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Title: A novel screening method for hydrogenase-deficient mutants in Chlamydomonas reinhardtii based on in vivo chlorophyll fluorescence and photosystem II quantum yield.

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Keywords: Chlamydomonas reinhardtii, anaerobic photosynthesis, hydrogenase, chlorophyll fluorescence, microalgae, hydrogen photoproduction

Corresponding Author: Mr. Pierre Cardol,

Corresponding Author's Institution:

First Author: Damien Godaux

Order of Authors: Damien Godaux; Barbara Emonds-Alt; Nicolas Berne; Bart Ghysels; Jean Alric; Claire Remacle; Pierre Cardol

Abstract: In Chlamydomonas reinhardtii, prolonged anaerobiosis leads to the expression of enzymes belonging to various fermentative pathways. Among them, oxygen-sensitive hydrogenases (HydA1/2) catalyze the synthesis of molecular hydrogen from protons and reduced ferredoxin in the stroma. In this work, by analyzing wild type and mutants affected in H2 production, we show that maximal PSII photosynthetic electron transfer during the first seconds of illumination after a prolonged dark-anaerobiosis period is linearly related to hydrogenase capacity. Based on the specific chlorophyll fluorescence induction kinetics typical of hydrogenase-deficient mutants, we set up an in vivo fluorescence imaging screening protocol allowing to isolate mutants impaired in hydrogenase expression or activity, as well as mutants altered in related metabolic pathways required for energy production in anaerobiosis. Compared to previously described screens for mutants impaired in H2 production, our screening method is remarkably fast, sensitive and non invasive. Out of 3000 clones from a small-sized insertional mutant library, five mutants were isolated and the most affected one was analyzed and shown to be defective for the hydrogenase HydG assembly factor.

*Cover Letter

Université de Liège

Liège, 07 november 2012

Dear Editor,

Please find here enclosed a revised version of our manuscript entitled 'A novel screening method for hydrogenase-deficient mutants in *Chlamydomonas reinhardtii* based on *in vivo* chlorophyll fluorescence and photosystem II quantum yield.' that had received a positive feedback from the first round of reviewing.

We are grateful to you for having encouraged us to resubmit quickly a modified manuscript. We have addressed all the comments raised by the reviewers on our first submission. A point to point answer has been submitted along with the MS. Main changes are : (i) the addition of a new experimental set of data (hydrogen evolution measurements in all mutant strain and some control experiments) and (ii) all data that were previously not shown are now included in the manuscript in main tables or as supplemental figures.

We hope that we have now improved our manuscript so you'll find it suitable for publication in International Journal of Hydrogen Energy.

Sincerely yours,

On behalf all coauthors,

Pierre Cardol

Pierre Cardol, PhD, FNRS research associate
Génétique des microorganismes – Institut de Botanique B22 Boulevard du Rectorat, 27 – Université de Liège- B-4000 Liège, Belgique
Tel/Fax +32 4 3663840
e-mail: Pierre.cardol@ulg.ac.be

***Detailed Response to Reviewers**

Reviewer #1:

Major points:

I. Please measure and show, respectively, the hydrogenase activity of the wild types or control strains (like respiratory mutants, pfl1-mutant) in your setup. Available literature shows that absolute values can differ significantly depending on exact experimental setup and strain. As the correlation between PSII quantum yield and hydrogenase activity is the major point of your study, this data is essential, and citing hydrogenase activities reported in literature is not sufficient.

Answer : the initial rate of hydrogen production has been measured for wild-type and mutant strains and plotted against photosystem II quantum yield measured in the same conditions. These data are now presented in figure 2D, Table2 and supplemental figure 2. The corresponding paragraphs of results and materials and methods sections have been modified accordingly.

II. Please avoid "data not shown" (see individual points below)

Answer : All data are now included in the core text or as supplemental figures (see individual points below and above) and some have been deleted.

III. Please avoid confirming your data with unpublished results; omit or re-phrase to "probably", "suggesting" or something like that (see individual points below)

Answer : done (see individual points below and above).

1. p.8/Fig. 1A, B: are the values shown in Fig. 1A, B also from 3 experiments? Please indicate standard deviation. Are the higher values depicted for the hydEF-1 mutant in Fig. 1A significant? Wouldn't that mean that the mutant is also somehow affected under aerobic conditions? Please clarify

Answer : yes, values are from 3 experiments, at least. This is now indicated in the legend of the figure. We prefer to show only the mean fluorescence curves because showing standard deviations for each individual point would make the figure difficult to read. Regarding PSII efficiency, standard deviations are however shown in table 2 and pannels C and D and should be sufficient to estimate the variability in electron transfer measurement between strains and conditions. Concerning the slight difference in induction curves presented in pannel A, we think that it could be due to a different genetic background. This is now mentioned in the text.

2. in this context: it would be a nice control to record fluorescence and/or ETR for several minutes and upon re-aeration. According to your hypothesis, values should become equal in wt and mutant in aerobiosis/prolonged PSII activity, when Calvin cycle is re-engaged and hydrogenase becomes inactivated in wt.

Answer : this is now shown in supplemental figure 1. The kinetics of reaerated cultures is very close to the kinetics of aerobic cells. We draw the attention of the reviewer on the fact that ETR during the first second of illumination in aerobiosis is mainly dependent of oxygen presence through the mehler reaction.

3. p.8, Figures 1B, D: please show or indicate in text hydrogenase activity (or the lack of in +O2) in wt (and confirm no hydrogenase activity in mutant) both in aerobiosis and anaerobiosis

Answer : an hydrogen production has been detected only for wild-type cells incubated over night in anaerobiosis. This is now indicated in the text.

4. p.8: "This is confirmed by the lack of significant PSI-P700 oxidation for hydEF-1 mutant cells in anaerobiosis (Ghysels, Matagne and Franck, personal communication)" -It's inappropriate to confirm your data with unpublished results. Maybe re-phrase

Answer . it was rephrased.

5. p.9, description of respiratory mutants ("In these two respiratory-deficient mutants, H2 production as well as HydA expression levels after 48h of sulphur starvation were less than 20% and 5%, respectively, of the wild-type values") - S-deprivation is a completely different condition; please describe or show hydrogenase activity of the respiratory mutants in your system (dark/anoxic)

Answer : the initial rate of evolved hydrogen upon illumination has been measured for strains depicted in figure 2A-C and plotted against photosystem II quantum yield measured in the same conditions. These data are now presented in figure 2D and supplemental figure 2. The corresponding paragraphs of results and materials and methods sections have been modified accordingly.

6. p.10, top: ".checked by spectrophotometric measurements that lower PSII activities in anaerobiosis were not due to PSII or PSI degradation (data not shown)." -please show data

Answer : PSI :PSII stoichiometry and PSI per chlorophyll values for representative mutants were added to Table 2.

7. p.10, second paragraph: "Similarly, PSII-dependant oxygen evolution upon illumination of dark anaerobic cells relies on hydrogenase abundance (Ghysels, Matagne and Franck, personal communication)" - see above, please don't confirm your data with unpublished results. Omit or re-phrase

Answer : this sentence has been deleted

7. p.10, "Again, the time-course of ?II followed the increase reported for HydA expression and hydrogenase activity during a similar protocol [19]." Please show hydrogenase activity in your system; the activities and time-courses differ significantly in different labs and set-ups; for your main conclusion, it is essential to show hydrogenase activity values in your lab

Answer : due to lack of time for revisions we did not perform this analysis. Figure 2D which only comprises the results obtained for mutant strains is sufficient to support our conclusions. However, we would like to keep the relationship between hydrogenase activity and fluorescence measurements during acclimation to anaerobiosis. The relationships between our data, data of Forestier et al., 2003 and a new set of data taken from a more recent paper from another group (Pape et al., 2012) are now shown in supplemental figure 3 and correlation coefficient for the linear regression are given in the text

8. see paragraph below "In an attempt to get a more quantitative correlation between the hydrogenase activity/expression and capacity of PSII electron transfer, we plotted ?II values at ~300 photons nm-2 . s-1 against hydrogenase activity, expression or H2 production (Figure 2F). The latter values were obtained from various sources in the literature [19, 23, 27-29, 44, 45]." It is not correct to use values from literature, please verify in your lab AND indicate which value you use - as you indicate A.U. in Fig. 2F and activity OR expression, the reader does not know what you mean at all

Answer : we now provided our own set of data (see above)

9. Fig. 2: please indicate number of replicates and show (or indicate) standard deviation

Answer : This is now indicated in the legend of the figure. We prefer to show only the mean fluorescence curves because showing standard deviations for each individual point would make the figure difficult to read. Regarding PSII efficiency, standard deviations are now given in Table 2.

