The rise of the photosynthetic rate when light intensity increases is delayed in *ndh* gene-defective tobacco at high but not at low CO2 concentrations

Mercedes Martín1, Dolores M. Noarbe2, Patricia H. Serrot1 and Bartolomé Sabater1*

1 Department of Life Sciences, University of Alcalá, Alcalá de Henares, Spain
2 Department of Physical Chemistry, University of Alcalá, Alcalá de Henares, Spain

*Correspondence: Bartolomé Sabater, Department of Life Sciences, University of Alcalá, Alcalá de Henares, Spain

e-mail: bartolome.sabater@uah.es

The 11 plastid *ndh* genes have hovered frequently on the edge of dispensability, being absent in the plastid DNA of many algae and certain higher plants. We have compared the photosynthetic activity of tobacco (*Nicotiana tabacum*, cv. Petit Havana) with five transgenic lines (*ΔndhF*, *pr-ΔndhF*, T181D, T181A, and *ndhF FC*) and found that photosynthetic performance is impaired in transgenic *ndhF*-defective tobacco plants at rapidly fluctuating light intensities and higher than ambient CO2 concentrations. In contrast to wild type and *ndhF* FC, which reach the maximum photosynthetic rate in less than 1 min when light intensity suddenly increases, *ndh* defective plants (*ΔndhF* and T181A) show up to a 5 min delay in reaching the maximum photosynthetic rate at CO2 concentrations higher than the ambient 360 ppm. Net photosynthesis was determined at different CO2 concentrations when sequences of 130, 870, 61, 870, and 130 μmol m−2 s−1 PAR sudden light changes were applied to leaves and photosynthetic efficiency and entropy production (*S*\textsubscript{g}) were determined as indicators of photosynthesis performance. The two *ndh*-defective plants, *ΔndhF* and T181A, had lower photosynthetic efficiency and higher *S*\textsubscript{g} than wt, *ndhF* FC and T181D tobacco plants, containing full functional *ndh* genes, at CO2 concentrations above 400 ppm. We propose that the Ndh complex improves cyclic electron transport by adjusting the redox level of transporters during the low light intensity stage. In *ndhF*-defective strains, the supply of electrons through the Ndh complex fails, transporters remain over-oxidized (specially at high CO2 concentrations) and the rate of cyclic electron transport is low, impairing the ATP level required to rapidly reach high CO2 fixation rates in the following high light phase. Hence, *ndh* genes could be dispensable at low but not at high atmospheric concentrations of CO2.

**Keywords:** dispensable genes, fluctuating light, high CO2, *ndh* genes, photosynthesis

INTRODUCTION

Some 30 years after their discovery (Ohyama et al., 1986; Shinozaki et al., 1986), the functional role of the 11 plastid-encoded *ndh* genes (which are homologous to genes encoding components of Complex I of the mitochondrial respiratory chain) is still a mystery. Among eukaryotic algae, only a few Prasinophyceae and all Charophyceae (the green algae related to higher plants) contain *ndh* genes (Martín and Sabater, 2010). Most photosynthetic land plants contain the *ndh* genes, which are absent in parasitic non-photosynthetic species of the genera *Cuscuta*, *Epiphagus*, *Orobanche* and the Orchidaceae family. This suggests that the thylakoid Ndh complex, encoded by the 11 plastid *ndh* genes and an as yet unknown number of nuclear genes, has a role in land plant photosynthesis. However, plastid DNAs of the photosynthetic Gymnosperms Pinaceae and Gnetales and of a few species scattered among angiosperm genera, families, and orders (e.g., *Erodium*, Ericaceae, Alismatæs, . . .) lack *ndh* genes (Braukmann et al., 2009; Blazier et al., 2011; Braukmann and Stefanović, 2012), which suggests that *ndh* genes could be dispensable under certain environments. The 11 *ndh* genes account for about 50% of all C to U editing sites identified in the transcripts of plastid genes (Tillich et al., 2005), which again suggests that dispensable *ndh* genes in the ancestors of extant plants accumulated inactivating mutations (Martín and Sabater, 2010). Of these, T to C mutations have been neutralized by C to U transcript editing in most extant plants which presumably recovers the functionality of the *ndh* genes. Thus, tottering on the edge of dispensability, the *ndh* genes provide an excellent opportunity to test the natural selection of photosynthesis-related genes during plant evolution.

