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REVIEW

Probing the photosynthetic apparatus noninvasively in the laboratory of Reto Strasser in the countryside of Geneva between 2001 and 2009

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Abstract

An overview is given of several studies on the fast chlorophyll (Chl) *a* fluorescence (OJIP) transient carried out in the laboratory of Reto Strasser between 2001 and 2009. At the beginning of this period the *HandyPEA* and *PEA-Senior* instruments were introduced by Reto Strasser and *Hansatech Instruments Ltd*. (UK) that gave a lot of experimental flexibility compared to the experiments that were feasible in the preceding years. These technical innovations, including the combination of 820-nm transmission measurements (for the determination of the P_{700} and PC redox states) and Chl *a* fluorescence [originating from photosystem II (PSII)], enabled us to establish the effects of electron flow through and at the acceptor side of photosystem I during a dark-to-light transition on fluorescence induction in leaves. These instruments further allowed us to show biological variability between various photosynthetic organisms and how several chemical treatments could modify the Chl *a* fluorescence kinetics. We also obtained new information on the effect of the inhibitor DCMU [3-(3',4'-dichlorophenyl)-1,1-dimethylurea] on Chl *a* fluorescence induction. In addition, the effects of heat stress on electron flow through PSII and the entire electron transport chain were investigated in detail. The article also reflects how our perception and interpretation of the OJIP kinetics changed over time.

Additional key words: conformational changes; drought stress; ionophores; photosynthesis; Photosynthetic Control.

Introduction

We, Szilvia Z. Tóth, Abdallah Oukarroum, and Gert Schansker, belonged to the last generation of people who worked in the laboratory of Reto Strasser before his retirement on 31 July, 2009. [For impressions on the period before 2001 and after 2010, *see* Govindjee *et al.* (2019)]. In 2001, we started a new phase in our scientific careers in the Laboratory of Bioenergetics of the University of Geneva in Lullier, a small village near the French border. Szilvia Z. Tóth and Abdallah Oukarroum started their PhD studies, whereas Gert Schansker joined the laboratory as maître assistant (first assistant). Our arrival in Geneva coincided with the introduction of two instruments, which Reto Strasser had developed in collaboration with *Hansatech Instruments Ltd*. (King's Lynn, UK): the *HandyPEA*, and a prototype of an instrument for the simultaneous measurement of chlorophyll (Chl) *a* fluorescence and 820 nm, called the *PEA-Senior*. A bit later, another version of the *HandyPEA*, the so-called *Fast HandyPEA* was devised by Reto Strasser and built by *Hansatech Instruments Ltd*. with a reduced minimum pulse length of 0.3 ms (compared to 300 ms for the basic instrument). *HandyPEA* instruments with a very strong light source [*ca.* $15{,}000 \times 10^{-6}$ mol(photon) m^{-2} s^{-1}] and a built-in FR (far-red) light source were also constructed in the year 2004. As

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Abbreviations: Asc – ascorbate; CET – cyclic electron transport around PSI; DCMU – 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; DFI – drought factor index; F_0, F_V, F_M – minimum, variable, and maximum Chl *a* fluorescence, respectively, of the dark-adapted state; Fd – ferredoxin; F_J, F_I – the fluorescence intensity at the J (~ 3 ms) and I (~ 30 ms) steps, respectively; FNR – ferredoxin-$NADP^+$ reductase; NPQ – nonphotochemical quenching (mainly due to dissipation of excitation energy as heat); OEC – oxygen-evolving complex; OJIP – the polyphasic fluorescence rise measured at high light intensity named after its defining points: O at ~ 20 μs, J at ~ 3 ms, I at ~ 30 ms, and P at 200–500 ms; P_{700} – PSI reaction center chlorophyll pair; PC – plastocyanin; $PI_{(abs)}$ – stress parameter derived from the OJIP transient; PQ – plastoquinone; PQH_2 – plastoquinol; q_P – photochemical quenching (due to conversion of excitation energy in chemical energy); RC – reaction center; S0, S1 – refer to redox states of the manganese cluster of the oxygen-evolving complex; α, β, γ phases – kinetic phases derived from the fluorescence induction kinetics measured in the presence of DCMU.

our paper will show, these instruments were essential for our studies on the fast Chl *a* fluorescence (OJIP) transient and, by employing these instruments, we covered a broad range of topics from the effect of 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) on Chl *a* fluorescence kinetics, electron donation by ascorbate (Asc) to the PSII donor side and the determination of the PQ pool redox state (Szilvia Z. Tóth), to the activation state of the acceptor side of PSI (Gert Schansker) and drought and biological variability (Abdallah Oukarroum) and several other topics that will not be discussed here.

The effects of DCMU on the OJIP kinetics of intact leaves

On her arrival, Reto Strasser proposed Szilvia Z. Tóth to investigate the relationship between the electron transfer reactions within PSII in DCMU-inhibited leaves by making use of the *HandyPEA*. Their analyses led to several new insights on the relationship between PSII and Chl *a* fluorescence, although it took some time to develop the relevant measuring protocols and to find the most suitable interpretation of the acquired data. DCMU had been applied in several studies on Chl *a* fluorescence. It displaces Q_B from its binding site at the D1 protein (Velthuys 1981, Wraight 1981); thereby, it prevents the reoxidation of Q_A^- by forward electron transport. Upon DCMU treatment, the Chl *a* fluorescence intensity at the J step (located at around 3 ms) strongly increases, and the I step (at 30 ms) disappears. Thus the OJIP transient is considerably simplified and the F_M is reached usually already at around 10 ms. The fluorescence kinetics of DCMU-treated leaves have a sigmoidal character during the first approx. 0.2 ms and in spite of its appearance, its kinetics remain complex (*see* below).