10. p.11: "During this procedure, the fluorescence parameters varied significantly between plates, probably because the anaerobic state was reached after a variable time of incubation in the dark." - why should that be? Didn't you use a commercially available system, and shouldn't this work rather uniformly? Could it rather be that your colonies were at different growth stages? Please explain and specify how you inoculated your plates and how long the colonies grew before the analysis

Answer : the text (methods and results) has been amended to better explain the plating procedure and the expected reason for variability between plates.

11. p.12, top "compared to the average induction curve of all mutants (around 40) of a same plate." Is that correct? If you use this approach, please show the 40 induction curves of one plate so that the reader can assess the variability. I think it would be better to have plates inoculated with wt in exactly the same way / same day / same plates for comparison, or pick some hygromycin resistant strains with wt-like photosynthetic parameters as control

Answer : the reviewer is maybe right but we cannot change anymore the screening procedure. For sure, we'll take his recommendation into account in future analysis. Concerning the variability of the 40 induction curves of each plate, this information was already given in table 3.

13. p.12, the quality of the manuscript would benefit profoundly by showing data about the other mutants found by the screening protocol - did they have a hydrogenase-impaired phenotype? If not, can you narrow down the photosynthetic defect somehow? (e.g. by chlorophyll fluorescence measurements, you could at least indicate if PSII activity itself or another complex/process is affected) - I won't insist on this information, but it would clearly enhance the quality of the paper

Answer : As written in the former version of manuscript, the other mutants identified through the screening procedure are under investigation and will be (hopefully) the subject of future publication(s). Some information regarding chlorophyll measurements and induction curves are however given in table 3.

14. p.12 bottom, p.13 top: "Our mutant, hydG-2, is thus allelic to the hydG-1 mutant. It behaves exactly has the hydEF mutant regarding photosynthetic activity in aerobiosis and anaerobiosis (data not shown), ." - please show data

Answer : the data are provided in table 2.

15. p. 12, hydG mutant: please show hydrogenase activity (or lack of it, respectively)

Answer : data are now shown in table 2 and raw records are shown in supplemental figure 2.

16. p.14, discussion, second paragraph: as far as I understood, Pape et al used an arylsulfatase screening, not a chemochromic sensor

Answer : No, Pape et al. used a chemochomic sensor to isolate hydG-1 and then used this new mutant to setup their screening procedure using arylsulfatase.

17. p.15, discussion, 2nd paragraph "Signal induction for hydrogenase has been suggested to be ATP generated by cyclic electron flow around PSI" I don't understand what you mean, please re-phrase

Answer. This sentence has been completely reformulated. We apologize for this unintelligible sentence.

18. and: "In respiratory-deficient mutants, reduced ATP levels in aerobiosis in the light are however sufficient to trigger the induction of H2 production under sulfur deprivation but the amount of hydrogenase rapidly declines, probably because energetic metabolism is too much affected in these mutants" - again, I don't know what you mean, please clarify

Answer. This sentence has been reformulated. We hope that it is now clear.

19. p.15, discussion, 3rd paragraph: "Even at very low light intensities (data not shown), PSII activity is fully inhibited" please avoid using "data not shown" - either show or don't mention

Answer : this minor part of the conclusion and corresponding “data not shown” were discarded since they don’t bring anything to the main core conclusion.

Minor points:

1. p.3, l.7: "that is branched to the photosynthetic electron transport chain" - "branched to", is that the correct phrase?

Answer : the sentence has been rephrased.

2. p.3, l.16: expression, activation AND activity

Answer : change has been made

3. p.3, l.23: proper "coordination"? Do you mean "assembly" or "maturation"?

Answer : coordination has been changed by maturation

4. p.5, materials and methods 2.1, "under continuous with light of 70 <mu>mol photons."; do you mean "white" light?

Answer : Yes, white light.Change was done.

5. p.5, materials and methods 2.2, "Cells were harvested during exponential growth phase (3-5 x 106 cells/ml) and resuspended at a concentration of 10 <mu>g chlorophyll/ml in TAP medium" - please indicate both densities either in cell number or in Chl

Answer : both units are now given

6. p.6, materials and methods 2.3; please explain why the cells are illuminated with green light

Answer : Green light (520 nm) is also suitable as actinic light : [On the advantages of using green light to study fluorescence yield changes in leaves. Rappaport F, Béal D, Joliot A, Joliot P. Biochim Biophys Acta. 2007 Jan;1767(1):56-65.]. No change was made in the text.

7. p.10: ".maximal value, reflecting transition to state II in anaerobiosis" - please show state-transitions or re-phrase ("Probably reflecting.")

Answer : Although Fm decrease in dark anaerobic conditions has been documented several time as related to State transitions, we added « probably. » as suggested by the reviewer,

8. p.10: "PSII quantum yield (?II) collapsed upon dark anaerobic adaptation and remained low during the first minutes." why is (?II) so low at the very beginning? Shouldn't it be higher (0h = pre-culture = illuminated/aerated?) please explain

Answer : sorry, the Yii value at time 0 for aerobic cells was missing. We now added the value for aerobic cells in panel 2E.

9. Highlights: >HYDG assembly factor is required for hydrogenase maturation process< is not novel

Answer : highlights were changed accordingly.

10. general: the text would benefit from editing by a native speaker

Answer : the text has been carefully scanned for English mistakes.

Reviewer #2:

a) That a manuscript on a method for identifying hydrogen production activity is performed without a single hydrogen production measurement is, let's admit it, rather strange as it is the figure 2F, a key figure in the manuscript and yet confined to one out of 6 panels, where the data are half experimental and half taken from literature. Why not to measure hydrogen production by gas-chromatography or even by volumetry?

Answer : as indicated above, hydrogen evolution measurements have been made and are now presented in Figure 2D, Table 2 and supplemental figure 2.

b) The draw-back of the existing method are highlighted and the simplicity of the method proposed in this ms is exaggerated. The present method does require a good competence in kinetic fluorometry of the system in study. A more equilibrated discussion would be useful.

Answer : we tried to equilibrate the discussion. A good competence in kinetic fluorometry is indeed necessary and we tried here to pave the way of the understanding of electron transfer in anaerobiosis. I don’t feel that the (non-)expertise of the scientists should be a limitating factor while discussing on the advantage of a method on another.

Editor's remarks: Please change your opening sentence. Hydrogen is not a "source" of energy like solar, wind, or oil; it has to be made from a primary source like solar, wind, or oil (nearly all commercial hydrogen is in fact made by steam reforming of natural gas). It is correct to call hydrogen an energy "carrier" in the same sense as electricity.

Answer : « source » has been deleted

***Highlights (for review)**

>In Chlamydomonas, PSII efficiency in anaerobiosis is hydrogenase-dependant.

>Chlorophyll fluorescence signature allows screening hydrogenase-deficient mutants

>The developed screening strategy is fast, sensitive and non invasive.

> HydG-2 mutant behaves the same as HydEF-1 mutant regarding photosynthesis.

Title

A novel screening method for hydrogenase-deficient mutants in *Chlamydomonas reinhardtii* based on *in vivo* chlorophyll fluorescence and photosystem II quantum yield.

Authors

Damien Godaux[a], Barbara Emonds-Alt[a], Nicolas Berne[a], Bart Ghysels[b], Jean Alric[c], Claire Remacle[a], Pierre Cardol[a*]

[a]Laboratoire de Génétique des Microorganismes, Institut de Botanique, Université de Liège, Belgique; [b]Laboratoire de Bioénergétique, Institut de Botanique, Université de Liège, Belgique ; [c] UMR 7141, CNRS et Université Pierre et Marie Curie (Paris VI), Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie 75005 Paris, France.

*corresponding author :

Pierre Cardol, PhD, FNRS research associate

Génétique des microorganismes – Institut de Botanique B22 Boulevard du Rectorat, 27 – Université de Liège- B-4000 Liège, Belgique

Tel/Fax +32 4 3663840

e-mail: Pierre.cardol@ulg.ac.be

Abstract

In *Chlamydomonas reinhardtii*, prolonged anaerobiosis leads to the expression of enzymes belonging to various fermentative pathways. Among them, oxygen-sensitive hydrogenases (HydA1/2) catalyze the synthesis of molecular hydrogen from protons and reduced ferredoxin in the stroma. In this work, by analyzing wild type and mutants affected in H_2 production, we show that maximal PSII photosynthetic electron transfer during the first seconds of illumination after a prolonged dark-anaerobiosis period is linearly related to hydrogenase capacity. Based on the specific chlorophyll fluorescence induction kinetics typical of hydrogenase-deficient mutants, we set up an *in vivo* fluorescence imaging screening protocol allowing to isolate mutants impaired in hydrogenase expression or activity, as well as mutants altered in related metabolic pathways required for energy production in anaerobiosis. Compared to previously described screens for mutants impaired in H_2 production, our screening method is remarkably fast, sensitive and non invasive. Out of 3000 clones from a small-sized insertional mutant library, five mutants were isolated and the most affected one was analyzed and shown to be defective for the hydrogenase HydG assembly factor.

