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REVIEW

Electrochromism: a useful probe to study algal photosynthesis

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Abstract In photosynthesis, electron transfer along the photosynthetic chain results in a vectorial transfer of protons from the stroma to the lumenal space of the thylakoids. This promotes the generation of an electrochemical proton gradient ($\Delta\mu_H^+$), which comprises a gradient of electric potential ($\Delta\Psi$) and of proton concentration (ΔpH). The $\Delta\mu_H^+$ has a central role in the photosynthetic process, providing the energy source for ATP synthesis. It is also involved in many regulatory mechanisms. The ΔpH modulates the rate of electron transfer and triggers deexcitation of excess energy within the light harvesting complexes. The $\Delta\Psi$ is required for metabolite and protein transport across the membranes. Its presence also induces a shift in the absorption spectra of some photosynthetic pigments, resulting in the so-called ElectroChromic Shift (ECS). In this review, we discuss the characteristic features of the ECS, and illustrate possible applications for the study of photosynthetic processes in vivo.

Keywords Spectroscopy · Electrochromism · Photosynthesis · Electrochemical proton gradient · ATP

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Introduction

In plants, algae and photosynthetic bacteria, the primary electron donors and the electron acceptors of the photosynthetic complexes are located on opposite sides of the membrane. Light exposure results therefore in a charge separation across the thylakoids, due to photochemistry of the reaction centres (the photosystems, or PS) (Witt. 1979) and electron flow in the cytochrome b_6f. According to the Q cycle hypothesis, the activity of this complex involves electron transfer in the "low potential chain" (Crofts et al. 1983), which spans the membrane due to the position of the two b_6 hemes in the complex (reviewed in Eberhard et al. 2008). Moreover, some electron flow steps are coupled to the uptake or the release of protons in the aqueous phase. The consequent movement of electrons and protons through the membrane generates a proton motive force (pmf or $\Delta\mu_H^+$) which comprises an electric field ($\Delta\Psi$) and a proton concentration gradient (ΔpH). Ultimately, the energy of the $\Delta\mu_H^+$ allows ATP synthesis by the activity of the ATP-synthase complex.

Electrochromism: the spectroscopic voltmeter

The presence of the electric field has also an effect on the photosynthetic pigments, the spectrum of which is modified, owing to the Stark effect. This phenomenon, known as the electrochromism, has been largely studied in photosynthetic membranes since its first observation by Duysens in the 1950s (reviewed in Witt 1979). Whilst exposure of chromophores to an electric field in solution results in various modifications in their physical properties, the main consequence in the case of pigments embedded in a lipid membrane is a shift of their absorption maxima. This effect, known as the "bandshift effect", can be described as follows (Fig. 1a): absorption of a photon results in the energetic transition of the pigment from its ground state to an excited state. If these two states are characterised by different dipole moments ($\vec{\mu}_e$ and $\vec{\mu}_g$) or polarisabilities (α_e and α_g), the energy difference (ΔE) between the two states will be changed by the electric field ($\vec{F}$) (see, e.g. Wraight et al. 1978; Lösche et al. 1988 for reviews), in a way described by the following relationship

$$\Delta\Delta E = -(\vec{\mu}_e - \vec{\mu}_g)\vec{F} - (\alpha_e - \alpha_g)\vec{F}^2$$

The absorption band of the molecule will be therefore shifted (Fig. 1b), the frequency (ν) being changed by

$$\Delta\nu = \frac{\Delta\Delta E}{h} = -\frac{1}{h}\left((\vec{\mu}_e - \vec{\mu}_g)\vec{F} + (\alpha_e - \alpha_g)\vec{F}^2\right)$$

where h is the Planck constant. If the shift is small when compared to the width of the absorption band (small perturbation), it is possible to obtain the spectrum of the "field-indicating absorption change", i.e. the Electro-Chromic Shift (ECS) signal, by generalising the equation above to the whole spectrum:

$$\Delta\varepsilon_{\nu 0} = \left(\frac{\partial\varepsilon}{\partial\nu}\right)_{\nu 0}\Delta\nu = -\frac{1}{h}\left(\frac{\partial\varepsilon}{\partial\nu}\right)_{\nu 0}\left((\vec{\mu}_e - \vec{\mu}_g)\vec{F} + (\alpha_e - \alpha_g)\vec{F}^2\right) \quad (1)$$

In this case, which is typical of photosynthetic systems, the theory of the ECS predicts two main features of the ECS: (i) The spectrum of the absorption change should follow the first derivative of the absorption band, and its shape should be independent of the field strength. Thus, the typical ECS spectrum obtained for a gaussian absorption band is a double-wave shape (Fig. 1b, c). In photosynthetic organisms, ECS spectra present several double-wave components (Fig. 2a, f), due to the superimposition of the ECS of different pigment absorption bands. The ECS can provide, therefore, extremely precise information about the pigment composition of a particular organism (see section "Influence of the pigment composition and pigment orientation on the ECS"). (ii) The magnitude of the ECS is the sum of a linear and a quadratic function of the applied field (Eq. 1 and Fig 1c). In principle the ECS can be calibrated to provide absolute values of the electric field in the chloroplast (discussed in Witt 1979, see also Takizawa et al. 2007 for a more recent discussion). However, this calibration is extremely difficult, unless the two components (the linear and quadratic ones) are present at the same time (see section "Quadratic components of ECS allow for the measurement of a pre-existing proton gradient").