Several authors had reported that a DCMU treatment causes an increase of F_0 and a decrease of F_M. The observed increase of F_0 in DCMU-treated thylakoids was explained by the presence of Q_B^- in some PSII reaction centers (RCs) even after a long dark adaptation. At a stromal pH of about 7.5, the electrons reside about 5% of the time on Q_A and 95% of the time on Q_B, which equates an equilibrium constant K of 20 for this reaction (Diner 1977). DCMU can only displace Q_B during the 5% of the time the electron is on Q_A and this results in a back-transfer of electrons from Q_B^- to Q_A (Velthuys and Amesz 1973, Rutherford *et al.* 1982, van Gorkom 1985), thus, a moderate increase of F_0 may occur, which becomes long-lived if recombination with the donor side of PSII is not possible. This happens when the PSII donor side is in the S0 or S1 state or when the oxygen-evolving complexes (OECs) are partially or completely inactive (Tóth *et al.* 2007a).

The decrease of F_M has been attributed to the quenching of fluorescence due to the presence of an oxidized PQ-pool (Vernotte *et al.* 1979, Neubauer and Schreiber 1987). In Tóth *et al.* (2005a), we showed that if the DCMU treatment is done in complete darkness overnight, and DCMU is allowed to diffuse slowly into the leaves, the F_0 and F_M values do not change relative to the control samples (*cf.* Fig. 1*A*). This result was reproduced many times after publication of our article in 2005; it requires the averaging of 20–25 measurements to minimize the effect of biological variability between the leaf samples. In the above-mentioned article, F_M-data +/–DCMU for four different plant species were determined with their standard deviations. Simultaneous 820-nm transmission measurements proved that in DCMU-treated leaves, linear electron transport was fully inhibited, thus the F_M values are equal in the presence of an oxidized or reduced PQ-pool. This allowed us to conclude that fluorescence quenching by the oxidized PQ-pool does not occur in intact leaves. On the other hand, in vacuum-infiltrated leaf discs and in isolated thylakoid membranes, the F_M values decreased upon a DCMU treatment. Therefore, we proposed that the F_M-decrease (*i.e.*, PQ-pool quenching) is caused by mechanical damage of the thylakoid membranes allowing

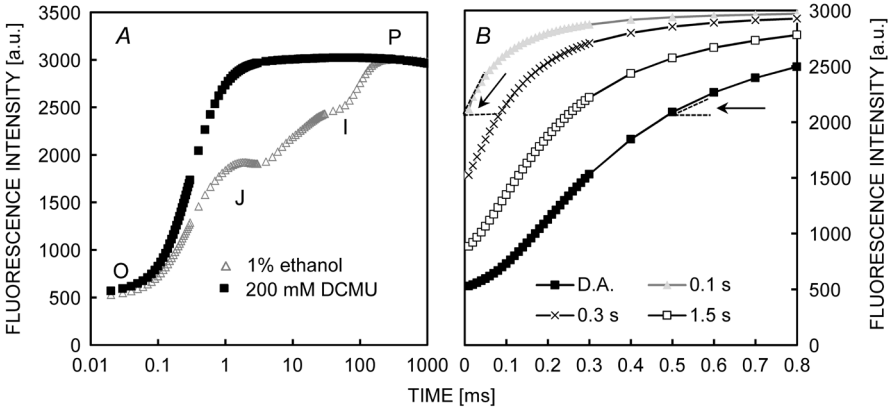

Fig. 1. Chl *a* fluorescence transients of intact pea leaves, incubated in 200 μM 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) solution overnight in complete darkness. Full, 1-s Chl *a* fluorescence transients of DCMU-treated and control (1% ethanol-treated) leaves (*A*). Chl *a* fluorescence of DCMU-treated leaves induced by double light pulses of 1 s with 0.1 to 1.5 s of darkness in between (*B*). The kinetics are plotted on a linear timescale and the values for the initial 0.8 ms are shown. *The arrows* indicate that upon a short dark period (0.1 s) enabling partial fluorescence relaxation, the initial slope of the fluorescence transient is much steeper than upon the first light pulse at the same fluorescence intensity value. Data are from Tóth *et al.* (2005a) and Tóth and Strasser (2005).

direct interaction between oxidized PQ molecules and the PSII antenna (Kurreck *et al.* 2000), which is absent in untreated leaves.

OJIP rise in the presence of DCMU and relations to conformational changes within PSII

The *HandyPEA* makes it possible to do double pulse experiments with a time interval between saturating pulses down to 100 ms. In such experiments, the first pulse reduces Q_A and the F_M level is reached; upon the second pulse the population of Q_A, which had become oxidized during the dark interval, is rereduced and the F_M level is reinduced as well. The double pulse experiments made it possible to study the Chl *a* fluorescence kinetics induced following different dark intervals between the first and the second pulse. We showed that when fluorescence induction measurements were carried out on DCMU-treated samples at short times after the first pulse, the initial slope of the fluorescence rise became considerably steeper than that of the fully dark-adapted sample (Fig. 1*B*). As noted by Reto Strasser, it looked as if Q_A reduction became faster by increasing the initial amount of Q_A^-. The α, β, γ PSII-heterogeneity analysis, based on the area-growth above the fluorescence transient (according to Melis and Homann 1975, 1976), gave 57.8% α-, 33.1% β-, and 6.2% γ-centers that are ascribed to different populations of PSII units. The antenna size of a β-center is supposed to be 2–3-fold smaller than that of the α-center. It is widely accepted that the α-centers are located mainly in the grana and the β-centers are in the stroma lamellae (Lavergne and Briantais 1996). It was also suggested that the three populations of PSII units may also differ in their connectivity properties (the α-centers are supposed to be grouped, whereas β- and γ-centers are not). The trapping efficiency of the γ-centers is thought to be lower probably due to a nonfunctional acceptor side; they were characterized in the laboratory of Reto Strasser using another experimental approach (Schansker and Strasser 2005). Apart from the properties mentioned above, the β phase had also been shown to be sensitive to stacking (Hodges and Barber 1983). Another important aspect of these measurements was that when employing a dark interval of less than 1 s, the sigmoidicity of the curves disappeared. At that time, Szilvia Z. Tóth and Reto Strasser explained these phenomena by connectivity and by antenna size heterogeneity of PSII units, with different dark-relaxation kinetics. However, too many things were unclear, and the results remained unpublished apart from conference proceedings (Tóth and Strasser 2005).