Keywords : *Chlamydomonas reinhardtii*, anaerobic photosynthesis, hydrogenase, chlorophyll fluorescence, microalgae, hydrogen photoproduction

1. Introduction

Hydrogen is thought to be a promising renewable energy for future because of its high energy content and its clean combustion into water. H_2 production is currently realized by chemical extraction from fossil hydrocarbons, water photolysis or biomass pyrolysis [1, 2]. Nevertheless, all of these processes consume a large amount of energy. Seventy years ago, Gaffron and co workers first reported the ability of some fermenting microalgae to evolve H_2 under illumination in anaerobic condition [3]. H_2 production in microalgae is possible due to the presence in the chloroplast of a [Fe-Fe] hydrogenase enzyme that catalyzes the reduction of two protons into H_2 and the reoxidation of ferredoxins reduced by the photosynthetic electron transport chain [4-7]. [FeFe] hydrogenases are also found in strictly anaerobic bacteria such as *Clostridium sp.* as well as in several eukaryotic protists such as *Trichomonas* where they can act as monomers or heteromultimers [8]. From X-ray crystallographic structural data available for two representative bacterial enzymes [9-11], their catalytic center (called H-cluster) is made of a [4Fe4S] coordinated by four cystein residues connected to a unique 2Fe center by a cysteinyl ligand. In addition to binding CO and CN, each of the iron atom coordinates a bridging organic ligand thought to be a di(thio-methyl)amine moiety [12]. An important limitation for this biohydrogen photoproduction is that hydrogenase only operates under anaerobic conditions because its expression, activation and activity is sensitive to oxygen [13-17]. In microalgae, the enzyme is quickly inactivated by photosystem (PS) II-dependent O_2 evolution upon illumination.

The green algal model *Chlamydomonas reinhardtii* possesses two hydrogenases called HydA1 and HydA2 [18, 19], HydA1 being responsible for the major hydrogenase activity under anaerobic conditions [20, 21]. To date, two assembly factors (HydEF and HydG) belonging to the "S-adenosylmethionine radical" protein super-family have been proved to be required for the proper maturation of the H-cluster of [FeFe] hydrogenase [22]. *Chlamydomonas* mutants devoid of HydEF or HydG are incapable of assembling an active [FeFe] hydrogenase [23-25]. Mutants defective in copper

response regulator 1 (Crr1) [26], in pyruvate formate lyase (Pfl1) [27], in chloroplast type II dehydrogenase (Nda2) [28], in starch accumulation (*sta7-10)* [25] or in mitochondrial respiratory chain complexes (*dum11, dum22*) [29] are also affected in *HYDA1* expression and/or H_2 production. These mutants provide links between H_2 production, fermentation, starch degradation, mitochondria bioenergetics and sensing of oxygen.

Among the mutants mentioned here above, *crr1-3*, *pfl1, hydEF-1* and *hydG-1* mutants have been isolated from a screening protocol based on chemochromic sensing of H_2 [30] while the other mutants were tested for H_2 production after their isolation based on other criteria. To date, three alternative screening systems have been established to allow identification of genes involved in H_2 metabolism and hydrogenase function: (i) another H_2-sensing system based on fluorescence emission of *Rhodobacter capsulatus* upon cell exposure to hydrogen [24]; (ii) a screening protocol called Winkler test which identifies strains in which the ratio of photosynthetic O_2 evolution and respiratory O_2 uptake is lowered so that these strains establish anaerobic conditions in the light, allowing them to express hydrogenase and to produce H_2 [31], and (iii) a screening system based on differential PSII chlorophyll fluorescence in state 1 and state 2 which allows the identification of mutants deficient in state transitions [32] and mutants impaired in cyclic electron flow around PSI, a process which competes with hydrogenases for electrons [33].

In this work, we describe a novel and fast screening protocol to identify players involved in the biosynthesis of [FeFe] hydrogenases or in the electron transfer towards H_2 production. We took advantage of the peculiar anaerobic chlorophyll fluorescence kinetics of hydrogenase-deficient mutants to evaluate by *in vivo* fluorescence imaging the photosynthetic electron flow linked to hydrogenase activity. This protocol allowed us to screen about 3000 transformants in one day and led to the isolation of a HydG deficient mutant *(hydG-2)*, thereby demonstrating a proof of concept for the screening for hydrogenase mutants.

2. Materials and methods

2.1. *Chlamydomonas reinhardtii* strains and growth conditions

All mutants used in this work are summarized in Table I. *mt+* and *mt-* wild-type strains used for insertional mutagenesis and genetic cross experiments (1' and 2' in our stock collection, respectively) derive from 137c reference wild-type strain.

Cells were routinely grown under mixotrophic conditions in Tris-acetate-phosphate (TAP) medium at 25°C under continuous white light of 70 µmol photons m^{-2} s^{-1}. Hygrogmycin-B was added to a final concentration of 25 mg/l.

2.2. Chlorophyll fluorescence yield kinetics

Cells were harvested during exponential growth phase (3–5 x 10^6 cells/ml or 8-12 µg chlorophyll/ml) and resuspended at a concentration of 10 µg chlorophyll/ml in fresh TAP medium. For the determination of chlorophyll concentration, pigments were extracted from whole cells in 90% methanol and debris were pelleted by centrifugation at 10000 x *g*. Chlorophyll a+b concentration was determined with a lambda 20 spectrophotometer (Perkin Elmer, Norwalk, CT) according to [34]. For *in vivo* spectrofluorimetry, liquid suspensions were sealed in air-tight spectrophotometric cuvettes. Anaerobiosis was reached after 1 hour of adaptation in the dark, due to cellular respiration, or after a few seconds following addition of catalase (1000 U/mL), glucose (10mM), and glucose oxidase (2mg/mL).

In vivo fluorescence measurements were performed at room temperature on cell liquid suspensions using a JTS-10 spectrophotometer (Biologic, France). Upon illumination, the fluorescence yield rises from a basal value (F_0) to reach a steady-state value (F_S) in ~1 second. After 3 seconds of illumination, a saturating pulse is given to fully reduce PSII acceptors and reach the maximal fluorescence yield (F_M). These three values allow us to calculate the maximal quantum yield of PSII photochemistry [$F_V/F_M=(F_M-F_O)/F_M$] and the PSII quantum yield at a given light intensity [$\Phi_{II} = (F_M-F_S)/F_M$] [35, 36].

Actinic light was provided by a continuous red (640 nm) LED source. Light intensity (I) ranged from 20 to 1250 photons . nm^{-2} . s^{-1}. The apparent PSII electron transport rate (ETR'_{II}) at a given light intensity was calculated as the product of Φ_{II} * I [36].

2.3. PSI/PSII stoichiometry

To determine the amount of p700, the primary electron donor of PSI, we measured light-minus-dark difference absorption at 705 using ε_{705nn} = 64 mm^{-1} cm^{-1} [37] with a JTS-10 spectrophotomer (Biologic, France). Oxidized p700 absorption was obtained for dark aerobic or reoxygenated anaerobic cells while reduced p700 absorption was measured after 250 ms under illumination of ~1250 photons . nm^{-1} . s^{-1} in the presence of 5 µM DBMIB to prevent p700 rereduction by cytochrome b_6f complex activity. PSI and PSII content was estimated spectroscopically from changes in the amplitude of the fast phase (600 µs) of the ECS signal (at 520–545 nm) upon excitation with a saturating laser flash [38] on dark aerobic or reoxygenated anaerobic cells. The PSII contribution can be calculated from the decrease in the signal amplitude upon the addition of DCMU (20 µM) and hydroxylamine (1 mM) that irreversibly block PSII charge separation once the sample has been pre-illuminated. Conversely, PSI was estimated as the fraction of the signal that was insensitive to these inhibitors.