In most of the photosynthetic systems, the ECS signal shows a linear response (Joliot and Joliot 1989). This feature is extremely interesting for the purpose of this review, because a linear ECS signal is an intrinsic membrane voltmeter, which rapidly responds to changes in the membrane potential. As the ECS can be measured spectroscopically in a non-invasive way, this technique can be

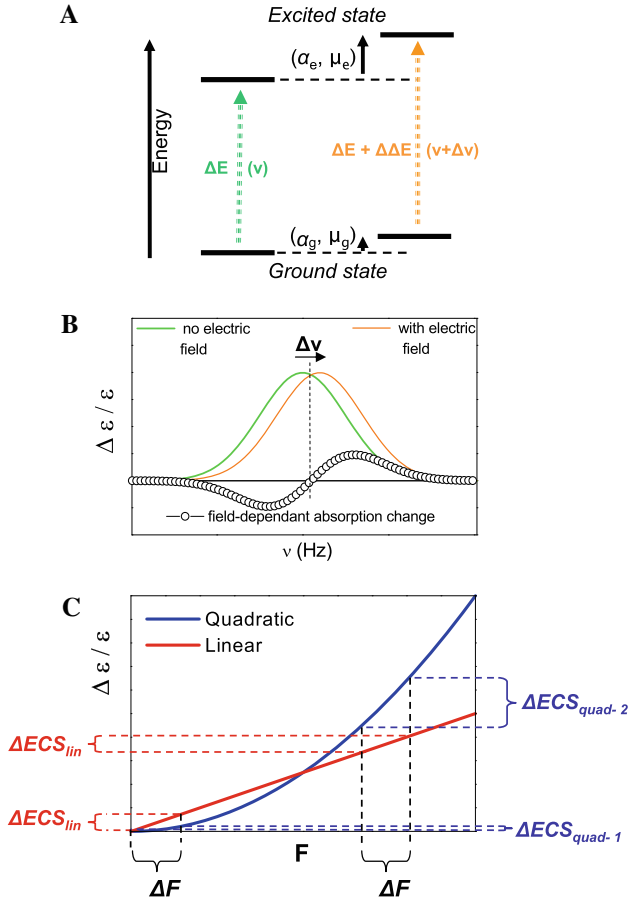

Fig. 1 Schematic representation of the electrochromism phenomenon. **a** Application of an electric field induces a change in the energy difference between the ground and the excited state of a chromophore. **b** This results in a shift of the absorption band, which leads to the appearance of a double-wave shape, typical of the ECS (*open circles*). **c** Dependency of linear (*red*) and quadratic (*blue*) components of the ECS upon the field, and effect of the pre-existing field on the flash-induced ECS signals

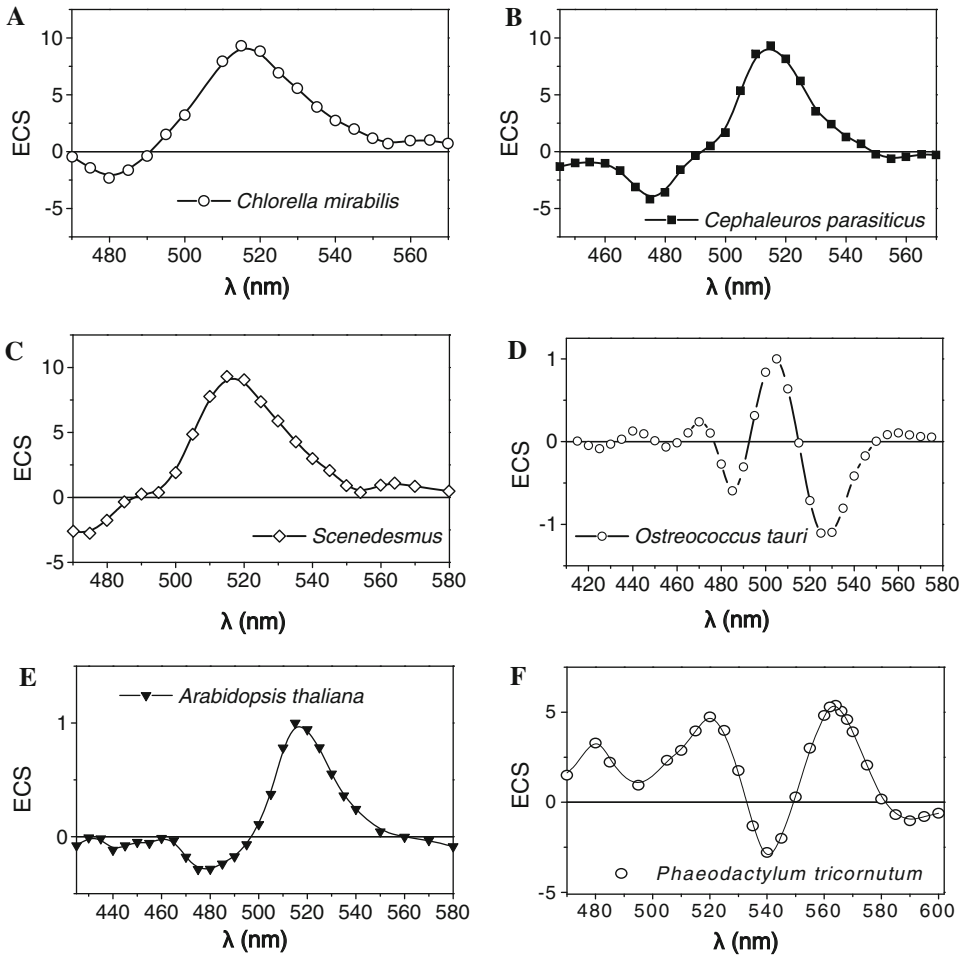

Fig. 2 ECS signals in different photosynthetic organisms. **a** *Chlorella mirabilis*, **b** *Cephaleuros parasiticus*, **c** *Scenedesmus*, *obliquus*, **d** *Ostreococus tauri*, **e** *Arabidopsis thaliana* and **f** *Phaeodactylum tricornutum*. Algae and leaves were illuminated with continuous light, and the ECS spectra are presented as the light minus the dark signal

employed to monitor the characteristics of living plants and algae under physiological or modified conditions.