Several years later, while working in the laboratory of Dr. Győző Garab in the Biological Research Centre in Szeged, Szilvia Z. Tóth determined the temperature dependence of the Chl *a* fluorescence induction kinetics of leaves inhibited with DCMU. This gave two new important insights: on lowering the temperature below –10°C, the sigmoidicity was diminished and at the same time the fast (α) and slow (β) phases of the fluorescence rise kinetics became better separated. Arrhenius plots of the α and β phases demonstrated that the fast rise phase was nearly temperature independent, whereas the β phase had a strong temperature dependence (Schansker *et al.* 2011). The difference in temperature dependence explained the kinetic separation of the two rise phases observed on lowering the temperature, suggesting that a large part of the sigmoidicity observed in the presence of DCMU is not due to transfer of excitation energy between PSII units. The difference in the temperature dependence between the α and β phases suggested that the α phase was associated with charge separations in functional PSII RCs (therefore, no temperature dependence) and the β phase was probably due to another, temperature-dependent and PSII-related process. These observations formed an important building block of the conformational change concept (Schansker *et al*. 2011, 2014): In this concept, stable charge separations, inducing Q_A reduction lead to a strong increase in fluorescence intensity (responsible for approx. 70% of F_V at room temperature), whereas the remaining 30% (traditionally seen as the β phase) is ascribed to a fluorescence yield increase driven by light-induced conformational changes in RCs with reduced Q_A^-. In other words, the β (temperature dependent) phase is also induced by light but requires the reduction of Q_A and, therefore, is only induced after a lag time (in the tens of μs in the presence of DCMU).

In view of the above, the fluorescence kinetics presented in Fig. 1*B* may be explained by the following mechanism: Upon a saturating pulse of $3{,}000 \times 10^{-6}$ mol(photon) m^{-2} s^{-1}, Q_A becomes reduced and conformational changes are induced within about 10 ms, resulting in F_M. During the short dark period (less than 1 s), the thermal phase relaxes, whereas Q_A^- remains largely reduced. Upon a subsequent light pulse, the generation of the conformational change, due to the presence of Q_A^- is rapid, explaining the steeper slope of the fluorescence rise than in dark-adapted samples. This mechanism is explained in detail in Schansker *et al.* (2011) and the arguments against a pure Q_A model were analyzed in a follow-up review paper (Schansker *et al.* 2014).

PQ pool redox state

The plastoquinone (PQ) pool plays an important role in the regulation of photosynthesis, in retrograde signaling and it also forms a crossing point for several pathways of electron transport (Heber and Walker 1992, Bennoun 2001, Haldimann and Tsimilli-Michael 2002, Joët *et al.* 2002, Yoshida *et al.* 2008, Borisova-Mubarakshina *et al.* 2015), thus determining its redox status *in vivo* is very important. At the Photosynthesis Congress in Montréal (2004), Jerzy Kruk and Stanislaw Karpinski presented an HPLC-based method to determine the PQ redox state (*see* Kruk and Karpinski 2006). They told us that, in their opinion, it was not possible to develop a Chl *a* fluorescence-based assay for the PQ-pool redox state. The data we had presented three years earlier at the Photosynthesis Congress in Brisbane (Strasser *et al.* 2001) had suggested the F_J level could be an indicator for the PQ-pool redox state – the problem was, how to prove the relationship experimentally. Subjecting intact leaves to anoxia by flushing them with nitrogen gas in a closed

chamber in the dark seemed to offer a viable approach. The plastoquinol oxidase responsible for the reoxidation of the PQ-pool is inhibited by the absence of oxygen (Bennoun 1982, Haldimann and Strasser 1999, Haldimann and Tsimilli-Michael 2002), and as a result of constitutive chlororespiratory activity (*e.g.*, Joët *et al.* 2002), the PQ-pool becomes reduced. The specificity of anaerobiosis allows studying the effect of the PQ-pool redox state on the fluorescence induction kinetics. Remarkably, F_J was linearly correlated both with the area between OJ and F_M and the area between JI and F_M. One of the implications of this observation is that connectivity does not affect the fluorescence intensity at the J step (F_J). Evaluating five parameters [$F_{20\mu s}$, initial slope ($F_{70\mu s} - F_{20\mu s}$), F_J, area above the OJ phase, and an 820-nm transmission parameter (I_{820nm} – 10 s FR)] as a function of the PQ-pool redox state, it was possible to show that in pea leaves a nearly fully reduced PQ-pool is in equilibrium with 20% Q_A^-, which provides an explanation for the relationship between F_J and the PQ-pool redox state. It also allowed us to propose an assay for the PQ-pool redox state in leaves with an inactive PSI acceptor side (Tóth *et al.* 2007b). The proposed assay, based on the F_J value for the quantification of the redox state of the PQ-pool has become a widely used tool since then (*e.g.*, Wunder *et al.* 2013, Bolychevtseva *et al.* 2015, Levitan *et al.* 2015, Wagner *et al.* 2016).

Heat stress and ascorbate as an alternative electron donor of PSII

In Tóth *et al.* (2007a), electron transport processes were investigated in leaves of which oxygen evolution was fully inhibited by a heat pulse (48 to 50°C, 40 s). The classical idea is that a complete destruction of Mn-clusters eliminates the electron donation capacity of PSII and we were interested in its consequences for Chl *a* fluorescence induction. The most remarkable effect of this rapid heat treatment is the appearance of a very clear K step at around 300 μs in the Chl *a* fluorescence transient – first described by a PhD student of Reto Strasser (Guissé *et al.* 1995) – reflecting Q_A reduction resulting from one charge separation and electron donation by Tyr_Z to P_{680}^+ (Fig. 2*A*). We observed, however, that additional Q_A^- accumulation occurred subsequently, in the 0.2–2 s time range. We showed by simultaneous Chl *a* fluorescence and 820-nm transmission measurements that this corresponds to partial reduction of the linear electron transport chain (Tóth *et al.* 2007a).