2.4. Hydrogen evolution. Hydrogen evolution was measured using an oxygen-sensitive Clark electrode (Oxygraph, Hansatech Instruments) modified to only detect hydrogen (Oxy-Ecu, Hansatech Instruments). Near-saturating actinic light was provided by a home-made light system composed of white and green leds. The entire setup was placed in a plastic tent under anoxic atmosphere (N_2) to avoid contamination of anaerobic samples by oxygen while filling the measuring cell of the oxygraph.

2.5. Insertional mutagenesis and fluorescence screening procedure

Insertional mutagenesis library was generated by transforming *Chlamydomonas reinhardtii* wild-type strain (1' in our stock collection) by electroporation [39] with *APHVII* resistant cassette derived from pHyg3 plasmid [40]. This hygromycin resistance cassette contains the coding sequence of bacterium

gene *aphVII* disrupted by the first intron of *RBCS2* gene under control of β2-tubulin promoter and *RBCS2* terminator. Each transformation was performed with 250 ng of hygromycin-B resistance cassette, 10 µg of carrier DNA and 2.5 10^7 cells into a disposable electroporation cuvette with a 1cm gap. Standard parameters applied for the electroporation were 800 V, 25 µF and 800 Ω using the electroporator [39]. Selection was performed during ten days on TAP agar medium containing 25 mg/l hygromycin-B. Individual colonies were then transferred on fresh TAP medium (about 40 colonies per plate) and grown for 3 days in the light. Anaerobiosis was then reached by placing Petri dishes containing transformants into air-tight transparent plastic bags for anaerobic conditions (GasPak™ EZ Pouch System, BD) in the dark. According to manufacturer, anaerobiosis occurs within the first two-three hours. After adaptation (>12h) to dark anaerobic conditions, colonies were subjected to illumination by a fluorescence imaging system (Speedzen, Beambio, France) described in details in [41]. This system measures time-resolved chlorophyll fluorescence in response to light. This image readout system, synchronized with actinic light and saturating pulses, allows a precise determination of F_o and F_M, which is required to monitor PSII activity. Illumination of colonies lasts three seconds with an actinic light of ~500 µmol photons at 520nm . m^{-2} . s^{-1}, before applying to colonies a saturating pulse to evaluate the photochemical yield of PSII (Φ_{II}).

2.6. Genetic analyses

Identification of insertion-linked sequences by thermal asymmetric interlaced (TAIL)-PCR was conducted as described in [42]. Using primers that are located at the 5' (APH7R3:AGAATTCCTGGTCGTTCCGCAG; APH7R4:TAGGAATCATCCGAATCAATACG; APH7R5: CGGTCGAGAAGTAACAGGG) and 3' end (Hyg Term 1:CGCGAACTGCTCGCCTTCACCT; Hyg Term 2: TCGAGGAGACCCCGCTGGATC; Hyg Term 3; CGATCCGGAGGAACTGGCGCA) of the coding sequence of the *APHVII* gene for the primary, secondary, and tertiary reactions, respectively, this technique (for details, see [43]) allows the amplification of DNA regions flanking the insertion site of the hygromycin cassette. PCR products were sequenced (Beckman Coulter Genomics) and similar nucleotide

sequences were searched into the *Chlamydomonas* genome sequence database (v5.3 available at http://www.phytozome.net).

Crossing between B3K56/212 mt^+ strain and a wt mt^- strain was conducted [44] and meiotic progeny was selected on TAP medium.

3. Results

3.1. Distinct chlorophyll fluorescence signatures in anaerobiosis between wild type and hydrogenase-deficient mutant

We compared mean chlorophyll fluorescence kinetics of the *Chlamydomonas reinhardtii* HydEF-deficient mutant and wild-type strains during the first seconds of illumination at ~300 photons nm^{-2} . s^{-1} (Figure 1A and 1B). This light intensity was chosen because the apparent PSII electron transport rate (ETR'_{II}) reaches a value which is close to its maximum (see below and Figure 1C). Prior to measurements, cells were acclimated to the dark for several hours in aerobic or anaerobic conditions. In aerobiosis, only a slight difference in the curve shape (Figure 1A) or in F_V/F_M and Φ_{II} values (Table 2) could be observed between wild-type and mutant strains. We attributed this minor difference to the fact that *hydEF-1* mutant was isolated from another wild-type strain. The various phases of the fluorescence induction rises reflect, as reviewed elsewhere [45], the reduction of the primary PSII acceptor (bound plastoquinone (PQ) Q_A), the reduction of PQ pool and the oxidation of PQ by cytochrome b_6/f complex. For dark anaerobic acclimated cells (Figure 1B), F_O is close to F_M which indicated that the PSII acceptor pool (Q_A, PQ) is largely reduced, probably due to the activity of type II NAD(P)H dehydrogenase (Nda2) and to the absence of plastoquinone oxidase (Ptox2) activity in anaerobiosis. In a few ms, the fluorescence reaches a maximal transient value which is close to F_M and then it decreases in both strains. After a few hundred ms the fluorescence yield is stable for wild-type cells and Φ_{II} is about half the one measured for aerobic cells (Table 2). In contrast, for *hydEF-1* mutant cells, fluorescence signal increases to reach a value which is close to F_M. Φ_{II} is thus very small, showing that in the absence of hydrogenase, the pool of PSII electron acceptors is very small and the rate of oxidation of these photosynthetic electron acceptors is very low. Several experimental results support this hypothesis. Firstly, when wild-type and *HydEF-1* mutant cells that have been incubated in anaerobiosis are reoxygenated for a few minutes, they show chlorophyll fluorescence kinetics (supplemental Figure 1) similar to those recorded for aerobic cells. Secondly a

lack of PSI-P_{700} oxidation for *hydEF-1* mutant cells in anaerobiosis has been observed (Ghysels, Matagne and Franck, personal communication). Finally, a significant hydrogen evolution could be only observed for anaerobic wild-type cells (2.05 ± 0.36 nmoles H_2 . min^{-1} . mg^{-1} chlorophyll at 300 photons . nm^{-2} . s^{-1}).

ETR'_{II} was then measured in aerobic and anaerobic conditions as a function of the light intensity (Figure 1C and 1D). Because of the absence of HydA enzymes in aerobiosis, no significant difference was observed between wild-type and mutant cells in the presence of oxygen (Figure 1C). In contrast, in anaerobiosis, while the PSII electron transfer rate reaches in wild type a maximal value which is about half the one measured in aerobiosis (Figure 1D), it is almost prevented in the hydrogenase-deficient cell line with a maximal value of 5%. At this stage, the nature of the electron sink in the mutant has not been evaluated. It could be due to a weak PSI activity because of the presence of residual oxygen or to NADPH or Fdx reoxidation by fermentation pathways.

3.2. Relationship between hydrogenase expression/activity and PSII electron transfer

In a next step and in order to determine whether the use of chlorophyll fluorescence induction curves could be used to isolate mutants with reduced HydA1 expression level, hydrogenase activity or H_2 production in anaerobiosis, we analyzed several mutants (Table 1) for which such a phenotype has been reported earlier in the literature. *pfl1* mutant is defective in pyruvate formate lyase and showed a 30-45 % residual hydrogenase activity and H_2 production compared to cc-124 wild-type strain [27, 46]. It was here compared to a parental cc-125 wild-type strain and to another fermentation mutant strain (*adh1*) lacking bifunctional acetaldehyde/alcohol dehydrogenase for which no hydrogenase deficiency has been reported [47]. Mutants lacking mitochondrial respiratory-chain complex III (*dum11*) or complexes I and III (*dum22*) have been also investigated. In these two respiratory-deficient mutants, H_2 production as well as HydA expression levels after 48h of sulphur starvation were less than 20% and 5%, respectively, of the wild-type values [29]. Knock-down mutant

for type-II NAD(P)H dehydrogenase Nda2, which showed a H_2 production diminished by about a half (Jans et al., 2008) was also investigated and compared to its wild-type parental strain (84).

No significant difference in the chlorophyll fluorescence induction curves was observed between various wild-type strains investigated (Figure 2). In contrast, *pfl1*, *dum11* and *Nda2-RNAi* mutant cells exhibited intermediary chlorophyll fluorescence induction curves (Figure 2) with reduced Φ_{II} values. This suggested that in these mutants, the photosynthetic electron transfer is limited by the availability of electron acceptors, probably due to lower hydrogenase levels. In wild type and most mutants, we checked by spectrophotometric measurements that lower PSII activities in anaerobiosis were not due to PSII or PSI degradation (Table 2). Respiratory-deficient d*um22* mutant cells showed a monotonous fluorescence curve, suggesting that PSII is fully inactive in this mutant, most probably because *dum22* cells cannot tolerate long period in dark and anaerobic conditions (see discussion).