It is widely accepted that the Ndh complex is located in the stromal thylakoids (Casano et al., 2000, 2004; Lennon et al., 2003), transfers electrons to plastoquinone and is involved in cyclic electron transport. However, two different electron donors have been proposed: reduced ferredoxin and NADH. Ferredoxin as the electron donor would imply a role of the Ndh complex providing a...
cyclic photosynthetic electron transport (Yamamoto et al., 2011) in addition to the commonly accepted model in which ferredoxin directly donates electrons to the PQ/cyt.b₅₆₇/F intermediate electron pool (Kurisu et al., 2003). However, as pointed out by Nandha et al. (2007) in a similar assay with the PGR5 protein, the rate of plastoquinone reduction is too low in the assay of the Ndh complex with reduced ferredoxin as electron donor. In contrast, spectrophotometric assays of activity and zymogram of NADH dehydrogenases after native electrophoresis, combined with immunodetection with antibodies raised against protein encoded by chloroplast ndh genes indicate NADH as the electron donor in different plants (Cuello et al., 1995; Cornelle et al., 1998; Sazanov et al., 1998; Casano et al., 2000; Diaz et al., 2007; Martin et al., 2009; Serrot et al., 2012). Accordingly, the Ndh complex, in concerted action with electron draining reactions (Meher, superoxide dismutase/peroxidase and terminal oxidase) in chlororespiration, would contribute to adjust (poise) the redox level of the cyclic photosynthetic electron transporters (Casano et al., 2000; Joët et al., 2002; Rumeau et al., 2007), hence optimizing (Heber and Walker, 1992) the transport rate necessary for cyclic photophosphorylation and, in general, the thylakoid polarization and lumen acidification that is also required to avoid the damages caused by excess light by dissipating energy through zeaxanthin (Esكلing et al., 2001; Karpinski et al., 2001; Minagawa, 2013) in the process of non-photocatalytic quenching. Accordingly, by poising the redox level of the cyclic electron transporters, the Ndh complex contributes to the protection against photodamage-related stresses (Martin et al., 2004; Sabater and Martin, 2013). The activity superoxide dismutase, which is key for electron draining, decreases in adult-senescence photosynthetic tissues, when the over-expression of the ndh genes results in an over-reduction of electron transporters which triggers the accumulation of reactive oxygen species inducing cell death (Zapata et al., 2005; Nashilevitz et al., 2010; Nilo et al., 2010; Sabater and Martin, 2013).

Related to the function of the Ndh complex in cyclic electron transport, questions remain on the extent the ndh genes improve (if such is the case) photosynthesis and on the environmental conditions that made ndh genes dispensable in certain plant lines. Apart from a certain weakening under different stress conditions, transgenic plants defective in ndh genes usually show normal growth (Burrows et al., 1998; Martin et al., 2004). However, the information provided by transgenic ndh-defective plants is sometimes debatable. Only a few of the claimed nuclear ndh genes have been unambiguously demonstrated to encode Ndh components (Darie et al., 2005; Rumeau et al., 2005; Shimizu et al., 2008). In fact, the basic respiratory complex I found in archaeal and eubacterial kingdoms may be functional with only 11 subunits (Moparthi et al., 2014) homologous to those encoded by the 11 plastid ndh genes. Frequently, Arabidopsis nuclear mutants defective in the thylakoid Ndh complex are affected in subunit assembly, plastid ndh transcript processing and, in general, processes that can have pleiotropic effects on several chloroplast functions (Meurer et al., 1996). On the other hand, the obtainment of homoplasmatic plastid ndh transgenics is highly improbable. Although efficient technologies are available that insert foreign sequences, the large background provided by 100s copies of plastid DNA (Rauwolf et al., 2010; Matsushima et al., 2011) in mesophyll cells makes the selection of homoplasmic transformed cells difficult, even after several culture cycles. DNA-blot hybridizations are not sufficiently sensitive to establish homoplasmity, and even more sensitive approaches such as PCR amplification could be insufficient. Rapid replication of non-transformed plastid DNA makes it difficult to maintain plants with a high proportion of plastid ndh defective DNA for several generations, unless selective culture conditions are regularly maintained during the 2 or 3 weeks after germination. Although not homoplasmic, the low level of non-transformed DNA has allowed us to investigate the functional properties of transformed tobacco plants that contain a high proportion of plastid DNA with defective ndh genes and show low amounts or malfunctioning thylakoid Ndh complex (Martin et al., 2004, 2009; Zapata et al., 2005).

The functioning of the photosynthetic machinery under rapidly changing environmental conditions (mainly light intensity) is recently receiving considerable research interest (Martin et al., 2009; Tikkanen et al., 2012; Garab, 2014). Photosystem I protection, cyclic electron transport and the control of reactive oxygen species require strategies that are being intensely investigated and are different from those under constant high light (Suorsa et al., 2013). However, little is known on the final effect of rapidly fluctuating light on net photosynthesis. The slight delay in reaching full photosynthetic rates in transgenic ndh-defective tobacco plants after sudden increases of light intensity (Martin et al., 2009) prompted us to investigate the contribution of the ndh genes to suppress that delay and the consequences on the photosynthetic efficiency and $S_g$, as measures of fitness (Sabater, 2006; Marin et al., 2014), at different CO₂ concentrations and rapidly fluctuating light intensity. To maintain the low entropy associated to leaf organization (Ksenzhek and Volkov, 1998; Davies et al., 2013; Marin et al., 2014) the entropy produced in photosynthesis must be exported, which increases the global entropy as required for all irreversible processes (Schrödinger, 1944). Therefore, comparisons of the entropy produced in photosynthesis by wt and ndh-deficient plants with the negative entropy associated to cell organization would help to evaluate the advantages provided by the ndh genes.