ECS and photosynthesis

The use of the ECS signal to study the photosynthetic apparatus is often limited by the difficulty of correctly deconvoluting this signal from other overlapping spectral changes. In vascular plants and green algae (*Chlorophyceae*, *Trebouxyophyceae* and *Ulvophyceae*), the maximal spectral change related to the ECS is around ~515 nm (Fig. 2a–d). Therefore, the kinetic analysis of the field-indicating absorption changes is carried out mostly at this wavelength. However, spectral changes at ~515 nm also result from the formation and decay of triplet states of carotenoids (e.g. Kramer and Mathis 1980). Moreover, accumulation of specific carotenoids in light-exposed plants and green algae (due to violaxanthin deepoxidation) leads to spectroscopic changes at 505 nm (Siefermann and Yamamoto 1975) and 535 nm (Heber 1969). Both signals are linked to the building of the ΔpH. A correct deconvolution of the ECS signal thus requires a global analysis, including both spectral and kinetic measurements. Alternatively, the ECS signal can be differentiated from other signals by its sensitivity to ionophores, i.e. chemicals that increase the membrane permeability and, therefore, promote a fast relaxation of the electric field (Witt 1979).

Despite the difficulty of correctly deconvoluting the ECS signal from field-independent spectral changes, previous studies have largely exploited this tool to assess the location and orientation of the pigments within the chlorophyll binding complexes, the origin of the electric field (generated in the dark or in the light), the topology of the primary electron donors and acceptors of PSII and PSI and the pathway for electron and proton transfer (reviewed in Witt 1979). The kinetic resolution and the signal-to-noise ratio of the ECS are largely improved when the photosynthetic sample is synchronised using repetitive flash spectroscopy (Witt 1979), a technique that relies on the use of single turnover xenon or laser flashes. Kinetic analysis of the ECS signal in *Chlorella sorokiniana* cells (Joliot and Delosme 1974) measured under these conditions has evidenced several phases (Fig. 3a). The first one ("a" phase <100 μs after the flash) is related to the rapid onset of the

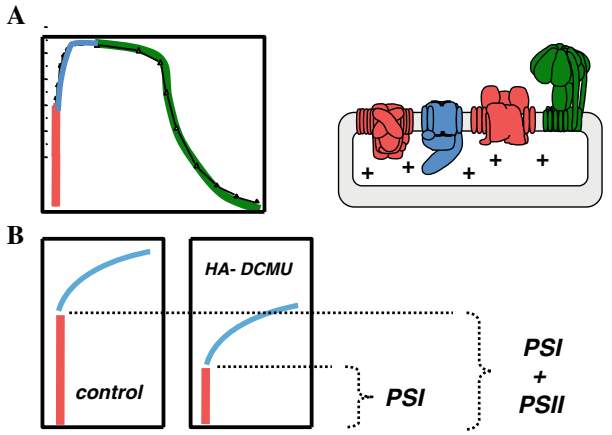

Fig. 3 Kinetics of the ECS measured in intact cells of the green alga *C. reinhardtii* upon excitation with a saturating laser pulse. Cells were adapted to anaerobic conditions (with nitrogen bubbling) to slow down membrane potential decay through the ATP-synthase, and better resolve the slow ECS rise in the ms range (e.g. Joliot and Delosme 1974). **a** The fast rise phase ("a" phase, *red*) corresponds to PSI and PSII charge separations, which occur on an unresolved timescale. The ms time range rise phase ("b" phase, *blue*) corresponds to electron flow in the cytochrome b chain of the cytochrome b_6f. The ECS decay ("c" phase, *green*) is due to charge leakage through the membrane, mainly H^+ via the ATP-synthase. **b** Application of flash-induced ECS signals to calculate PS stoichiometries. Inhibition of PSII activity (right panel through addition of DCMU and hydroxylamine) results in a decrease of the amplitude of the "a" phase. The relative decrease of the amplitude is directly proportional to the PSI/(PSI + PSII) ratio, and allows the stoichiometry of functional reaction centres to be estimated

electric field due to charge separation by the PS. This is followed by a slower phase ("b" phase), occurring in the ms time range, which reflects cytochrome b_6f activity (Joliot and Delosme 1974). After completion of this phase, variable decay lifetimes are seen ("c" phase, Joliot and Delosme 1974), which reveal the breakdown of the field by ionic flux. In native chloroplasts, this process essentially stems from H^+ flux through the ATP-synthase complex, and variations in the decay lifetime of this phase (from a few milliseconds to several seconds) reveal different levels of activity of this enzyme.

The ECS signal measured using repetitive flash spectroscopy provides precise information on the function of the photosynthetic complexes and on the pmf: (i) the amplitude of the "a" phase is proportional to the number of active PS, and its change upon addition of specific PSII inhibitors allows access to the PSI/PSII stoichiometry (see section "Application of the ECS to study acclimation of the photosynthetic apparatus"). (ii) The "b" phase provides kinetic information on the cytochrome b_6f and can be exploited to derive information on the size of the $\Delta\mu_H^+$ in the dark in living cells (see section "Quadratic components of ECS allow for the measurement of a pre-existing proton gradient"). (iii) Measurements of the rate of the relaxation phase of the ECS ("c" phase) can be exploited to estimate the activity of the ATP-synthase in vivo (Lemaire and Wollman 1989) or to evaluate the two components of the pmf in the dark (the $\Delta\Psi$ and the ΔpH, see section "The ECS approach to study the pmf composition under illumination").

