlorophyll fluorescence when an individual mechanism is expected to dominate I deleted 'semi'. Due to word limit this full explanation was not included in the abstract, but added to the protocol description.

Reviewer 2

Table 1, another major player for CEF in *C. reinhardtii* is PGR5 (see Buchert et al., 2020). PGRL1 is rather required for PGR5 expression and its stability (Petroutsos et al. 2009). As the only Cys in PGR5 is not required for its functions, the mechanistic role of PGRL1 remains elusive.

Author

I added pgr5 mutant and Buchert et al., 2020 as a reference for the cyclic electron flow in Table 1.

Reviewer 2

Fig. 2 the qE capacity is rather low, possibly because the cells were shifted from TAP conditions into photo-autotrophic conditions. The full expression of LHCSR3 takes at about 12 h to 24 h. Moreover, the presence of acetate suppresses LHCSR3 expression. Please clarify in the text.

Author

I agree that photoautotrophic needs mentioning here, but since accumulation of LHCSR proteins can be influenced by several factors (i.e. reactive carbonyls, electron flow inhibitors, photorecpetors etc.) I did not want to over-complicate the text with too many details. Therefore, I decided to leave the text as it was, but added 'photoautotrophic' to the Figure legend since cells were acclimated to low light and photoautotrophic for 24h.

Reviewer 2

STT7 does not only phosphorylate LHCII but also LHCSR3 as well as CEF effector proteins such as PETO.

Author

I added STT7-mediated LHCSR3 phosphorylation to text and cited Bergner et al., 2015.

Reviewer 2

Fig. 3A. FLVs rather work transient (Chaux et al. 2017)? What is the evidence of Mehler reaction? O2 uptake is rather low in flvB-21.

Author

I agree (see Table 1 and text on pg. 5), although the Mehler here is not specifically referring to flv mutants. Both Mehler and flv activities result in oxidation of PSI and PETC, as is the intention to show in the Figure. No changes have been made.

Reviewer 2

Interconnection between LHCSR3 and PGRL1 has been already show via (Chaux et al. (2017). Mol Plant 10, 216-218) and Kukuczka et al. (2014). Plant physiology 165, 1604-1617).

Author

Yes, I know, but am missing the relevance of this statement. Text refers to overlapping NPQ mechanisms, but not interactions of NPQ with electron flow.

Reviewer 2

For the interpretation of chlorophyll fluorescence measurements, the physiological of cells need to be considered. This is particularly important as even different WT cells acclimate with different speed to changes in the trophic status, light regime and nutrient conditions. This needs to be explained in regard to protocols for quantifying NPQ phases with PAM chlorophyll fluorescence in liquid *C. reinhardtii* cultures.

Author

Good point. I have added "Importantly, consideration needs to be given to acclimation time to any change in trophic status or light intensity before performing experiments. At least 24 h is recommended if cells are to be considered acclimated to a specific state but breaking or training circadian rhythms can take longer (Mittag et al. 2005)."

Reviewer 2

The author should mention in the text that the stt7-9 mutant is not only leaky but also has different regulatory control mechanisms due to the ARG insertion into the STT7 kinase.

Author

I added the text: "Of note, the stt7-9 mutant is 'leaky' and can still phosphorylate LHCSR3, but insufficiently phosphorylates other LHC to enable qT (Bergner et al. 2015).