Measurements of net photosynthetic rates revealed that the increase of the rate of photosynthesis when the intensity of light suddenly increases is delayed in ndh-defective plants when compared with wt and control transformed (ndhF FC) plants at high but not at low concentrations of CO₂. Probably, by balancing the redox level of transporters, the Ndh complex maintains high rates of photosynthetic cyclic electron transport during the low light intensity stage to maintain thylakoid polarization and the ATP level required to protect photosynthetic machinery and the rapid response of photosynthetic rate when light suddenly increases in the following stage. The consequence is comparatively low photosynthetic efficiency and high $S_g$ in ndh-deficient tobacco plants under rapid fluctuating light and high concentrations of CO₂, which suggests that the ndh genes could be dispensable at low atmospheric concentrations of CO₂, but not at higher CO₂ concentrations.
MATERIALS AND METHODS

PLANTS CULTURE

Most assays were performed with wt tobacco (Nicotiana tabacum, cv. Petit Havana) and transgenics defective in the ndhF gene by intragenic insertion of the spectinomycin-selection gene aadA (Koop et al., 1996; Martin et al., 2004, 2009; ∆ndhF and pr-∆ndhF described later). Additional assays were carried out with different tobacco plants where the aadA selection gene was inserted upstream of the ndhF gene (Martin et al., 2009) maintaining intact ndhF gene (ndhF FC, control) or site-directed mutated: T181A and T181D, in which the 541ACT543 triplet encoding the phosphorylatable Thr-181 of the NDH-F subunit has been substituted by GCT.

Tobacco plants were cultured as described Martin et al. (2009). Seeds from non-transformed wt tobacco were sown in pots with compost soil substrate, germinated and grown in a glasshouse. Seeds from transformed plants were aseptically germinated and grown for 1–2 months in sterile Murashige/Skoog (MS) agar-solidified medium supplemented with 600 mg L⁻¹ spectinomycin. Plantlets were transplanted to compost soil substrate in pots under controlled glasshouse conditions and irrigated with MS.

The genetic identity of the different tobacco plants was regularly tested by primer-directed amplification of appropriate plastid DNA regions, size determination, and sequencing (Martin et al., 2004, 2009). Since 2002 (for ∆ndhF) and 2007 (for the other transgenics), new seed generations of each transformed tobacco plant were produced at least once a year by completing the life cycle of the original transformed plants (Martin et al., 2004, 2009), obtained as detailed in Supplementary Materials. Sample seeds of ∆ndhF, T181D, and ndhF FC are available from authors upon request.

MEASUREMENT OF NET PHOTOSYNTHESIS, TRANSPIRATION RATES, AND CHLOROPHYLL FLUORESCENCE INDUCTION

Photosynthesis and transpiration rates were determined in the glasshouse at 25°C in 6.25 cm² regions of intact fully expanded healthy leaves (containing ~20 μg chlorophyll cm⁻²), of the mid-stem of plants at the beginning of flowering. Leaf was placed on the chamber of the LiCor+ portable photosynthesis system (ADC BioScientific Ltd., Hertfordshire, UK) as previously described Martin et al. (2009) and Marín et al. (2014), except for the CO₂ concentration that, having been programmed as fixed during the light sequence treatment was varied. Net photosynthetic activity (in μmol consumed of CO₂ m⁻² s⁻¹) and transpiration rate (in mmol of H₂O m⁻² s⁻¹) were measured during the light sequence treatment where the intensity (in μmol m⁻² s⁻¹ PAR, at leaf surface) abruptly changed according to the sequence: 15 min acclimation at 130, 6 min at 870, 6 min at 61, 6 min at 870, and 6 min at 130 μmol m⁻² s⁻¹ PAR. Data collected each min and at light intensity transitions were directly represented using the GraFit Software (Surrey, UK) as previously described.

Photon flux (in W m⁻²) at leaf surface was corrected by the 7% transmission of the glass of the chamber. The light intensities were set to the following values: 0.15 μmol m⁻² s⁻¹ PAR (dim light), 0.30 μmol m⁻² s⁻¹ PAR (low light), 0.60 μmol m⁻² s⁻¹ PAR (moderate light), 1.0 μmol m⁻² s⁻¹ PAR (high light), and 2000 μmol m⁻² s⁻¹ PAR (full light).

As detailed previously (Marín et al., 2014), photosynthetic efficiency (η, the fraction of absorbed radiant energy converted to biomass chemical energy) is: η = 100 E₅₇/Eᵢ₅₇, where E₅₇ is the chemical Gibbs energy of the net photosynthesis products stored as biomass (CH₂O) and Eᵢ₅₇ the absorbed PAR measured at leaf surface and corrected by the 7% transmitted. The entropy generated (S₈) was calculated from net CO₂ fixation data, dimensions of the experimental design, Gibbs free energy and entropy values in data banks and conventional thermodynamics. The entropy generation was expressed per J (Joule) of biomass chemical energy generated: S₈/E₅₇.