ECS measurements under continuous illumination can be employed to characterise alternative electron flow processes (see section "Application of the ECS to study acclimation of the photosynthetic apparatus") and/or to assess the partition between the light-induced $\Delta\Psi$ and ΔpH in plants and unicellular algae (see section "The ECS approach to study the pmf composition under illumination").

In the following, we provide a more detailed description of the different applications of the ECS in vivo in the case of some unicellular algae.

Applications

Influence of the pigment composition and pigment orientation on the ECS

Besides being a natural voltmeter, the ECS signal can be used as a non-invasive tool to study the nature and environment of the photosynthetic pigments, provided that they undergo a band shift. This aspect is highlighted by the comparison between the ECS features of plants leaves (*Arabidopsis thaliana*) and of the unicellular alga *Ostreococcus tauri* (Fig. 2e, d, respectively). In leaves, the rather complex features observed in the blue-green part of the ECS spectrum reflect the combined contribution of chlorophylls *a* and *b* and of carotenoids to the observed shift (e.g. Schmidt et al. 1971). Conversely, the peaks observed in the red region of the spectrum (Witt 1979), which are more symmetrical, suggest that chlorophylls are the only pigments undergoing a band shift in this region. In the prasinophyte *O. tauri*, the blue–green ECS spectrum is a typical carotenoid band shift, suggesting that chlorophylls are less sensitive to the applied field in this alga. Therefore, the ECS features in *O. tauri* can be used to study the nature and orientation of carotenoids in the photosynthetic membranes. The ECS spectrum of *O. tauri* shows two major peaks at ~505 and ~530 nm, asymmetrically centred on 510–515 nm (Fig. 4d). This latter value clearly corresponds to the maximum difference in the absorption spectra of the light harvesting complexes of Arabidopsis (LHCII) and *O. tauri* (LHCP) (Fig. 4b, c), suggesting that an Ostreococcus-specific carotenoid (e.g. prasinoxanthin the most abundant carotenoid in this alga, Six et al. 2005) is responsible for the ECS. The spectrum measured in *O. tauri* is also illustrative of the principle that the ECS

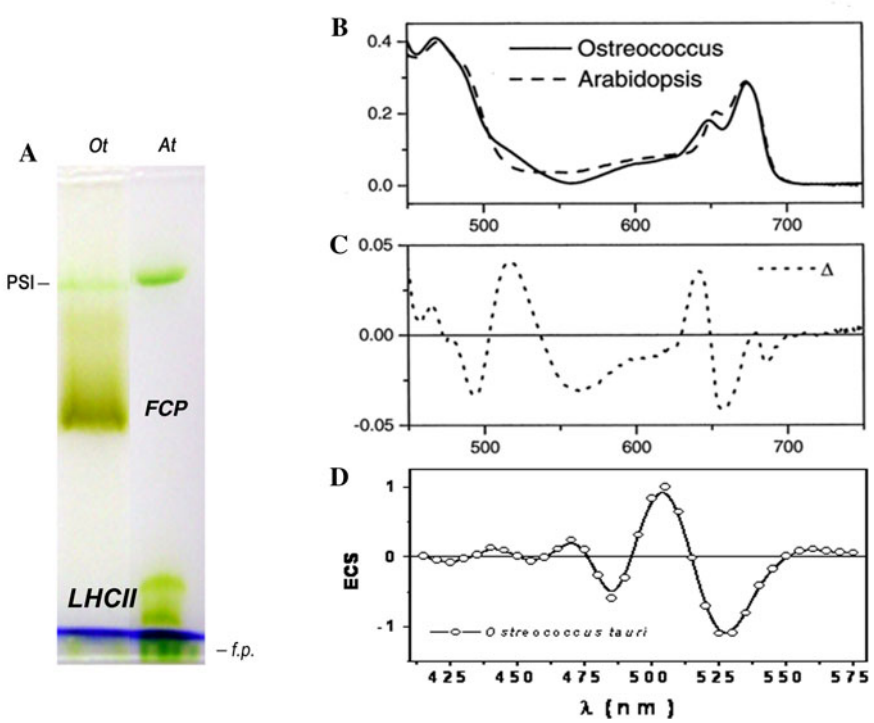

Fig. 4 Absorption spectrum of the LHC bands from vascular plants (*A. thaliana* leaves) and *Ostreoccus*, ecotype OTH95. **a** Non-denaturating, 10% SDS-PAGE electrophoresis gel run at 4°C of *OTH95* cells (Ot), and *A. thaliana* (At) thylakoids. Thylakoids were prepared using standard procedures and 10 µg Chl of 2% SDS solubilised material were loaded. The difference in apparent molecular weight between LHCP and LHCII reflects a stronger resistance of LHCP trimer to SDS denaturation, as LHCP and LHCII migration on either BN- or denaturing SDS-PAGE is comparable (not shown). **b** Absorption spectrum of the antenna bands in the 450–750 nm region. **c** Absorption spectrum difference between the *A. thaliana* and *OTH95* light harvesting complexes. Absorption spectra were normalised at 670 nm before calculating their difference spectrum. **d** ECS spectrum of *OTH95*, measured in living cells upon excitation with a saturating laser pulse. The light minus dark spectrum is shown. *fp* free pigments

characteristics reflect the environment experienced by the field-responding pigments. Whilst in plants the light-induced generation of an electric field results in the red shift of the spectrum, a transition towards the blue is observed in *O. tauri.* This suggests that the orientation between the field and the transition moment of the carotenoid is antiparallel in this alga (see Eq. 1), i.e. a rather unusual configuration for photosynthetic organisms (e.g. Lösche et al. 1988). This finding implies that the spatial arrangement of carotenoids within the LHCP complex must be very different from what is observed in LHCII (Liu et al. 2004), consistent with previous conclusions based on linear and circular dichroism analysis of the LHCP complex from the prasinophyte *Mantoniella squamata* (Goss et al. 2000).