In order to test the origin of this secondary fluorescence rise, Szilvia Z. Tóth had the idea to employ short (5-ms) double pulses with brief dark intervals. Upon the first 5 ms-pulse one charge separation and reoxidation of Q_A^- during the light pulse occurs (manifested as the K peak, followed by a decrease of fluorescence intensity to the F_0 level; Fig. 2*B*). Upon the subsequent light pulse, Q_A reduction (*i.e.*, fluorescence rise) will only be possible, if there are electrons available on Tyr_Z enabling Q_A reduction, thus a putative electron donor had rereduced Tyr_Z^+. Determining this electron donation process became possible by the *Fast HandyPEA* instrument, which enabled us to develop a protocol consisting of two short 5-ms light pulses spaced 2.3–500 ms apart. Based on these fluorescence measurements, we showed that 'alternative' electron sources donate electrons to Tyr_Z^+ within PSII, with a $t_{1/2}$ of about 30 ms in pea leaves (Tóth *et al.* 2007a).

In another experimental approach, DCMU-inhibited leaves were exposed to a heat pulse and subsequently fluorescence induction was measured. Such kinetics consisted of a fast fluorescence rise phase followed by a much slower rise, suggesting that recombination reactions were taking place during the fluorescence rise. It is likely that while Tyr_Z was in the oxidized state, charge recombination between Tyr_Z^+ and Q_A^- could take place rather efficiently, whereas upon the reduction of Tyr_Z^+ by the alternative electron donors, charge recombination was prevented and F_M could be reached.

Later in the laboratory of Győző Garab in Szeged, using Asc-deficient *vtc2* mutants (*VTC2* encodes GDP-L-galactose phosphorylase, a key enzyme of Asc biosynthesis), Szilvia Z. Tóth has proven that this alternative electron

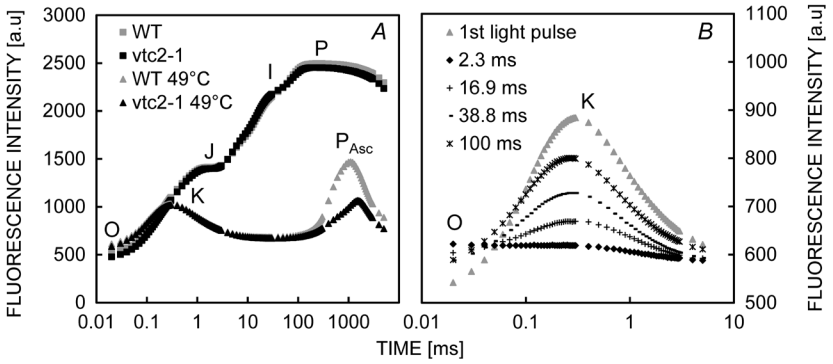

Fig. 2. The effects of heat stress (49°C, 40 s in water bath) on the fast Chl *a* fluorescence transient of intact *Arabidopsis* leaves. Upon a heat pulse inactivating all OECs, the K peak appears which is followed by a decrease and then a remarkable increase in fluorescence intensity (*A*). This second peak, appearing at around 1 s in the fluorescence transient, is strongly diminished in the ascorbate (Asc)-deficient *vtc2-1* mutant. The halftime of electron donation by Asc to PSII can be determined by double pulses of 5 ms, enabling a single charge separation when the OECs are inactive (*B*). Data are based on Tóth *et al.* (2009).

donor is Asc (Tóth *et al.* 2009); the physiological role of this process is to mitigate the effects of heat stress and it also enables a faster recovery of PSII RCs in the light (Tóth *et al.* 2011). More recently, it was shown that upon sulphur deprivation of green algae, Asc accumulates and reaches the mM range; at this concentration level, Asc may inactivate the OEC and then provides electrons to Tyr_Z^+ at a relatively slow rate (Nagy *et al.* 2016, 2018).

The activation state of the acceptor side of PSI

Gert Schansker was given the opportunity to work with the prototype of the *PEA-Senior*, developed by Reto Strasser as part of a very fruitful collaboration with *Hansatech Instruments Ltd*. The advantage of the *PEA-Senior* compared to the instruments sold by *Heinz Walz GmbH* at the time, was, that it was the only instrument capable of the simultaneous recording of the fast Chl *a* fluorescence transient and 820-nm transmission with µs-resolution. Thus studying the electron transport processes occurring during the OJIP transient became possible. In the *PEA-Senior* instrument, 820-nm light is used to detect changes in the redox states of plastocyanin (PC), P_{700}, and ferredoxin (Fd) together. With such instruments it is possible to monitor electron flow through PSI, but it is not suitable for the determination of PSI quantum yield. The first, application-oriented paper was published in 2003 (Schansker *et al.* 2003), in which a red–far-red–red protocol was introduced, enabling the determination of the maximum 820-nm transmission changes and the analysis of the rereduction kinetics of PC^+ and P_{700}^+ following a 10-s far-red pulse. In this paper, we also observed that the acceptor side of PSI [in all likelihood ferredoxin-$NADP^+$ reductase (FNR)] was inactive in darkness and that its activation state has a significant effect on the fluorescence kinetics.

The purpose of the quenching analysis is to separate photochemical quenching (the reduction of Q_A) and nonphotochemical quenching. To achieve this separation, saturating pulses of light of 700–800 ms were used. Doing such experiments and reading the literature in the first half of the nineties Gert Schansker had gotten the impression that a saturating pulse of 700–800 ms reduced only Q_A. In the almost complete absence of clear data on the relationship between light intensity and excitation rate, he realized only in the lab of Reto Strasser that this was a misconception. Actually, it was commonly known and accepted before ~ 1990 that the acceptor side of PSI is inactive in the dark and that it affects Chl *a* fluorescence. The phenomenon was first published in German by Kautsky *et al.* (1960). John Munday translated it and the paper was updated (Munday and Govindjee 1969) and around 1980 FNR had been identified as the inactive factor (Satoh and Katoh 1980, Satoh 1981, Carrillo *et al.* 1981). In a more recent paper, the biochemical data on FNR were discussed (Benz *et al.* 2010). The authors propose that only soluble FNR is active in electron transport and that the role of binding of FNR to the membrane is mainly to protect the enzyme against degradation at the relatively acidic pH value of the stroma in darkness. Fd is an electron donor to several processes and the authors further propose that the activation state of FNR, as gate keeper of the electron transport to the Calvin-Benson cycle, may play a role in the distribution of the electrons to the different Fd-dependent processes.