For each photosynthesis assay, the integrated net CO₂ consumed over the last 24 min of light incubation and the intermediate 12 min light phases (at 61 and the following at 870 μmol m⁻² s⁻¹ PAR) were converted to C-equivalent biomass (CH₂O) according to the reaction:

\[ \text{CO}_2 \text{(gas)} + \text{H}_2\text{O(liquid)} \rightarrow 1/6\text{C}_6\text{H}_{12}\text{O}_6\text{(solid)} + \text{O}_2\text{(gas)} \]

The integrated fixed CO₂ (mol m⁻²) was multiplied by the equivalence:

\[ E' = \Delta G = \Delta G^0 + RT \ln \left( \frac{P_{O_2}}{P_{\text{CO}_2}} \right) \]

\[ = 475.9 - RT \ln \left( 10^{-6} \times \left[ \text{CO}_2 \right] \right) \text{kJmol}^{-1} \]
DNA copies contained in a single mesophyll cell. The presum-
non-transformed (\(ndh\) absent in fluorescence increase characteristic of only slightly permitted the recovery of the clear post-illumination amplified 1,928 and 515 bp bands should approximate and respec-
twt DNA of et al., 2004; Zapata et al., 2005) of the non-transformed plastid amplified band (found among descendants of the \(ndh\) and T181D tobacco plants (Martín et al., 2009), we assayed par-
tially reverted phenotypes of \(ndhF\) tobacco (Figure 1A). The frequency of pr-\(\Delta ndhF\) phenotype is increasing in successive off-
spring derived from the original \(\Delta ndhF\) tobacco (despite the presence of spectinomycin during the initial culture of \(\Delta ndhF\)). Conceivably, unknown factors favor the replication of the few remaining copies of \(wt\) plastid DNA in \(\Delta ndhF\) tobacco over the transformed molecules defective in the \(ndhF\) gene. Although we are not yet able to control its emergence or its inheritance, the finding of pr-\(\Delta ndhF\) phenotype provides an additional retro-
mutant control that confirms the involvement of \(ndh\) genes in photosynthesis and other processes. In the future, the ability to control (and determine by quantitative PCR) the \(wt\) to \(\Delta ndhF\) plastid DNA ratio will provide a deeper understanding of the influ-
ence of the copy proportion of the plastid \(ndh\) gene in different processes.

PHOTOSYNTHETIC RATES UNDER FLUCTUATING LIGHT INTENSITIES. EFFECT OF THE CONCENTRATION OF CO2
In the field, leaves are exposed to frequent and rapid changes in light intensity (0 to about 2,500 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) PAR) due to tran-
sitory shadow produced by clouds, by other leaves fluttering in the wind and by wandering animals (Kühlheim et al., 2002). To investigate the effect of rapid light intensity variations on the pho-
sotosynthetic performance of leaves, we established a reference light fluctuation incubation consisting of 15 min of leaf acclimation at 130, followed by four 6-min light phases of 870, 61, 870, and 130 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) PAR at leaf surface. Rates of net photosynthesis varied for the same plant from 1 day and leaf to another. How-
ever, relative photosynthetic rates for different CO2 concentrations were highly reproducible for a same tobacco line with differences not higher than 5% and result in similar shape of the rate-time curves characteristic for each CO2 concentration. Therefore, we determined photosynthetic rate curves for up to five different CO2 concentration assays carried out successively with the same leaf section by changing the setting of the CO2 concentration. The 15 min acclimation was repeated for each CO2 concentration and the order of the assays with different CO2 concentrations (increasing or decreasing) did not affect the responses of photosynthesis rates. Experiments were repeated without significant differences 2–10 times and each graph in the following Figure 2 corresponds to one representative group of assays carried out in the same day with each plant and variable concentrations of CO2.

Figure 2 shows photosynthetic rates during the four light phases after the 15 min acclimation of \(wt\), one pr-\(\Delta ndhF\) pheno-
type and \(\Delta ndhF\) tobacco plants at different CO2 concentrations. In most repeated experiments (Figure S2), \(wt\) showed the highest and \(\Delta ndhF\) the lowest photosynthetic rates but, as stated above, no definitive conclusion could be drawn from differences of activ-
ity among the three plants. However, differences in the time with which each plant reaches maximum activity after light intensity increased to 870 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) were highly reproducible. In con-
trast to \(wt\), which rapidly reached the maximum photosynthetic
rate when light increased from 130 or 61 to 870 μmol m⁻² s⁻¹ (Figure 2, upper box), ΔndhF (middle box) showed a considerable delay in reaching the maximum photosynthetic rate at CO₂ concentrations higher than the ambient 360 ppm and, paradoxically, the photosynthetic rate of this transgenic was higher at 475 (-Δ-) than at 615 (-Δ-) ppm CO₂ during the first 3 min after the transition from acclimation 130 to 870 μmol m⁻² s⁻¹. Furthermore, during the first 3–5 min after the transition from 61 to 870 μmol m⁻² s⁻¹ (12 to 18 min in Figure 2) the photosynthetic rate was higher at 365 (-Δ-) than at 475 (-Δ-) and 615 (-Δ-) ppm CO₂. By comparison, during the fluctuating light incubation, the rate of photosynthesis in wt was always higher the higher the concentration of CO₂. At low CO₂ concentrations, 260 to 312 ppm (-Δ-) and 260 to 312 ppm (-Δ-), respectively, there were no detectable differences between wt and ΔndhF tobacco plants in the increase of the rate of photosynthesis after transition to high light.

Figure S2 shows photosynthetic rate versus time curves for several groups of assays (each group corresponds to experimental determinations carried out within the same day and leaf) with wt (four groups), pr-ΔndhF (two groups), and ΔndhF (three groups) tobacco plants. Figure S2 shows the phenotypic variability of the various ΔndhF strains. Rate curves are highly reproducible within the same day and leaf (see groups wt-3, wt-4, and pr-ΔndhF-2 in Figure S2) for the same CO₂ concentration, although absolute rate values for the same plant and similar CO₂ concentration significantly vary among the different days of assay (compare in Figure S2 the high activity at 470 ppm CO₂ in wt-1 with the low activity in wt-4 group of assays).