In an attempt to get a quantitative correlation between hydrogenase activity and PSII electron transfer, we plotted Φ_{II} values against initial rate of hydrogen evolution measured under illumination light of ~300 photons . nm^{-2} . s^{-1} (Figure 2D and Supplemental Figure 2). From this analysis, we concluded that the PSII quantum yield (Φ_{II}) measured after 3 s of near-saturating illumination in anaerobiosis reflects accurately hydrogenase activity at the onset of illumination (R^2 = 0.88 for a linear regression) and could be used to screen mutants with an altered H_2 production.
In a complementary approach, we monitored the chlorophyll fluorescence kinetics of wild-type cells as a function of the incubation time in anaerobiosis. It has been previously shown that in anaerobiosis, HydA1 expression level and hydrogenase activity reached a maximal value after ~150 min with a half-time of ~30 min [19]. In Figure 2E, the main fluorescence parameters (F_M, F_v/F_M, and Φ_{II}) were calculated and plotted against the time of incubation in anaerobiosis. F_v/F_M progressively decreased indicating a reduction of PSII direct acceptors and F_M quickly dropped to 60% of its maximal value, probably reflecting transition to state II in anaerobiosis [48]. PSII quantum yield (Φ_{II}) collapsed upon dark anaerobic adaptation and remained low during the first minutes in anaerobiosis,

reflecting the lack of PSI acceptors (oxygen through the Mehler reaction or $NADP^+$ following inactivation of Calvin cycle) but recovered and reached a maximal value after 2-3 hours (half-time ca. 30 min) (Figure 2E). Again, the time-course of Φ_{II} followed the increase reported for HydA expression and hydrogenase activity during same protocols [19, 26]. This is illustrated in Supplemental Figure 3 where we plotted the Φ_{II} values that we measured during acclimation to data obtained in previous independent studies for hydrogenase activity/expression [19] and for *in vitro* hydrogenase activity [26] (R^2 = 0.96 and 0.93 for linear regressions, respectively).

3.3. Other mutants impaired in photosynthetic electron transport chain

To check the specificity of the chlorophyll fluorescence induction signature attributed to a deficiency in hydrogenase activity, several other mutants affected in various components of the photosynthetic electron transfer chain were analyzed. As shown in Figure 2F, fluorescence kinetics of non-photosynthetic mutants defective in photosystem I (Δ*psaB*) [49] or in cytochrome b_6f complex (Δ*petB*) [50] are distinct from the one observed for *hydEF-1* mutant. A single phase was observed and it consisted in an increase of fluorescence yield in a few milliseconds to reach F_M. Chloroplastic plastoquinol oxidase-deficient strain (*ptox2*) which is different from WT under aerobic conditions [51] is rather expectedly identical to WT under anaerobic conditions (Figure 2C). Similarly, the phosphoribulokinase-deficient mutant strain *prk* [52] which is impaired for the Calvin cycle showed a wild-type fluorescence signature (Figure 2F). Thus mutants impaired in linear photosynthetic electron flow display specific chlorophyll fluorescence induction curves, different from the one described for hydrogenase-deficient mutant strains.

3.4. Setup of a new fluorescence screen to isolate mutants impaired in hydrogenase activity.

Because of the specific chlorophyll fluorescence kinetics of mutants impaired in hydrogenase function, we thus designed a screening procedure based on time-resolved video imaging of

chlorophyll fluorescence. After transformation of a wild-type strain using a hygromycin resistance cassette (*AphVII*), 3000 transformants resistant to hygromycin were plated on TAP solid medium, acclimated to dark and anaerobic conditions for at least 12 h, and further analyzed with respect to their chlorophyll fluorescence signature. During this procedure, the fluorescence parameters varied significantly between plates (Table 3), probably because the anaerobic state was reached after a variable time of incubation in the dark due to important variation of air volume contained in each pouch before sealing. For this reason, fluorescence induction curve of each mutant was compared to the average induction curve of all mutants (around 40) of a same plate. From this analysis, nine colonies showed Φ_{II} values below 40 % of the average value of transformants of the same plate (Table 3). Among them, five harbored a chlorophyll fluorescence induction signature in anaerobiosis similar to the one recorded for hydrogenase-impaired mutant strains (Figure 1) while four showed a monotonous flat fluorescence yield close to the one recorded for mitochondrial *dum22* mutant or for PSI and cytochrome b_6f-deficient mutants (Figure 2). Figure 3 shows the fluorescence induction kinetics of the most affected hydrogenase deficient-like mutant (B3K56/212) (panel A) and a false-color image of Φ_{II} values for the B3K56 series of transformants (panel B).

3.5. Characterization of the B3K56/212 mutant strain

In the last part of the work, to validate the screening procedure, we focused our efforts on the characterization of B3K56/212 mutant strain. In order to determine whether the insertion of the cassette responsible for hygromycin resistance was linked to the fluorescence phenotype in anaerobiosis, a genetic cross between B3K56/212 mt^+ strain and wt mt^- strain was conducted. From 352 meiotic progeny analyzed, all hygromycin-resistant strains (59 % of the progeny) were photosynthetically inactive in anaerobiosis while all hygromycin-sensitive strains (41%) showed a Φ_{II} value close to the one measured for wild-type parental strain. This indicated that only one cassette was inserted in the mutant genome and that the resistance cassette was responsible for the photosynthetic phenotype in anaerobiosis.

Since the mutant was tagged with the insertional marker, we sought to identify the interrupted locus by TAIL-PCR analysis (see methods section for details). This allowed us to localize the hygromycin resistant cassette in the first exon of the *HYDG* gene (cre06.g296700.t1.1, chromosome_6: 6998683 - 7003289), after 48 nucleotides in the 5' untranslated region (Figure 3C). This insertion is accompanied by a deletion of 104 bp and is most probably different from the one found in another mutant isolated very recently [24]. Our mutant, *hydG-2,* is thus allelic to the *hydG*-1 mutant. It behaves exactly has the *hydEF* mutant regarding photosynthetic activity in aerobiosis and anaerobiosis (Table2), thus confirming that *in vivo* both HydG and HydEF assembly factors are required for an efficient hydrogenase activity [23, 25].

4. Discussion

The effect of anaerobic pre-incubation in the dark on photosynthesis reactions had been already studied forty years ago in microalgal species naturally provided with or devoid of an hydrogenase [53-56]. Oxygen evolution and chlorophyll fluorescence measurements indicated an inhibition of PSII after anaerobic incubation of algae deprived of hydrogenase. In this respect, the data obtained for *Chlamydomonas reinhardtii* are very similar to those obtained for *Scenedesmus obliquus.* On the one hand chlorophyll fluorescence induction curves obtained for *Chlamydomonas* wild-type strains match those obtained for *Scenedesmus* after long adaptation (>60 min) to anaerobiosis, when hydrogenase was probably expressed. On the other hand fluorescence kinetics of *Chlamydomonas hydG-2* or *hydEF-1* mutants are similar to those shown in *Scenedesmus* for shorter anaerobic adaptation periods, when hydrogenase was not expressed (>20 min) [53]. Altogether, these results thus support the idea that H_2 photoproduction by hydrogenase is the process which enables algae to oxidize and thereby activate their photosynthetic electron transport chain after anaerobic incubation.

Chlorophyll fluorescence is commonly used to monitor photosystem II (PSII) photochemistry, linear electron flux, or CO_2 assimilation *in vivo* in algae and plants [57]. *In vivo* chlorophyll fluorescence imaging also proved to be a powerful tool to isolate mutants of the photosynthetic machinery and

associated mechanisms in *Chlamydomonas reinhardtii* [33, 41, 51, 58, 59]. Compared to other screening methods previously developed to identify mutants defective in H_2 production or hydrogenase activity/expression [24, 30-32], the present screen based on distinctive fluorescence kinetics of hydrogenase-deficient mutants has three main advantages. (i) This is a non-invasive procedure since *Chlamydomonas* cells can recover from exposures to dark anaerobic conditions of several days (data not shown). (ii) The protocol is quite fast (a few thousands transformants analyzed a day) by using a camera with a high sensitivity which enables the direct screen of colonies. (iii) Because our data indicate that PSII activity upon the first seconds of illumination is linearly related to hydrogenase activity, screening mutants for PSII quantum yield would allow the recovery of mutants with decreased as well as increased hydrogenase activity/expression. In fact, as shown in Figure 1C and D, the electron transport rate of wild-type cells is at least two times slower in anaerobiosis than in aerobiosis. The anaerobic yield of PSII being probably limited by hydrogenase, an increased hydrogenase activity is therefore likely detectable as an increase of the yield of PSII.