Methylviologen (MV) is an electron acceptor capable of transferring electrons from the FeS-clusters of PSI to O_2 (Ke 2001). In this way, it bypasses FNR and diminishes the IP phase, lowering F_M (Munday and Govindjee 1969, Neubauer and Schreiber 1987). Based on the light intensity dependence of OJIP transients of MV-treated pea leaves (Schansker *et al.* 2005), Gert Schansker noted that the maximum fluorescence intensity did not extrapolate to F_M at infinite light intensities (*see* the inset of figure 1*B* in Schansker *et al.* 2005). He further observed that a suppression of the IP phase was invariably associated with the absence of, or an incomplete rereduction of P_{700}^+ and PC^+ following the initial oxidation paralleling the OJI-rise. Using the high intensity head built as a collaboration between Reto Strasser and *Hansatech Ltd*., it was possible to test if these observations still held at much higher light intensities. It was shown in Schansker *et al.* (2006), that the suppression of the IP phase in MV-treated leaves or in light-adapted pea leaves could not be overcome by applying light intensities as high as $15{,}000 \times 10^{-6}$ mol(photon) m^{-2} s^{-1}. Further, the regeneration of the IP phase in light-adapted pea leaves needed ~ 15 min of darkness. These data strongly suggested that it was not possible to reach the 'true' F_M once the acceptor side of PSI is activated. Sometime later, an even more clear-cut experiment was found, which allowed us to confirm these observations. We discovered that ginkgo leaves and pine needles have a special property that make them particularly suitable to determine if it is possible to induce the 'true' F_M' once the acceptor side of PSI has been activated. In gymnosperms, a single saturating pulse of about 0.2–0.5 s is sufficient to make the IP phase of a fluorescence transient induced by a subsequent strong pulse of light disappear (Fig. 3). In the case of *Pinus halepensis* we observed that regeneration of the IP phase following a saturating pulse needs a full hour of darkness (Schansker *et al.* 2008), which means that the IP phase is still suppressed long after the relaxation of any pH difference over the thylakoid membrane and of the electric field, as well as the reoxidation of the electron transport chain back to its dark-adapted state. Further, in the context of the quenching analysis, it is assumed that a saturating pulse does not induce NPQ/q_N (van Kooten and Snel 1990) and, therefore, we can assume that the dark-adapted F_M after at least 1 h of dark adaptation can be used as reference F_M. For the skeptical reader, it may be noted that the double pulse experiment on pine needles is easy to perform and it is, therefore easy to confirm the observations discussed here. Since each additional pulse will again lead to a full suppression of the IP phase, the practical consequence is that a quenching analysis will erroneously suggest the presence of a very persistent form of NPQ. The 820-nm measurements showed that the suppression of the IP phase is accompanied by a suppression of the rereduction of P_{700}^+ and PC^+, indicating that it is in fact a photochemical effect, and not due to nonphotochemical quenching (described in detail by Schansker *et al.* 2006).

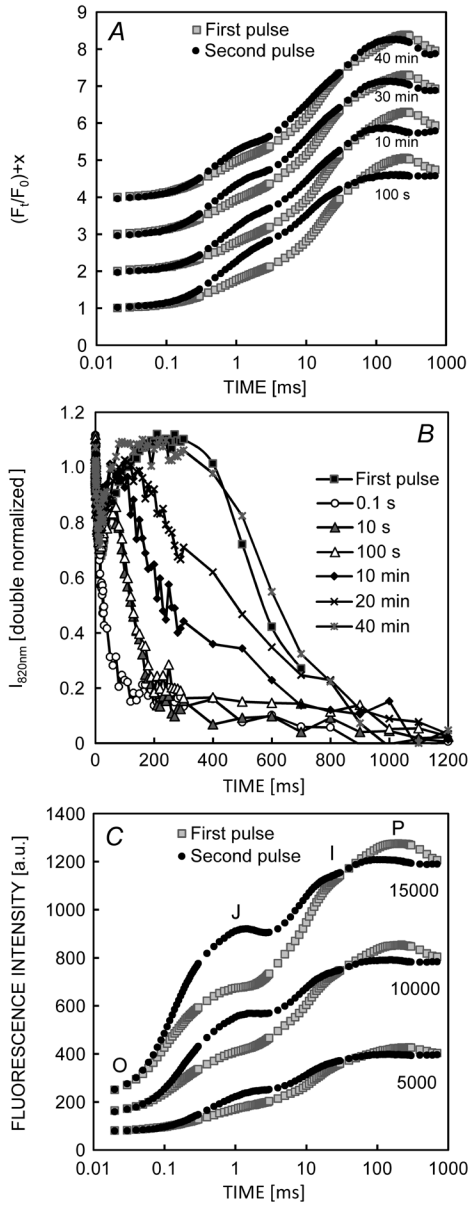

Fig. 3. Double pulse experiments on pine needles. Chl *a* fluorescence induced by a double 0.7-s 1,800 × 10^{-6} mol(photon) m^{-2} s^{-1} light pulse with 0.1–2,400-s dark interval on *Pinus halepensis* needles (*A*). 820-nm transmission kinetics measured on pine needles simultaneously with the data shown in panel A (*B*). Chl *a* fluorescence induced by a double 0.7-s light pulse with 200-s dark interval and varying pulse intensity [5,000–15,000 × 10^{-6} mol(photon) m^{-2} s^{-1}] (*C*). To overcome the amplitude differences between measurements due to biological variability, the data in (*A*) were divided by F_0; in (*B*), the initial transmission signals were double normalized between 1 and 0. In (*A*) and (*C*), logarithmic and in (*B*), linear time scales were used. For (*A*) and (*B*), $n = 3$ and for (*C*), $n = 15$. Data are based on Schansker *et al.* (2008).