The rates of photosynthesis in pr-ΔndhF were determined at CO₂ concentrations of 365 ppm and higher (Figure 2, bottom box) and the curves were closer to those of wt or ΔndhF, probably dependant on the relative ndhF gene dose with respect to ΔndhF tobacco. Therefore, these results support that the products of intact functional ndh genes improve the photosynthetic performance at fluctuating light intensities at higher than ambient CO₂ concentrations. The slow increase of the rate of photosynthesis at high concentrations of CO₂ in ΔndhF is not due to an indirect effect of plastid DNA transformation because the control ndhF FC transgenic tobacco, containing the inserted aadA gene and intact ndhF gene, did not show delay in reaching high photosynthetic rate when assayed up to 535 ppm CO₂ (Figure S3).

The slow increase of the rate of photosynthesis at high concentrations of CO₂ cannot be attributed to an impaired stomatal response of ΔndhF with respect to wt. We found (Marín et al., 2014) that in wt tobacco, under the successive 6 min periods at 870, 61, 870, and 130 μmol m⁻² s⁻¹ PAR and different concentrations of CO₂, the rate of photosynthesis varied strongly and rapidly between a minimum and an approximately 5-fold higher maximum; a result similar to that shown in Figure 2. In contrast, the rates of transpiration and the stomatal conductance changed slowly with a maximum that barely doubled minimum values. In this work, we found similar results for all tobacco plants: the rates of transpiration and the stomatal conductance change slower than the rates of photosynthesis and the span between maximum and
minimum values is significantly narrower for transpiration and conductance than for photosynthesis. As an example, Figure 3 shows that at 470 ppm CO₂ the changes in transpiration (increase at high light and decrease at low light intensities) were slower in ΔndhF than in wt tobacco. Transpiration rates mirrored stomatal conductance changes determined in parallel assays (not shown). Similarly to photosynthesis, the transpiration responses in partially reverted pr-ΔndhF tobacco lay between those of wt and ΔndhF plants. The comparison with Figure 2 indicates that, in both wt and ΔndhF, rate responses are slower for transpiration than for photosynthesis. In general, there is an inverse relation between the internal CO₂ concentration in the leaf and the stomatal opening and, consequently, transpiration (Wheeler et al., 1999). As the internal concentration of CO₂ is lower at higher photosynthetic activity, it seems likely that the slower photosynthetic response in ΔndhF than in wt is responsible for the even slower transpiration response of ΔndhF than of wt, and not the opposite.

**DETERMINATIONS OF PHOTOSYNTHETIC EFFICIENCY AND ENTROPY PRODUCTION**

We evaluated the efficiency of the conversion of radiant energy to chemical energy (biomass) and the Sₑ of wt and mutant plants both during the total 24 min and for the intermediate 12 min light treatments (6 min at 61 and 6 min at 870 μmol m⁻² s⁻¹ PAR). Obviously, the relative differences between wt and ΔndhF were higher for the intermediate 12 min than for the 24 min incubation.

wt, ΔndhF, pr-ΔndhF, point mutant transgenics T181A and T181D and control ndhF FC (containing the aadA spectinomycin resistance gene near the 5’ end of the unmodified ndhF gene) plants were assayed. In T181A and T181D, the phosphorylatable threonine-181 is substituted by alanine and aspartic acid, respectively. Thus, the thylakoid Ndh complex in T181A cannot be activated by phosphorylation, whereas the negative charge of aspartic acid (D) in T181D mimics the activation effect of the threonine phosphorylation in wt, resulting in a highly active Ndh complex (Martín et al., 2009). As Figure S2 (for wt, pr-ΔndhF, and ΔndhF), S3 (for ndhF FC), and S4 (for T181D and T181A) show rate curves obtained from several groups of assays with T181D and T181A tobacco plants. Interestingly, T181D shows slight delay to reach full photosynthesis rate at low CO₂ concentration (T181D-1 in Figure S4), but similarly to wt and in contrast to ΔndhF (Figure 2) and T181A (T181A-1, T181A-3, and T181A-4 in Figure S4) no delay at high CO₂. Therefore, in agreement with previous enzyme determinations (Martín et al., 2009), the Ndh complex of T181D is probably always active due to the negative charge of aspartate (D), while the Ndh of wt tobacco requires 181-threonine phosphorylation. Conceivably,
de-regulated hyperactive Ndh complex in T181D tobacco could over-reduce cyclic electron transporters at low CO₂ concentrations, when the electron draining by Benson–Calvin cycle is low. The consequences would be low rate of cyclic transport and over production of reactive oxygen species. In this aspect, T181D tobacco would provide an interesting tool for further investigation of the redox and protein phosphorylation control of photosynthetic electron transport. The higher photosynthetic performance of T181D than of non-phosphorylatable T181A tobacco at high CO₂ concentration is also appreciated when comparing efficiency and Sₜ at different CO₂ concentrations.