To date, only three direct actors (HydEF, HydG and Crr1) for hydrogenase transcription and maturation have been described in *Chlamydomonas* [23, 24, 26] and they all have been identified with help of a chemochromic sensor [30]. According to the authors [see also [60] for a review], the achievement of such a sensor is labored and several problems could arise with this method. In particular, the sensitivity limit of the sensor is at least 4 nmoles of H_2 evolved per colony and this is typically achieved within two minutes of illumination. If illumination is too long or too high, PSII activity will rapidly lead to the inactivation of hydrogenase enzyme by O_2 evolution and the amount of produced H_2 will then be insufficient to turn on the sensor. Although our screening system requires a sophisticated fluorescence imaging system, it circumvents the problems depicted hereabove for the chemochromic sensor : it does not require a direct measurement of evolved H_2, it is relevant on a wide range of light intensities, and it requires only an illumination period of few seconds which prevents hydrogenase inactivation.

Various metabolic alterations can lead to hydrogenase deficiency and to the prospected chlorophyll fluorescence signature including an altered PSII quantum yield upon illumination in anaerobiosis. Pyruvate formate lyase-deficient strain has been extensively studied [27, 46] and it exhibits a rearranged anaerobic metabolism in which *HYDA1* transcript and HydA1 protein levels are very low, showing that pyruvate formate lyase activity has a significant impact on H_2 metabolism. In contrast, *adh1* mutant strain defective in acetalhedyde/alcohol dehydrogenase, although also exhibiting important metabolic changes in anaerobiosis is not affected in hydrogenase expression [47] or activity and thus displays a wild-type fluorescence signature.

Mitochondrial mutant (*dum11*) deficient for respiratory-chain complex III has a similar fluorescence profile compared to HydEF-deficient strain while *dum22* that lacks complex I in addition to complex III has a more drastic fluorescence phenotype. Both mutants are almost fully impaired in hydrogen evolving capacity. Expression of hydrogenase has been suggested to be dependant of ATP, notably through cyclic electron flow around PSI [61]. Although ATP levels in aerobiosis in the light are lower in respiratory-deficient mutants compared to wild type [62], they are sufficient to trigger the induction of H_2 production under sulfur deprivation but the amount of hydrogenase rapidly declines [29]. In principle, anaerobic state should compensate for respiration deficiencies, but since ATP, neutral lipids and starch contents are much lower in mutant cells cultivated in aerobiosis in the light compared to wild-type cells [29], glycolysis probably cannot sustain anaerobic metabolism for long period in darkness.

Non-photosynthetic mutants also display specific features after dark anaerobic adaptation. Chlorophyll fluorescence induction curves of PSI and cyt b_6f deficient strains can be explained by an immediate reduction of the few oxidized electron acceptors in the absence of enzymes capable of oxidizing the PQ pool.

Further experiments, easy to perform even on a large mutant collection like checking the growth phenotypes in heterotrophic conditions or recording chlorophyll fluorescence induction curves in

aerobiosis can be undertaken to discriminate mutants affected in mitochondrial respiration [62] or in aerobic photosynthesis [59]. And if necessary, since most non-photosynthetic mutants are acetate-requiring and photosensitive (*e.g*. [42, 63, 64]), they could be simply discarded by selecting the transformants on inorganic medium in the light.

5. Conclusion

In summary, the present screening strategy is based (i) on differential fluorescence signatures that display hydrogenase-deficient and wild-type strains after adaptation to dark and anaerobiosis, and (ii) on the linear relationship in the light between PSII electron transfer and hydrogenase activity *in vivo*. It has been developed in alternative to the chemochromic sensor protocol [30] and offers several major advantages. This screening procedure should allow to identify new players in hydrogenase transcription, translation, maturation and regulation processes, but also to point out metabolic pathways critical for energetic balance (ATP and redox) in anaerobiosis. Other mutants recovered through the screening of the first small transformant collection described in this paper are under investigation.

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Figure legends

Figure 1: PSII activity of *Chlamydomonas reinhardtii* wild-type and hydrogenase-deficient (*hydEF-1*) strains after acclimation to dark aerobic and anaerobic (>12 h) conditions. ***A-B,*** Mean chlorophyll fluorescence induction curves (>3 independent experiments) upon illumination at ~300 photons (λ=640 nm) . nm^{-2} . s^{-1}. Arrows indicate the moment when the saturating light pulse was given. Maximal fluorescence yield (F_M) of aerobic wild-type cells was normalized to 1. ***C-D,*** PSII apparent electron transfer rate as a function of the light intensity (I, 640 nm photons . nm^{-2} . s^{-1}); ETR'(II), PSII apparent electron transfer rate (é . s^{-1} . nm^{-2}). Means and standard deviations of >3 independent experiments.

Figure 2: **A-D** Mean chlorophyll fluorescence induction kinetics (>3 independent experiments) of *Chlamydomonas reinhardtii* mutants after acclimation to dark anaerobic conditions for at least 12 h. I ~ 300 photons (λ=640 nm) . nm^{-2} . s^{-1}. Arrows indicate the moment when the saturating light pulse was given. ***A,*** fermentative pathway mutants ; ***B,*** mitochondrial respiratory-chain mutants ; ***C,*** chlororespiratory pathway mutants ; ***F,*** non-photosynthetic mutants. Maximal fluorescence value of wild-type control cells was normalized to 1. ***D,*** Relationship between PSII quantum yield values and initial rates of hydrogen production of mutant and control strains presented in panel A-C. Each point represents a mean value for a wild-type or a mutant strain (see Table 2 for mean numerical values and standard deviations). ***E,*** Chlorophyll fluorescence parameters of wild-type cells upon acclimation to dark anaerobic conditions. Main fluorescence parameters (F_M, Fv/F_M and Ψ_{II}) were calculated and plotted against time incubation in anaerobiosis (light intensity of ~300 photons (λ=640 nm) . nm^{-2} . s^{-1}). Means and standard deviations of >3 independent experiments.

Figure 3: ***A,*** Chlorophyll fluorescence induction kinetics of B3K56/212 mutant compared to the average curve of all transformants of the same plate. Arrow indicates the moment when the saturating light pulse was given. ***B***, false colour image of B3K56 transformants series plated on Petri dish. The color gradient scale represents the value for PSII quantum yield (Ψ_{II}) (min 0 in blue-dark;

max 0.25 in orange-red). ***C,*** Exon-intron structure of *HYDG* gene as annotated in the *Chlamydomonas reinhardtii* genome v5.3 available at http://www.phytozome.org (cre06.g296700.t1.1, location: chromosome_6: 6998683 - 7003289) (exon, red boxes; introns, black lines; untranslated regions, blue boxes) and localization of the inserted hygromycin resistance cassette between nucleotide 48 and 153 (dark boxes, regulatory sequences; light grey box, first intron of *RBCS2* gene; white boxes, coding sequence of bacterium *aphVII* gene).

Supplemental Figure 1. Mean chlorophyll fluorescence induction curves of wild-type and *hydEF-1 mutant* cells upon illumination at ~300 photons (λ=640 nm) . nm^{-2} . s^{-1}. **A.** dark anaerobic cells (>12h); **B.** dark anaerobic cells (>12h) reoxygenated for ~5 min. Arrows indicate the moment when the saturating light pulse was given. Maximal fluorescence yield (F_M) of wild-type cells was normalized to 1 (>3 experiments).

Supplemental Figure 2. Short-term hydrogen photoproduction under prolonged (>12h) anaerobiosis. Hydrogen production was measured using a hydrogen-sensitive clark-type electrode (see methods for details). Arrows indicate the moment when light was switched on at ~300 photons . nm^{-2} . s^{-1}. Representative traces for hydrogen evolution in the wild type (A), *hydG-2* (B), *dum11* (C) and *pfl1* (D) mutants are shown. Chlorophyll concentrations were adjusted to 30 µg . ml^{-1}. Mean values and standard deviations for initial rates are given in table 2 and figure 2D.

Supplemental Figure 3. Relationship between PSII quantum yield values calculated from data shown in Figure 2E and (A) hydrogenase activity/expression [19] or (B) *in vitro hydrogenase assay* [26]. Maximal values reported in the previous studies were normalized to 1.

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Table 1

Table1: Overview of *Chlamydomonas reinhardtii* mutants used in this work (parental strain, mutation and reference).