Application of the ECS to study acclimation of the photosynthetic apparatus

The non-invasive character of the ECS, together with the possibility to synchronise the photosynthetic turnover (see section "ECS and photosynthesis"), allows information about changes in the architecture of the photosynthetic chain to be derived, which follow acclimation to environmental stimuli. Changes in the photosynthetic apparatus are typically observed in photosynthetic organisms subjected to nutrient deficiencies, where photosynthesis decreases due to changes in the activity and stoichiometry of the photosynthetic complexes (Merchant et al. 2006). Photosynthetic acclimation to nutrient availability is particularly well characterised in the case of iron starvation, where changes in both light absorption and electron flow capacities of the cells are observed (reviewed in Merchant et al. 2006). Iron is of primary importance in biological systems because it is a central constituent of hemes and iron–sulphur clusters. The activity of many types of enzymes, particularly the ones involved in energy conversion processes, relies on these redox centres. Therefore, several mechanisms exist to ensure iron homeostasis in plants and algae (for reviews see Curie and Briat 2003; Briat et al. 2007). Iron mobilisation is critical for microalgae in the iron-limited open ocean and for terrestrial plants, which are exposed to limited bioavailability of iron because its prominent form in soil in the presence of oxygen, Fe^{3+}, is poorly soluble at neutral and

alkaline pH. In photosynthesis, PSI has the highest iron content (12 Fe per reaction centre) and is, therefore, particularly sensitive to iron deficiency. Deprivation of this metal induces changes at the level of both the PSI light harvesting apparatus and the reaction centre. In cyanobacteria, Fe starvation induces the expression of IsiA, a chlorophyll binding protein similar to CP43, which binds the PSI cores (Singh and Sherman 2007). Similar changes are observed in the halotolerant eukaryotic alga *Dunalliela salina* (Varsano et al. 2006), where the light harvesting protein homologue TidI accumulates in iron-limited conditions. Whilst modifications in PSI antenna are also observed in Fe-deficient *Chlamydomonas reinhardtii* cells (Moseley et al. 2002), no significant changes in the PSI antenna of plants have been reported so far (Timperio et al. 2007). In general, the most important effect of iron deprivation is a marked decrease in PSI centres, relative to other molecular constituents of the photosynthesis apparatus, which results in a reduced capacity to reoxidise the plastoquinone pool in the light. The decrease in PSI complexes is particularly severe in cyanobacteria, leading in some instances to a fourfold decrease in the PSI/PSII ratio (e.g. Guikema and Sherman. 1984; Sandstrom et al. 2002). Large changes in PSI content have also been observed in photosynthetic eukaryotes, including *C. reinhardtii* (Moseley et al. 2002) and centric diatoms (Strzepek and Harrison 2004). Recently, the employment of the ECS approach has allowed a precise quantification of the PSII/PSI stoichiometry in Fe-starved unicellular algae, including diatoms (Allen et al. 2008), Ostreococcus (Cardol et al. 2008) and *C. reinhardtii* (Petroutsos et al. 2009). Detecting changes in the PSII/PSI stoichiometry with the ECS measurement requires single turnover flash spectroscopy. In conditions where all the active PS perform one charge separation, i.e. after excitation by a saturating laser flash, the "a" phase of the ECS is indicative of the total number of active reaction centres in the chloroplasts. The addition of DCMU and hydroxylamine to completely block the PSII turnover makes the "a" phase proportional to the sole number of PSI. PSII contribution can then be calculated from the decrease in the signal amplitude upon the addition of DCMU and hydroxylamine, whereas PSI can be estimated as the fraction of the signal that is insensitive (see Fig. 3b). In contrast to other techniques employed before, such as biochemical assessments of complex subunits (e.g. Anderson et al. 1995), evaluations of oxygen evolution versus P_{700} oxidation extents (Melis 1989) and EPR measurements (Danielsson et al. 2004), the ECS approach provides a more reliable quantification procedure (e.g. Chow et al. 2000; Fan et al. 2007) as it does not require any normalisation between the different parameters employed to assess the amounts of PSII and PSI. On the other hand, a correct estimate of the PSI/PSII ratio requires the utilisation of laser flashes, to avoid occurrence of double PSI turnovers, which would otherwise lead to over-estimation of this complex. In most photosynthetic organisms, recovery of photo-active PSI centres is limited by the rate of re-reduction of P_{700}^{+}, the primary electron donor of PSI. This process can be fast, however, if plastocyanin and/or cytochrome c_6 (the soluble PSI donors) are pre-bound to the reaction centre. Pre-bound PSI donors can reduce P_{700}^{+} in less than ten microseconds (e.g. Delosme 1991), i.e. a time that is shorter than the average lifetime of commercial xenon sources (20–30 μs). A xenon flash can, therefore, excite a PSI with a soluble donor bound, inducing a first charge separation, and then, after P_{700}^{+} rereduction by bound PC or cyt c_6, excite it a second time during the illumination by the flash (especially if the flash intensity is high, thus providing more than one photon per PSI per flash). In green algae, it has been estimated that the maximum extent of double PSI turnover in saturating light is ~30–40% (Farah et al. 1995).