The implication of all these experiments on the IP phase was that in a light-adapted leaf it is not possible to reach F_M, no matter how high the light-intensity is, due to the activation of the acceptor side of PSI, two rate limiting steps away from Q_A. To put it in kinetic terms, the fluorescence transients behave like the Blackman curves well known from CO_2 assimilation experiments (Blackman 1905). Further, we noted that the amplitude of the IP phase depended on the pulse intensity: at ~ 2,000 × 10^{-6} mol(photon) m^{-2} s^{-1}, it represented about 25% of F_V, whereas above 10,000 × 10^{-6} mol(photon) m^{-2} s^{-1}, it contributed by about ~ 13% to F_V (Schansker *et al.* 2006). These observations imply that the IP phase of the OJIP rise depends on (this does not mean 'is caused by') a biochemical process. In summary, if the photosynthetic electron transport chain cannot be reduced completely due to the outflow of electrons at the acceptor side of PSI, the F_M(') cannot be reached.

The effects of valinomycin on the OJIP transient

The electric field generated across the thylakoid membrane has also been suggested to play a role in the OJIP kinetics, more specifically during the IP phase. Vredenberg and Bulychev (2003) showed that valinomycin, a compound that is known to dissipate the electric field, suppresses the IP phase of the fluorescence transient. We then tried to reproduce these observations.

In our valinomycin experiments, the IP phase was suppressed as well in a MV-like way (Fig. 4*A*), but, we observed that valinomycin also prevented the rereduction of P_{700}^+ and PC^+ (*see* Fig. 4*B*). This indicated that valinomycin affected the acceptor side of PSI and that the suppression of the IP phase was not a direct consequence of the dissipation of the electric field by valinomycin as Vredenberg and Bulychev (2003) had assumed. Unfortunately, we were not able to infiltrate the newly bought valinomycin, which we had to buy after the old stock ran out, into the leaf. As a consequence we did not manage to reproduce IP suppression effect of valinomycin during our stay in Geneva; thus the observation remained unpublished.

The effects of nigericin on the OJIP transient

Finazzi *et al.* (2004) observed in barley leaves that 100 × 10^{-6} mol(photon) m^{-2} s^{-1} caused a strong but transient induction of NPQ that relaxed within 3–4 min of illumination. Essentially no NPQ was induced at the same light intensity, during the same time interval, if the leaves had been infiltrated with nigericin. Finazzi and coworkers assumed that the observed transient NPQ was ΔpH dependent, because it was abolished by nigericin. We investigated the possibility that nigericin could have a side effect. Nigericin eliminates the acidification of the lumen, but at the same time, it also has an (inverse) effect on the pH of the stroma. The activation of FNR has been suggested to depend on the stroma pH (Carrillo *et al.* 1980, 1981). In Fig. 5*A*,*B*, the Chl *a* fluorescence induction kinetics of nigericin-infiltrated leaves illuminated with 100 and 300 × 10^{-6} mol(photon) m^{-2} s^{-1} actinic light are shown, similar to the method employed by Finazzi *et al.* (2004). In the presence of nigericin, the fluorescence intensity

remains for at least 150 s close to the F_M level (approx. 85–90% of F_M, *see* Fig. 5*B*) and the 820-nm transmission measurements indicate that during the same period of time, PC and P_{700} remain in the reduced state (Fig. 5*C*). In other words, nigericin inhibits, or at least slows down the activation of FNR, a side effect, which was not taken into account by Finazzi *et al.* (2004).

(In)activation of the PSI acceptor side

An issue that deserves closer attention is the relationship between the Kautsky kinetics from dark adapted to steady state and the associated biochemical processes. Foyer *et al.* (1992) biochemically determined changes in the reduction state of the $NADP^+$ pool during a dark-to-light transition and observed that the reduction of the $NADP^+$ pool in pea leaves takes tens of seconds of illumination (with 35% of $NADP^+$ reduced at time zero). Similar measurements can be done spectroscopically on, *e.g.*, intact chloroplasts. However, as shown for pea leaves (Schansker *et al.* 2006) and needles of *Pinus halepensis* (Schansker *et al.* 2008), the regeneration of the IP phase following light acclimation takes ~ 15 min and 60 min, respectively. We have interpreted this process to reflect the inactivation of FNR in darkness (Schansker *et al.* 2006). This means that

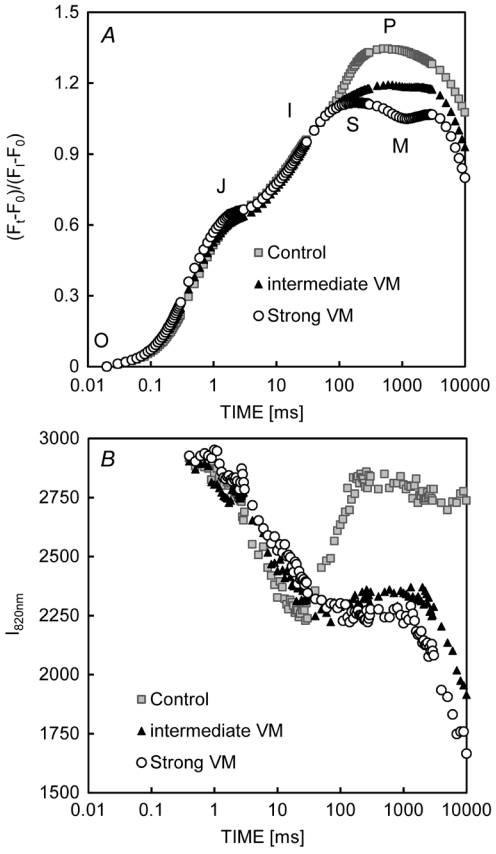

Fig. 4. Effect of valinomycin (VM) in intact pea leaves. The OJIP transients (*A*) and the 820-nm transmission induction transient (*B*) were induced by 1,800 × 10^{-6} mol(photon) m^{-2} s^{-1} 650-nm light. Examples of an intermediate and strong valinomycin effect are shown. The pea leaves were left overnight in a 25 µM valinomycin solution containing 2.5% ethanol. S and M refer to fluorescence steps defining the fluorescence decline beyond P.