Since the experimental approach did not provide a reliable comparison of absolute photosynthetic measurements on different days, a reference assay at 360 ppm of CO₂ (sometimes interpolated from data at very similar concentrations) was always carried out within each group of experiments. Therefore, integrated net CO₂ fixations, over the last 24 min or the intermediate 12 min of light incubation time, with the other 2–4 CO₂ concentrations (assyed in the same group of experiments) were referred to the CO₂ fixation at 360 ppm CO₂ and photosynthetic efficiency and Sₜ were expressed as percentages of the respective values at 360 ppm CO₂. This approach allowed us to combine results from about 35 groups of assays carried out different days with the six tobacco lines totaling 139 rate-time curves.

Photosynthetic efficiency η increased almost linearly with the concentration of CO₂ (upper boxes of Figure 4) up to 400 ppm in an impressively very similar manner in all plants, which supports the statistical relevance of the approach. For CO₂ concentrations higher than 400 ppm significant h differences were observed among tobacco lines: in most assays pr-ΔndhF (□) and T181A (Δ) showed lower η (and consequently higher Sₜ/EBI) during the 24 min incubation period (left boxes; compare with the gray line corresponding to plants with full functional ndhF gene described in the legend of Figure 4). For the intermediate 12 min incubation period (right boxes) when a strong rise from 61 to 870 μmol m⁻² s⁻¹ PAR took place, all assays with ΔndhF (□, three assays) and T181A (Δ, one assay; encircled) failed to significantly increase η at concentrations of CO₂ higher than 400 ppm. For the intermediate 12 min period at higher than 400 ppm CO₂, pr-ΔndhF (□) only showed slightly lower efficiencies when compared with all the assays with tobacco plants containing full functional ndhF gene. Conversely, the entropy produced (Sₜ/EBI) lower boxes of Figure 4) decreased as the concentration of CO₂ increased. The decrease above 400 ppm CO₂ was less pronounced, especially for determinations in the intermediate 12 min (lower right box), for ΔndhF (□) and T181A (Δ; encircled; around 90% of the 360 ppm value) than for the other tobacco plants (around 75% of the 360 ppm value). Variable ndhF gene dose of pr-ΔndhF phenotypes in the different assays (□) could explain the variable, although generally lower, efficiency at CO₂ higher than 400 ppm of pr-ΔndhF than ndhF FC (■) which, by containing the spectinomycin resistance gene, is more representative control than wt (●).

DISCUSSION
As shown in Figure 2, the delay of Ndh complex-deficient ΔndhF tobacco plants in reaching high photosynthetic rates when exposed to sudden increases of light intensity becomes clear at CO₂ concentrations above the 400 ppm predictable in the next decades. Under the sun fleck conditions common in open environments, the delay could impair the net photosynthetic performance of plants lacking ndh genes when compared with normal plants.

With the exception of the plastid ndhF gene disruption, no genome alteration has been found in ΔndhF tobacco (Martín et al., 2004, 2009; Zapata et al., 2005), which strongly indicates that the low proportion of ndhF gene copies and the resulting low level of the Ndh complex are solely responsible for the low photosynthetic performance of ΔndhF tobacco plants at high CO₂ concentrations. The rapid photosynthetic response (Figure S3) of the plastid transgenic control ndhF FC, which has the same aadA gene insertion as ΔndhF but outside the ndhF reading frame, indicates that the ΔndhF phenotype is not due to an indirect effect of plastid transformation. The intermediate (although variable) photosynthetic performance of pr-ΔndhF tobacco plants (Figures 2 and 4) provides additional evidence of the necessity of the ndh genes and the thylakoid Ndh complex for rapid photosynthetic responses to light at high CO₂ concentrations. On the other hand, the differences in the transpiration response (Figure 3) with regard to the photosynthetic response (Figure 2) to sudden light increases between wt and ΔndhF plants indicate that the ndh deficiency directly affects photosynthesis and is not mediated by a primary effect on the stoma machinery.

As described in preliminary results (Martín et al., 2009), the similar photosynthetic responses of ΔndhF and T181A at high CO₂ concentrations suggests that the impossibility of site-181 phosphorylation impairs the photosynthetic performance in T181A under rapidly fluctuating light intensities.

Redox balance of the electron transfer chain is critical for optimizing photosynthesis under fluctuating light in cyanobacteria and higher plants (Tikkanen et al., 2012; Allahverdiyeva et al., 2013), in Chlamydomonas (Alric, 2014), as in higher plants (Heber and Walker, 1992), redox equilibration is required for the maximal rate of cyclic electron flow. Therefore, the effect of the Ndh complex accelerating the photosynthetic response when light intensity suddenly increases could be due to its proposed role in optimizing the cyclic photosynthetic electron transport by adjusting the redox level of transporters (Casano et al., 2000; Joët et al., 2002). Electrons supplied by the Ndh complex prevent the over-oxidation of cyclic electron transporters that would occur due to the low supply of electrons from photosystem II between two high light intensity phases (Figure 2), especially at high CO₂ concentrations that would rapidly deplete electrons from transporters by consuming NADPH in the Benson–Calvin cycle. In the case of ΔndhF, the supply of electrons from NADH through the Ndh complex fails, consequently the photosynthetic electron transporters remain over-oxidized and the rate of cyclic electron transport around photosystem I is low, impairing the thylakoid membrane potential and the ATP level required to rapidly reach high CO₂ fixation rates in the following high light phase. As reported by Kanazawa and Kramer (2002), in comparison with the linear electron transport, the contribution of cyclic electron transport in maintaining thylakoid polarization is negligible at low CO₂
concentrations, which could explain the similar photosynthetic rate responses between ndh-defective and wt tobacco plants at low CO2 concentrations.