Application of the ECS approach to study alternative electron flow processes

Measurements of the ECS signal under continuous illumination are most suitable to study the overall function of the photosynthetic chain. To measure the ECS in continuous light, it is necessary to deconvolute this signal from other overlapping optical changes (Witt 1979). As discussed in the introduction, this can be achieved; thanks to the different relaxation times of the different signals: whilst relaxation of the triplet of carotenoids is faster than the ECS decay, both the 505 and the 535 absorption changes are much slower than the ECS changes (see above). Thus, techniques have been developed to study the ECS in continuous light, including the so-called DIRK (dark interval relaxation kinetics, Sacksteder et al. 2000) or the "dark pulse" (Joliot and Joliot 2002) methods. In both cases, the ECS signal is probed at appropriate wavelengths, both when steady-state photosynthesis is established by continuous illumination and when light is switched off for a short time (in general less than 1 min). In steady state, the contributions of PSI, PSII, cytochrome b_6f and of ion leak through the membrane are not distinguishable on a kinetic basis. The overall rate of membrane potential formation $\left(V_{\mathrm{ph}} = R_{\mathrm{ph}} + R_{b_6f} - R_{\mathrm{leak}}\right)$ is the sum of the photochemical rate of membrane potential formation R_{ph}, of the cyt b_6f specific rate R_{b_6f} and of the rate R_{leak} of ion leaks through the membrane (Fig. 5a). When the light is switched off, R_{ph} immediately falls to zero whereas R_{leak} and R_{b_6f} are unchanged. R_{ph} can, therefore, be evaluated from the difference $(S_{\mathrm{L}} - S_{\mathrm{D}})$ between the slopes of the ECS signal measured immediately before (S_{L}) and after (S_{D}) the light is switched off (Joliot and Joliot 2002).

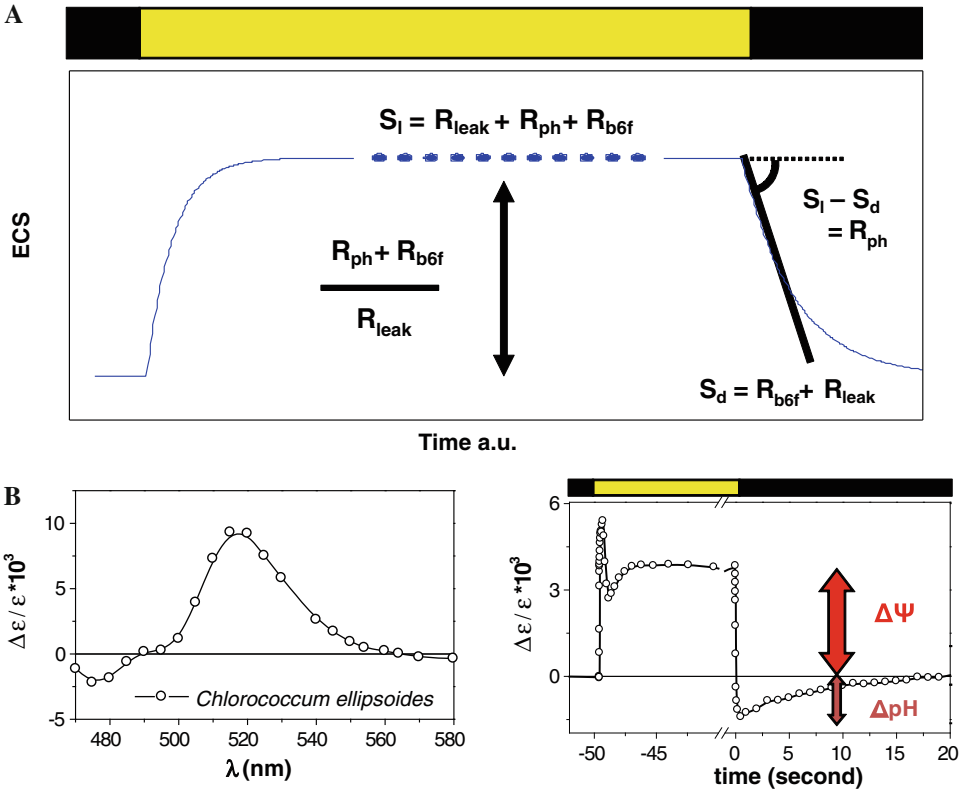

Fig. 5 The ECS signal allows the turnover of the photosynthetic chain to be measured in continuous light. **a** By measuring the transient changes of the ECS under steady-state illumination or upon switching the light off, it is possible to accurately estimate electron transfer kinetic and thermodynamic parameters, as shown. See text for further details. **b**. ECS spectrum (*left panel*) and ECS changes kinetics (*right panel*) in *Chlorococcum ellipsoides*. After switching the light off, a fast decay followed by a slower rise phase are seen, which have been interpreted in terms of the different rates of relaxation of the two components of the pmf (ΔΨ and ΔpH, *red arrows*) and lead to an inversion of the ECS signal. Black and yellow bars represent dark and light phases, respectively

In steady state, electron transfer mainly stems from linear electron flow (LEF), in which PSI and PSII work in series with very similar photochemical rates, leading to CO_2 assimilation. However, when the Calvin cycle is mostly inactive (e.g. at the onset of illumination, Ort and Baker 2002; Johnson 2005), LEF becomes limited by the low rate of NADPH reoxidation. A significant fraction of the photogenerated electrons is available, therefore, for other processes, leading to different PSI and PSII activities. Measuring different PSI and PSII photochemical rates is a criterion employed in the past to reveal the existence of electron diversion towards other sinks than CO_2 in vivo (Harbinson and Foyer 1991). In particular, cyclic electron flow (CEF) activity is considered to be predominant in plants (e.g. Joliot and Joliot 2006) at the onset of illumination. The high efficiency of the CEF is interpreted as the consequence of a redox modulation of the relative efficiencies of the linear and cyclic processes (see Allen 2003). Using the DIRK approach, the fraction of reaction centres involved in CEF can be taken into account whilst estimating the membrane potential formation rate due to photochemistry (Joliot and Joliot 2002):

$$\begin{aligned} &R_{ph} = R_{PSII} + R_{PSI-linear} + R_{PSI-cyclic} \\ &\text{with } R_{PSI-linear} = R_{linear} = R_{PSII} \\ &\text{therefore, } R_{PSI-cyclic} = R_{ph} - 2 \times R_{PSII}. \end{aligned} \quad (2)$$