Mutant	**Parental strain**	**Mutation**	**Reference**
hydEF-1	CC-425	Insertion of *ARG7* gene into the coding sequence of hydrogenase maturation gene *HYDEF-1*	[23]
pfl1	CC-124	Insertion of *APHVIII* gene into the coding sequence of pyruvate formate lyase gene *PFL1*	[27]
adh1	CC-124	Insertion of *APHVIII* gene into the coding sequence of bifunctional acetaldehyde/alcohol dehydrogenase gene *ADH1*	[45]
ptox2	Jex4	Insertion of *APHVIII* gene into the coding sequence of plastidal terminal oxidase 2 gene *PTOX2*	[49]
dum11 (strain 194 in our stock collection)	1' (137c)	1.5kb deletion encompassing the mitochondrial left telomere and the *cob* gene. Loss of complex III	[63]
dum22 (strain 662 in our stock collection)	1' (137c)	Deletion of 3.5kb encompassing the mitochondrial left telomere, the *cob* and *nd4* genes. Loss of both complex I and complex III	[64, 65]
Nda2-RNAi (g4 in our stock collection)	Cell-wall less wild-type strain (84 in our stock collection)	Random insertion of *NDA2*-RNAi construction. Knock down of chloroplastic NAD(P)H dehydrogenase Nda2 protein expression	[28]
ΔpsaB	137C	Non-photosynthetic mutant deficient in photosystem I by site-directed mutagenesis strategy	[47]
ΔpetB	137C	Non-photosynthetic mutant deficient in b_6f complex by site-directed mutagenesis strategy	[48]
prk	137C	Phosphoribulokinase-deficient strain isolated after chemical mutagenesis with methyl methanesulfonate	[50]

Table 2

Table 2: Photosynthetic parameters of dark aerobic or anaerobic wild-type and mutant strains. PSII quantum yield before (Fv/F_M) and after (Φ_{II}) 3s of illumination at ~300 photons (λ=640 nm) . nm^{-2} . s^{-1}. H_2, initial rate of hydrogen evolution (nmoles H_2 . min^{-1} . mg^{-1} chlorophyll) upon illumination at ~300 photons (λ=640 nm) . nm^{-2} . s^{-1}. PSI, p700 content (nmoles . mg^{-1} chlorophyll) was estimated from p700 redox difference spectroscopy. PSI/PSII ratio was estimated from PSI and PSII charge separation capacity. Nd, not determined. Means and standard deviations of >3 independent experiments.

		Fv/F_M	Φ_{II}	H_2	PSI	PSI :PSII
aerobiosis	**Wt (1')**	0.64 ± 0.08	0.33 ± 0.17	0.00 ± 0.00	3.47 ± 0.57	1.2 ± 0.2
	hydEF-1	0.66 ± 0.01	0.30 ± 0.13	0.00 ± 0.00	nd	1.3 ± 0.2
	hydG-2	0.64 ± 0.06	0.31 ± 0.07	0.00 ± 0.00	3.28 ± 0.45	1.1 ± 0.3
anaerobiosis	**WT (1')**	0.12 ± 0.08	0.20 ± 0.03	2.07 ± 0.36	3.88 ± 0.15	1.2 ± 0.3
	hydEF-1	0.16 ± 0.06	0.05 ± 0.01	0.04 ± 0.02	3.3 ± 0.13	1.3 ± 0.2
	hydG-2	0.14 ± 0.08	0.03 ± 0.01	0.04 ± 0.03	3.99 ± 1.46	1.0 ± 0.1
	dum11	0.26 ± 0.02	0.13 ± 0.09	0.51 ± 0.74	3.41 ± 0.44	1.0 ± 0.3
	WT (cc125)	0.01 ± 0.01	0.23 ± 0.02	2.12 ± 0.42	3.21 ± 0.55	1.5 ± 0.2
	pfl1	0.39 ± 0.05	0.11 ± 0.02	0.56 ± 0.08	3.63 ± 0.35	1.2 ± 0.3
	adh1	0.01 ± 0.02	0.22 ± 0.03	1.58 ± 0.48	3.55 ± 0.13	1.6 ± 0.3
	WT (Jex4)	0.03 ± 0.03	0.21 ± 0.03	2.00 ± 1.08	4.18 ± 0.12	1.6
	Nda2-RNAi	0.31 ± 0.13	0.16 ± 0.02	1.73 ± 0.31	3.91 ± 0.09	nd

Table 3

Table 3. Photosynthetic activity of the most affected hygromycin-resistant transformants. Mean Φ_{II} value represent the mean value and the standard deviation of transformants of a same plate (40 to 42 transformants per plate). Chlorophyll fluorescence signatures refer to kinetics described in Figures 1 and 2.

strains	Φ_{II}	**mean Φ_{II}**	**Chlorophyll fluorescence signature**
B3K56/212	**0.03**	0.21 ± 0.03	hydrogenase-deficient
B1F133/52	**0.04**	0.16 ± 0.04	hydrogenase-deficient
B7F45/316	**0.07**	0.25 ± 0.07	hydrogenase-deficient
B1F133/73	**0.05**	0.16 ± 0.04	hydrogenase-deficient
B1F1/430	**0.12**	0.35 ± 0.04	hydrogenase-deficient
B3G31/153	**0.03**	0.27 ± 0.05	non-photosynthetic
B7F45/350	**0.03**	0.25 ± 0.07	non-photosynthetic
B7F1/164	**0.04**	0.28 ± 0.05	non-photosynthetic
B6H1/172	**0.08**	0.27 ± 0.07	non-photosynthetic

Figure 1

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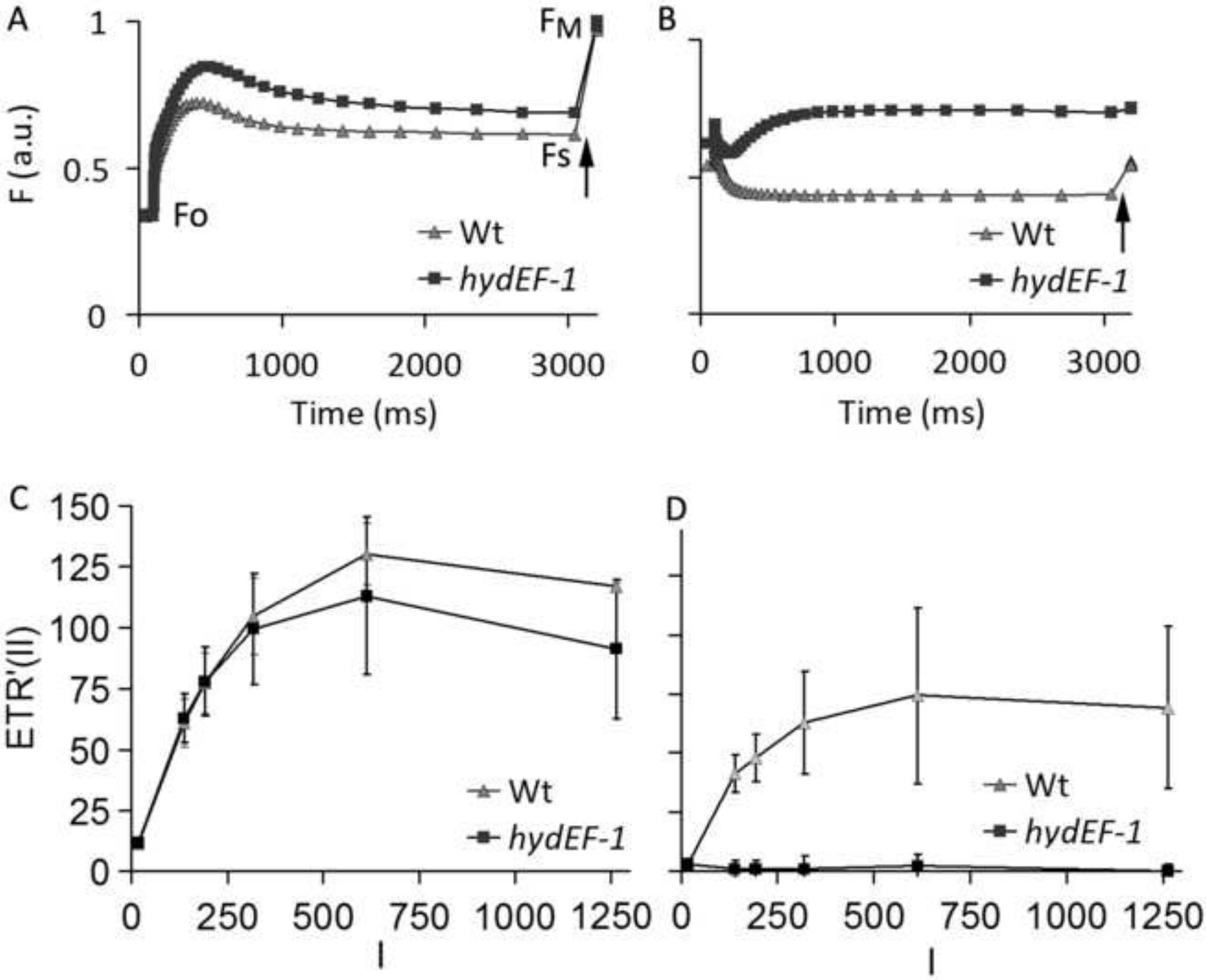