The over-oxidation of cyclic electron transporters should be more pronounced the lower the light intensity and, accordingly, the delay in reaching a full photosynthetic rate at high CO2 concentrations in ΔndhF was longer for the 61 to 870 than for the 130 to 870 μmol m⁻² s⁻¹ PAR transition (Figure 2). Thus, the strong influence of the light intensity in the middle low intensity phase seems more compatible with a role of the Ndh complex improving cyclic electron transport by adjusting the redox level of transporters than by providing an additional cyclic electron transport chain, as proposed by Yamamoto et al. (2011), where the light intensity applied to the middle phase conceivably does not affect the subsequent photosynthetic rise of ΔndhF. The necessity to maintain the redox balance of the photosynthetic electron transport chain under fluctuating light has also been reported (Allahverdiyeva et al., 2013) in cyanobacteria, in which the flavo-diiron proteins Flv1 and Flv3 involved are dispensable under constant but not under fluctuating light conditions. Therefore, under fluctuating light, the inability of ndh-defective plants to provide enough electrons to balance the redox level of transporters (specially at high concentrations of CO2) could determine a low rate of cyclic electron transport under low light intensity. The low rate impairs the thylakoid polarization and ATP level required for a rapid response of the net photosynthetic rate when the light intensity suddenly increases in the following stage. Transitory shortage of ATP would

FIGURE 4 | Effect of the concentration of CO2 on photosynthetic efficiency (η) and Sg/E_BI of different tobacco plants. As explained in the thermodynamic background, photosynthetic efficiency is the ratio of chemical energy (E_BI) stored as photosynthesized biomass to the PAR energy absorbed by the leaf. Sg/E_BI is the ratio of the total entropy produced to the chemical energy (E_BI) stored as photosynthesized biomass. Calculations were performed for the total 24 min (left boxes) and the 12 min intermediate (right boxes) light incubation treatments. The represented values of η and Sg/E_BI at different CO2 concentrations are the percentages with respect to corresponding values at 360 ppm CO2 (references at 360 ppm are in the range of 3.6% η and 0.08 K⁻¹ Sg/E_BI for all plants). The concentrations of CO2 represented are those determined by the LCpro+ photosynthesis system in the leaf chamber and could slightly change with respect to those programmed. As repeated determinations for very similar CO2 concentrations in a same tobacco produced very close points, only the mean value is represented. Therefore, points in the figure result from 139 different assays and several are the mean of 2–4 independent determinations. Inserted gray lines were obtained by second degree polynomial fitting of the experimental points corresponding to wt, ndhF FC, T181D tobacco plants that contain full dose of functional ndhF gene. Encircled points in left boxes correspond to those of Δ1ndhF and T181A at high CO2 concentrations.
decrease the rate of NADPH oxidation in the Benson–Calvin cycle at the next exposure to high light intensity, resulting in hyper-reduction of the electron transfer chain and PSI photodamage. Significantly, the PGR5 protein is also involved in the redox poising of photosynthetic electron transport (Nandha et al., 2007) and probably plays a role in the protection of PSI from photodamage under fluctuating light (Suorsa et al., 2013).

The effect of ndh gene products accelerating the increase of photosynthetic rates during the high intensity phases of fluctuating light reasonably indicates a definitive role for the Ndh complex. However, the mechanism involved and its relation with other factors, such as the PGR5 protein, require further investigations.

The differences in η and $S_\beta/E_{\beta}$ between wt and $\Delta ndhF$ were higher for the intermediate 12 min incubation than for the total 24 min incubation (Figure 4) and, plausibly, would be greater still under the rapid and more frequent changes of light in natural environments.

As the result of many assays, a 0.08 K$^{-1}$ $S_\beta/E_{\beta}$ value was estimated at 360 ppm CO$_2$ and approximately 80 K$^{-1}$ was estimated for the S$_\beta$ per leaf Kg and min, an amount 100-fold higher than the decrease of entropy associated to solute compartmentalization in cell organelles (Martin et al., 2009). Although roughly estimated, these values indicate that the ndh-associated S$_\beta$ reduction in less than 1 s at CO$_2$ concentrations higher than 400 ppm is in the range of structural leaf entropy values and could be evolutionarily significant as a selectable trait (Sabater, 2006) as found in other systems (Ding et al., 2011; Davies et al., 2013). In this regard, higher yields of energy conversions are equivalent to lower S$_\beta$ and both are plausible selectable traits in photosynthesis.

**CONCLUDING REMARKS**

The functional relevance of the thylakoid Ndh complex has been investigated by determining the photosynthetic response under fluctuating light and several CO$_2$ concentrations in different tobacco plants affected in the plastid ndhF gene that encodes the NDH-F subunit of the Ndh complex. In contrast to wt, ndhF-defective plants show an up to 5 min delay in reaching the maximum photosynthetic rate at CO$_2$ concentrations higher than the ambient 360 ppm. Accordingly, ndhF-defective tobacco plants show a lower photosynthetic efficiency and higher S$_\beta$ under rapidly fluctuating light intensities and high CO$_2$ concentrations than wt. Based on our results and the previous results of other groups, it is postulated that the activity of the Ndh complex maintains high rates of photosynthetic cyclic electron transport by providing electrons to balance the redox level of transporters during the low light intensity stage and could be dispensable at low but not at high atmospheric concentrations of CO$_2$ and under less intensively fluctuating lights. For the first time, these results establish a definitive connection between ndh gene products and photosynthetic performance and predict the influence of changing atmospheric CO$_2$ concentrations on the evolutionary conservation of the ndh genes.