As stated above, R_{ph} is derived from ECS measurements. R_{PSII} can be estimated from fluorescence measurements using the Genty parameter (Genty et al. 1990):

$$R_{PSII} = ki_{PSII} \times (Fluo_{ss} - Fluo_0)/(Fluo_{max} - Fluo_0),$$

where $Fluo_{max}$ is the maximum fluorescence emission by PSII, $Fluo_{ss}$ is the steady-state emission, $Fluo_0$ is the minimum fluorescence emission in dark-adapted samples, and ki_{PSII} is the photochemical rate of PSII.

Previous study has established that the Calvin cycle requires ATP and NADPH in a stoichiometry of at least 1.5, i.e. a ratio that cannot be entirely fulfilled by the sole operation of LEF, which provides an insufficient proton to electron balance when compared with the stoichiometry of H^+ required to fuel ATP synthesis by the chloroplast ATP-synthase (Allen 2003, see, however, Steigmiller et al. 2008 for a different conclusion). Alternative electron flow could contribute to an additional proton gradient across the thylakoid membranes, and, therefore, to additional ATP synthesis to adjust the ATP/NADPH stoichiometry for proper carbon assimilation (reviewed in Eberhard et al. 2008). Besides CEF, the water to water cycle (Mehler 1951) could also contribute to the synthesis of "extra ATP" in cyanobacteria (e.g. Zhang et al. 2009), plants (e.g. Forti and Elli 1995) and unicellular algae (e.g. Johnson et al. 2010). In particular, a different version of the water to water cycle has been recently observed in marine *Synechococcus* under Fe starvation (Bailey et al. 2008) and in a particular Ostreococcus ecotype (*Ostreococcus RCC809*, Cardol et al.

2008), where a significant fraction of electrons generated by PSII are consumed by a plastid plastoquinol terminal oxidase (probably PTOX).

This alternative electron flow, which bypasses PSI, should also lead to the generation of a proton gradient without NADPH production, by boosting PSII activity. Indeed, although the direct contribution of PTOX to the generation of a pmf is controversial, it is clear that the combined oxidation of water by PSII and of plastoquinol by PTOX must lead to the vectorial transfer of H^+ from the stroma to the luminal space (e.g. Rumeau et al. 2007, Eberhard et al. 2008). This possibility was experimentally tested using the ECS tool in the case of *Ostreococcus RCC809*. In this strain, inhibition of PSII by DCMU completely abolished the generation of the ECS. Conversely, addition of the cytochrome b_6f inhibitor dibromothymoquinone (DBMIB), blocking both LEF and CEF, allowed a substantial fraction of the ECS signal to be maintained. Further addition of propylgallate, a PTOX inhibitor, completely suppressed this remaining ECS signal. The different effects of DCMU and DBMIB on the ECS suggest, therefore, that the cytochrome b_6f-independent pmf generation is related to the combined PSII and PTOX activities.

The magnitude of this process can be estimated by introducing a new term related to PTOX activity in Eq. 2. This yields to $R_{ph} = R_{PSII\text{-}linear} + R_{PSII/PTOX} + R_{PSI\text{-}linear} + R_{PSI\text{-}cyclic}$.

Considering that $R_{PSI\text{-}cyclic} \sim 0$, and that $R_{PSI\text{-}linear} = R_{PSII\text{-}linear}$ one obtains $R_{PSII/PTOX} = R_{ph} - 2\ R_{PSII\text{-}linear}$.

Knowing that $R_{PSII/PTOX} \sim 0.5\ R_{ph}$ (Cardol et al. 2008), one obtains $R_{PSII/PTOX} \sim 2\ R_{PSII\text{-}linear}$, implying that only 1/3 of the electrons from PSII are directed to PSI in *RCC809*, i.e. a value in agreement with the estimates of the PSII/PSI ratio in this ecotype ($\sim$3/1, Cardol et al. 2008). This suggests that linear flow is strictly limited by the PSI availability in this alga. It is tempting to propose that diversion of electrons to oxygen downstream of PSII, but before PSI, may reflect a useful strategy in marine phytoplankton to bypass the constraints imposed by light and/or nutrient limitation and allow successful colonisation of the open-ocean environments.

The ECS approach to study the pmf composition under illumination

When the DIRK method is extended to longer dark period, an interesting phenomenon is observed: after the first decay, a slower rise is seen, which leads to an inversion of the ECS (e.g. Cruz et al. 2001, see Fig. 5b in the case of the green alga *Chlorococcus elipsoideum*). This inversion has been interpreted in terms of the different rates of relaxation of the two components of the pmf. After illumination, the electric component of the pmf is expected to relax faster than the ΔpH, due to the low dielectric constant of the thylakoid membranes (Vredemberg. 1976), the high H^+ buffering capacity of the lumen (Junge and McLaughlin 1987), and the slow rate of charge redistribution along the membranes (see Cruz et al. 2001 for a further discussion). Therefore, the fast transfer of positive charges from the lumen to the stroma should allow the pmf to relax to its dark-adapted level. This would reduce the ΔΨ component (i.e. the ECS signal) to a level below its dark-adapted level, to compensate for the presence of a buffered ΔpH. During the second phase, the slow relaxation of the buffered ΔpH would be compensated by a flux of counterions, leading to the slow rise of the ECS signal. Eventually, this phase will end once both the ΔpH and ΔΨ have reached their dark equilibrium state (see Kramer et al. 2004 for further discussion).