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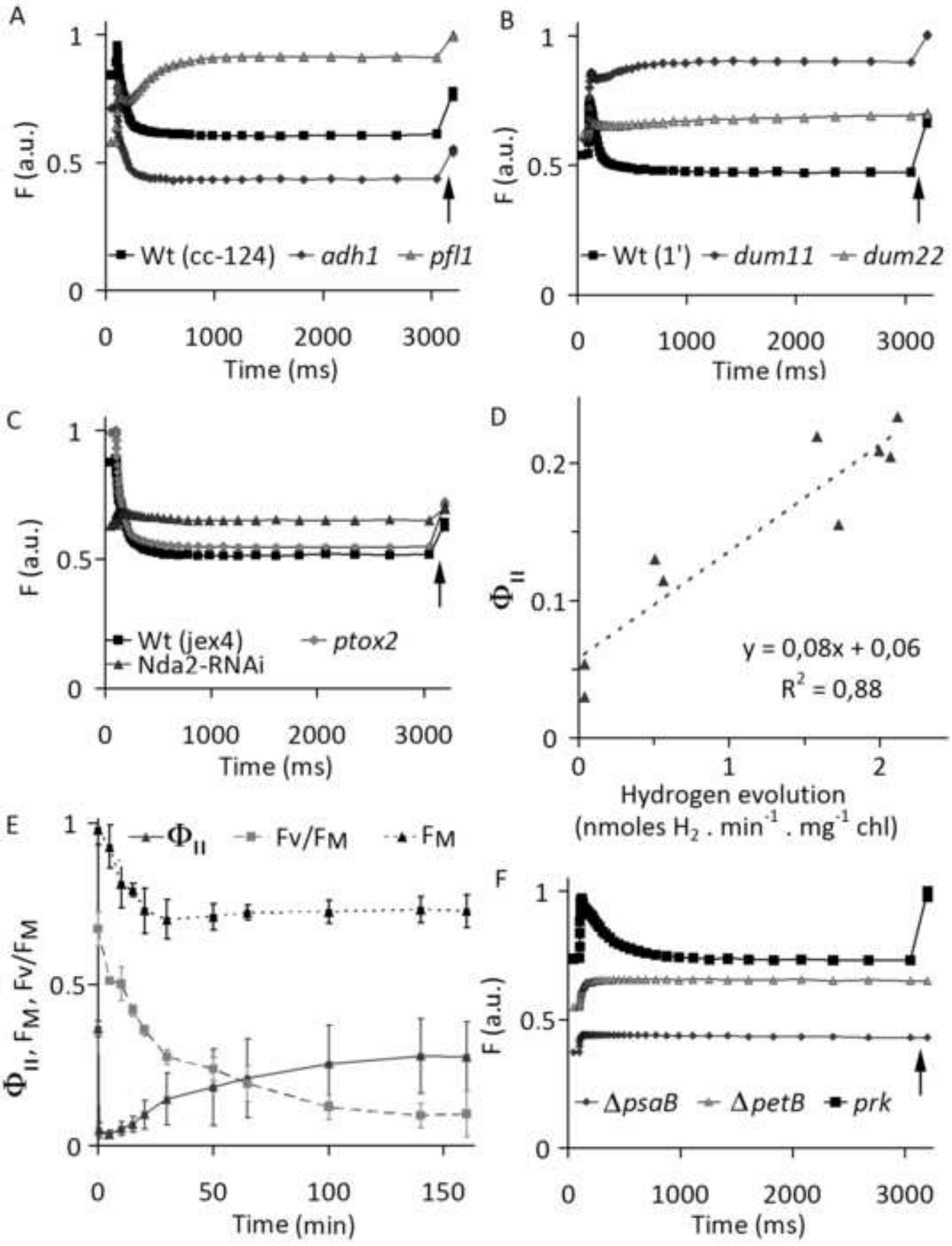

Figure 3

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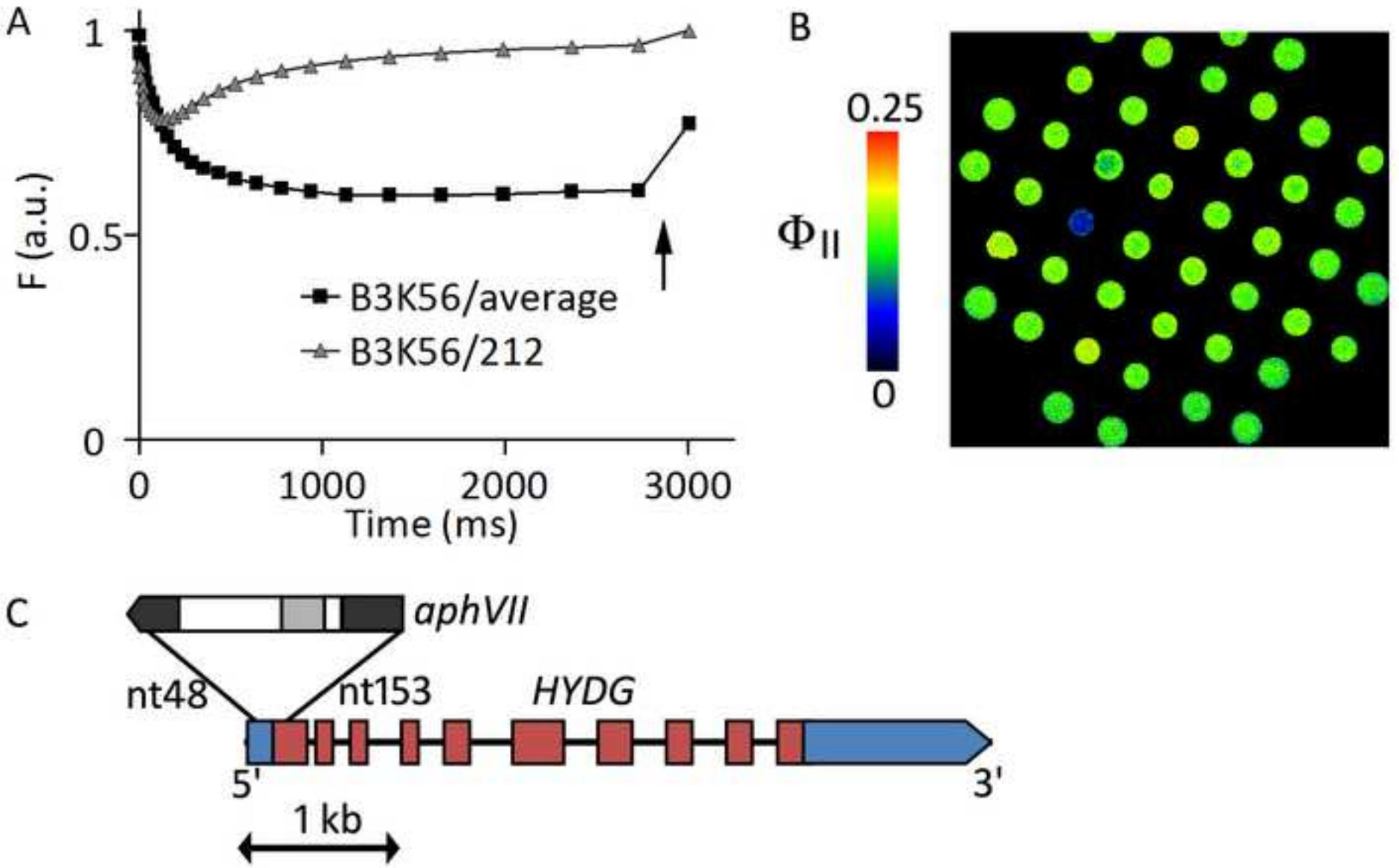

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