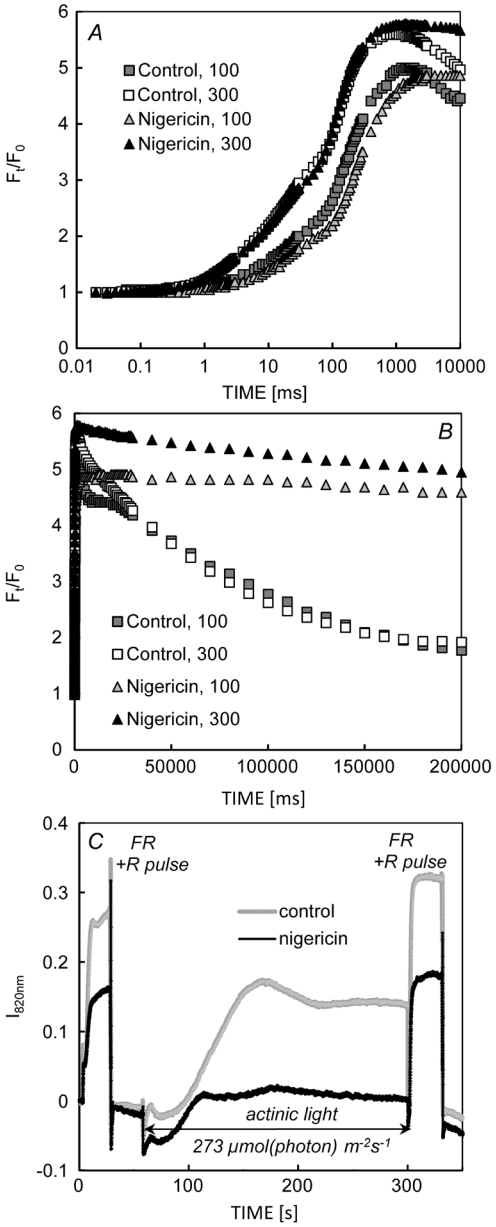

Fig. 5. Effect of nigericin on Chl *a* fluorescence and 820–870-nm induction transients of intact pea leaves. Chl *a* fluorescence transients induced by 20 s of 100 and 300 × 10^{-6} mol(photon) m^{-2} s^{-1} 650-nm light (*HandyPEA*, *Hansatech Instruments Ltd.*, UK) shown on a logarithmic (*A*) and linear time scale (*B*). The effect of nigericin on the A_{820}–$A_{870\text{-nm}}$ kinetics induced by 273 × 10^{-6} mol(photon) m^{-2} s^{-1} red light (measured by a *DUAL-PAM-100* instrument, *Heinz Walz GmbH*, Germany). The measurement was started and finished by a saturating pulse consisting of far red illumination followed by a strong pulse of red light to determine the maximum absorption changes. The transients of the nigericin-treated leaves were scaled to the maximum absorption changes determined for the control leaves. Pea leaves were incubated for 7 h in 100 µM nigericin solution to allow passive infiltration.

repetition of spectroscopic measurements requires either very long dark-adaptation times between measurements or the use of a new sample for each measurement. A 1-min dark interval as used by Schreiber and Klughammer (2009) is, in this respect, insufficient.

The comparison we made between Kautsky transients of pea leaves and *Pinus halepensis* needles (Schansker *et al.* 2008) and between pea leaves and thalli of the lichen *Parmelina tiliacea* (Oukarroum *et al.* 2012) demonstrated that the fluorescence decrease beyond the P level differs strongly between an angiosperm species like pea on the one hand and gymnosperm and lichen species on the other hand. The faster fluorescence decrease is accompanied by a much faster P_{700}+PC oxidation beyond P. We have always interpreted these faster kinetics beyond P as a faster activation of FNR. Benz *et al.* (2010) note in their FNR paper that only angiosperms have a chaperone like protein: TIC62 (factor interacting with the translocon at the inner envelope of the inner envelope of chloroplasts) and TROL (thylakoid rhodanese-like protein) that can bind FNR to the membrane in darkness and possibly protect it against degradation under the more acid stroma conditions in darkness. This binding may also be a reason for the slower FNR activation kinetics observed in angiosperms. It should be noted though that both in gymnosperms and lichens there is a clear IP phase on a dark-to-light transition. In these species a 200-ms illumination at high light intensities is needed, as well, to induce a fast oxidation of the PSI acceptor side. The recent discovery of flavodiirons as a safety valve that can funnel electrons away from a reduced PSI acceptor side, especially under fluctuating light conditions (*e.g.*, Allahverdiyeva *et al.* 2013), has become an alternative explanation for the observed fast kinetics beyond P (Ilík *et al.* 2017). To what extent this pathway will affect the fluorescence induction kinetics on a dark-to-light transition also depends on the question if the electron donor to these flavodiirons is NADPH, as suggested by Allahverdiyeva *et al.* (2015), or reduced Fd. However, as noted above, in either case, there is a 200-ms activation period.

Drought tolerance of barley plants assessed by Chl *a* fluorescence transient

Abdallah Oukarroum arrived in Lullier and started his PhD studies in the early autumn of 2001. Coming from Morocco, a country with limited water resources and high summer temperatures, a study of drought and heat stress was a natural choice. From Morocco he brought ten barley varieties and landraces differing in their drought tolerance, which he used as a source of biological variability.

For water deficit stress, several indicators are used (photosynthesis, stomatal conductance, Chl content, water potential, *etc*.) to elucidate the effects of water deficit on the physiological state of the plant. Chl *a* fluorescence studies have shown that PSII is tolerant to water deficit (Lu and Zhang 1999, Cornic and Fresneau 2002, Oukarroum *et al.* 2007, 2009) and only becomes affected in response to a prolonged drought stress (Saccardy *et al.* 1998). The most popular parameter derived from the Chl *a* fluorescence transient is the F_V/F_M, which is often used as a proxy for the maximum quantum yield of primary photochemistry of PSII (Baker 2008, Krause and Weis 1991). This parameter is and has been used extensively to monitor various environmental stress effects; however, there are two problems with this parameter: In the first place, stress factors that do not affect PSII activity cannot be monitored by it. Secondly, various processes may be responsible for a decrease in the F_V/F_M value. Photoinhibition causes an inactivation of individual PSII RCs and an associated loss of variable fluorescence (van Wijk and Krause 1991, Schansker and van Rensen 1999, Matsubara and Chow 2004), destruction of the Mn-cluster strongly reduces the PSII donor side capacity and prevents a full reduction of the electron transport chain (Tóth *et al.* 2005b, 2007), whereas destacking causes a lower F_V/F_M value due to increased spillover (Trissl and Wilhelm 1993, Caffarri *et al.* 2014) and finally, any process that increases the (apparent) F_0 value such as a partially reduced PQ pool or detached antenna units (*e.g.*, Tian *et al.* 2015).