Based on the overall amplitude of the total and of the inverted ECS signals, attempts have been made to estimate the partitioning between the light-induced ΔpH and ΔΨ components of the pmf in plants (Kramer et al. 2004; Cruz et al. 2005a, b; Takizawa et al. 2007) and green algae (Fig. 5b, Finazzi et al. 2006; Cardol et al. 2008). These estimates have indicated that the size of the ΔpH is by far lower than what was previously estimated based on in vitro measurements (e.g. Rumberg and Siggel 1969). Thus, Kramer and colleagues have proposed a "moderate" lumen pH hypothesis (Sacksteder et al. 2000), which assumes that the lumen pH is maintained in a range where it can regulate light capture whilst not damaging the photosynthetic apparatus. The predicted lumen pH should range between 5.7 and 7.8 (Kramer et al. 2004). On the other hand, measurements of the ECS in the dark (see section "Quadratic components of ECS allow for the measurement of a pre-existing proton gradient") have revealed the existence of a pmf across the thylakoid membranes in both dark-adapted algae (Finazzi and Rappaport 1998) and plants (Joliot and Joliot 2008), which comprises a ΔpH of 0.5–1 unit (Heldt et al. 1973). These findings may provide a means to reconcile the in vitro and in vivo ΔpH estimates. Indeed, the sum of the dark- plus the light-induced proton gradient in vivo closely resembles the pH gradient estimated in vitro.

Quadratic components of ECS allow for the measurement of a pre-existing proton gradient

Owing to the linearity between the ECS and the applied field, the ECS techniques only allow measuring changes of the pmf but not its absolute value, as required to study ATP synthesis and regulation of the photosynthetic process.

However, as discussed above, the ECS signal can in principle be calibrated, leading to the estimate of the absolute value of the ΔΨ in the light (Witt 1979; Joliot and

Joliot 1989). Indeed, the amplitude of the spectral shift undergone by a pigment when submitted to an electric field (F) is proportional to F or F^2 depending on the polarisation state of the pigment (Eq. 1). The amplitude of the linear ECS response (ΔECS_{lin}) observed upon a flash-induced increase of the electric field (ΔF, due to charge separation) is constant, independent of the value of the field preexisting the perturbation (Fig. 1c). In contrast, the amplitude of the quadratic ECS response (ΔECS_{quad}) depends on the value of the preexisting field. Therefore, the amplitude of the flash-induced quadratic ECS, when present, can be used to probe the absolute value of the electric field. This has been experimentally evidenced in *C. sorokiniana* and *C. reinhardtii* mutants devoid of the light harvesting complexes. In these mutants, most of the pigment-binding proteins are absent and both the linear and quadratic probes are observed, in contrast with wild type cells, where the linear response is predominant (Joliot and Joliot 1989).

Using these mutants, the amplitude of the electric component (the $\Delta\Psi$) of the large electrochemical gradient built in darkness has been evaluated and found to be half the ΔpH, assessed from the kinetics of the "b" phase (Finazzi and Rappaport 1998). The amplitude of the pmf in the dark ($\sim$110–140 mV) fits well with estimations based on the ATP/ADP ratio measured in these algae, suggesting that it is built at the expense of ATP hydrolysis by the ATPsynthase–ATPase complex. A pmf of reduced size was found in anaerobic (respiration inhibited) conditions compared to aerobic (respiration active) conditions, confirming that the ATP comes from respiration.

The dark pmf could have several functions in vivo: it could help maintaining a constitutive amount of activated ATP-synthase complexes, thereby facilitating ATP synthesis upon illumination. Indeed, activation of the ATP-synthase upon a light transition is mediated by the building of a pmf (Junesh and Gräber 1985). The dark $\Delta\mu_H^+$ could also allow the enzymes involved in the xanthophylls cycle (the activation of which is ΔpH dependent) to be maintained close to their active states. This would facilitate photo-protection during a dark-to-light transition, by speeding up the onset of non-photochemical quenching of absorbed energy (Horton et al. 1996; Niyogi 1999). Finally, the existence of a $\Delta\mu_H^+$ in the dark could provide the energy source for protein import in the dark, thus maintaining an active photosynthetic apparatus (reviewed in Jarvis and Robinson 2004).

Perspectives

As discussed above, employment of the ECS tool allows the energetics of the chloroplast in vivo to be studied, opening the possibility to investigate several processes related to the physiology of photosynthesis (the balance between ATP synthesis and generation of reducing equivalents, chloroplast assembly and degradation, acclimation to environmental conditions, etc.). However, this approach can be applied only to organisms which display an ECS signal. As a general rule, photosynthetic organisms possessing intrinsic light harvesting complexes should show an ECS band shift when exposed to illumination. This is true in the case of green algae (Fig. 2a, b, c, d) and diatoms (Fig. 2f, see also Szabo et al. 2008). Recent measurements of the ECS in the pennate diatom *Phaeodactylum tricornutum* indicated the coexistence of linear and quadratic probes within the same light harvesting complex (Bailleul and Finazzi unpublished). This finding may prove very useful, as the presence of pigments responding to F and F^2 allows the absolute value of the $\Delta\Psi$ and ΔpH to be assessed and, therefore, the dynamics of the pmf to be followed in a living organism.

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