**AUTHOR CONTRIBUTIONS**

Bartolomé Sabater, Mercedes Martín, and Dolores M. Noarbe conceived and designed research. Bartolomé Sabater, Mercedes Martín, and Patricia H. Serro conducted experiments. Bartolomé Sabater and Dolores M. Noarbe determined thermodynamics parameters. Bartolomé Sabater wrote the manuscript. Patricia H. Serro English edited the manuscript. All authors read and approved the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2015.00034/abstract

**REFERENCES**

Allahverdiyeva, Y., Mustila, H., Ermakova, M., Bersanini, L., Richaud, P., Ajlani, G., et al. (2013). Flavodiiron proteins FvI and FvII enable cyanobacterial growth and photosynthesis under fluctuating light. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4111–4116. doi: 10.1073/pnas.1221194110

Alic, J. (2014). Redox and ATP control of photosynthetic cyclic electron flow in *Chlamydomonas reinhardtii*. (II) Involvement of the PGR5–PGRL1 pathway under anaerobic conditions. *Biochim. Biophys. Acta* 1837, 825–834. doi: 10.1016/j.bbabio.2014.01.024

Blazier, J. C., Gaisenger, M. M., and Jansen, R. K. (2011). Recent loss of plastid-encoded ndh genes within *Erodium* (Geraniaceae). *Plant Mol. Biol.* 76, 263–272. doi: 10.1007/s11103-011-9753-5

Braukmann, T. W. A., Kuzmina, M., and Stefanović, S. (2009). Loss of all ndh genes in Gnetales and conifers: extent and evolutionary significance for seed plant phylogeny. *Carr. Genet.* 55, 323–337. doi: 10.1007/s00294-009-0249-7

Braukmann, T., and Stefanović, S. (2012). Plastid genome evolution in mycoheterotrophic Ericaceae. *Plant Mol. Biol.* 79, 5–20. doi: 10.1007/s11103-012-9884-3

Burrows, P. A., Sazanov, L. A., Svab, Z., Maliga, P., and Nixon, P. I. (1998). Identification of a functional respiratory complex in chloroplasts through analysis of tobacco mutants containing disrupted plastid ndh genes. *EMBO J.* 17, 868–876. doi: 10.1093/emboj/17.4.868

Casano, L. M., Lascano, H. R., Martin, M., and Sabater, B. (2004). Topology of the plastid Ndh complex and its NDH-F subunit in thylakoid membranes. *Biochem. J.* 382, 145–155. doi: 10.1042/BJ20031828

Casano, L. M., Zapata, J. M., Martin, M., and Sabater, B. (2000). Chlororespiration and poising of cyclic electron transport: plastocyanin as electron transporter between thylakoid NADH dehydrogenase and peroxidase. *J. Biol. Chem.* 275, 942–948. doi: 10.1074/jbc.275.2.942

Cornelle, S., Courmarc, L., Guedeney, G., Havaux, M., and Pelletier, G. (1998). Reduction of the plastocyanin pool by exogenous NADH and NADPH in higher plant chloroplasts. Characterization of a NAD(P)H-plastocyanine oxidoreductase activity. *Biochim. Biophys. Acta* 1363, 59–69. doi: 10.1016/S0005-2728(97)00074-1

Cuello, J., Quiles, M. J., Albacete, M. E., and Sabater, B. (1995). Properties of a large complex of NADH dehydrogenase from barley leaves. *Plant Cell Physiol.* 36, 265–271.

Davies, P. C. W., Rieper, E., Jack, A., and Tuszynski, I. A. (2013). Self-organization and entropy reduction in a living cell. *BioSystems* 11, 1–10. doi: 10.1016/j.biosystems.2012.10.005

Darie, C. C., Biniossek, M. L., Winter, V., Mutschler, B., and Haehnel, W. (2003). Isolation and structural characterization of the Ndh complex from mesophyll and bundle sheath chloroplasts of *Zea mays*. *FEBS J.* 272, 2705–2716. doi: 10.1011/j/jb/biosystems.2003.04685.x

Díaz, M., de Haro, V., Muñoz, R., and Quiles, M. J. (2007). Chlororespiration is involved in the adaptation of *Brassica* plants to heat and high light intensity. *Plant Cell Environ.* 30, 1578–1585. doi: 10.1111/j.1365-3040.2007.01735.x
NADH dehydrogenase-like complex of Arabidopsis. Plant Cell 23, 1480–1493. doi: 10.1105/tpc.110.080291

Zapata, J. M., Guéra, A., Esteban-Carrasco, A., Martín, M., and Sabater, B. (2005). Chloroplasts regulate leaf senescence: delayed senescence in transgenic ndhF-defective tobacco. Cell Death Differ. 12, 1277–1284. doi: 10.1038/sj.cdd.4401657

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