The insensitivity of the F_V/F_M ratio to water deficit and its inability to differentiate between plant varieties regarding their water deficit tolerance has been reported in several studies (Araus *et al.* 1998, Lu and Zhang 1999, Panković *et al.* 1999, Oukarroum *et al.* 2007, Boureima *et al.* 2012). For example, relationships between different parameters derived from Chl *a* fluorescence kinetics and the yield of 144 durum genotypes were studied under three different water regimes (Araus *et al.* 1998); the parameter that showed the best genetic correlation with the grain yield was the half-rise time from F_0 to F_M: $t_{1/2}$ (r = 0.92), followed by F_0 (r = 0.88), F_M (r = 0.74), and F_V (r = 0.71), while F_V/F_M (r = 0.34) gave the worst correlation coefficient, suggesting that effects on F_0 and F_M cancel out when the ratio is taken. In contrast to the F_V/F_M, the photosynthetic performance index [$PI_{(abs)}$] was shown to be a sensitive fluorescence parameter with respect to water deficit (Oukarroum *et al.* 2007, 2009). This is a JIP-test parameter developed by Reto Strasser and co-workers (Strasser *et al.* 1999), which combines three functional components: photochemical energy absorption, trapping of excitation energy, and conversion of excitation energy in electron transport. The $PI_{(abs)}$ parameter integrates in the equation not only F_0 and F_M, but also F_J and the initial slope of the OJIP transient. Significant variation of the $PI_{(abs)}$ between the ten barley varieties formed the basis for the definition of a new parameter called Drought Factor Index (DFI): $\log(PI_{(abs)week\ 1}/PI_{(abs)control}) + 2 \times \log(PI_{(abs)week\ 2}/PI_{(abs)control})$ (Oukarroum *et al.* 2007), where week 1 and 2 refer to the length of the water deficit treatment. The calculated DFIs of the different barley varieties were found to correlate well with the known field tolerance to water deficit. In another study, Boureima *et al.* (2012) used the DFI-principle to characterize and classify 21 mutant germplasms of sesame in terms of their drought stress tolerance.

At the end of his stay in Geneva, Abdallah Oukarroum worked among other things on the photosynthetic behavior of the above-mentioned barley varieties exposed to water deficit conditions in the light acclimated or steady state

and the effect of water deficit on cyclic electron transport around PSI (CET). In Tóth *et al.* (2007) it had already been shown that a heat pulse inactivated the OEC effectively, whereas the induction of CET activity was rather low. Similarly, in the case of water deficit conditions, stress-induced stimulation of CET was hardly detectable (Oukarroum and Schansker, unpublished data). A similar lack of drought-induced stimulation of CET was observed in spinach leaves (Jia *et al.* 2008), even though it is widely assumed in the literature that stimulation of CET upon water deficit and heat stress occurs (Havaux 1996, Golding and Johnson 2003, Golding *et al.* 2004).

The effects of water-deficit on Photosynthetic Control were much more notable. The reoxidation of PQH_2 by the cyt b_6f complex depends on the lumen pH. As a consequence of a more acidic lumen, Q_A becomes more reduced and P_{700} becomes more oxidized (Tikhonov *et al.* 1981, Kramer *et al.* 1999, Chaux *et al.* 2015, Tikkanen *et al.* 2015). Our 820-nm transmission measurements were designed to determine the P_{700}+PC redox state under steady-state conditions: two strong light pulses were given, the first one under steady state conditions and the second one 2 s after lights off. Upon turning off the actinic light, the (partially) reduced PQ-pool will rereduce all PC^+ and P_{700}^+ within 100 ms. The initial transmission value after 2 s of darkness is, therefore, our I_{max} value, the level reached after 20–30 ms of strong light was used as I_{min}, and the $(PC+P_{700})_{red}$ state under steady-state conditions was obtained as the initial transmission level in the steady state (I_0). In other words, $(I_0 - I_{min})/(I_{max} - I_{min}) \times 100$ is $(PC+P_{700})_{red}$ [%] under steady-state conditions, where high values mean that P_{700} and PC are largely reduced and Photosynthetic Control is low, whereas low values point to largely oxidized P_{700} and PC and strong Photosynthetic Control (*see* Fig. 6*C*).

In parallel, NPQ also increases in response to lumen acidification (Rees *et al.* 1992, Ruban 2016). Leaves of four of the ten barley cultivars differing in their water deficit tolerance were brought in steady state by exposing them to 15 min of 340×10^{-6} mol(photon) m^{-2} s^{-1}. For the light intensity used, the drought stress leads to a decreased q_P value (Fig. 6*A*), an increased NPQ value (Fig. 6*B*) and more oxidized P_{700}+PC (Fig. 6*C*), indicating that the lumen pH became lower. This may, for example, be caused by a lower ATP utilization and an associated lower ATP synthase activity. In this context, it may be noted that a drought-induced decrease in ATP synthase activity has been reported for wild water melon (Kohzuma *et al.* 2009). Fig. 6*C* shows that the drought-induced change in Photosynthetic Control is relatively small in Tarodant and Lannaceur and quite large in Aït Baha and Immouzer. Using F_0 quenching (*e.g.*, Bilger and Schreiber 1986, Horton and Ruban 1993) as a measure for dissipation processes in the antenna of PSII (Fig. 6*D*), we observed that water deficit affected this parameter only in the cultivars Lannaceur and Immouzzer (the two least water deficit tolerant plants, *see* Oukarroum *et al.* 2007). This means that water deficit induced changes in NPQ and F_0 quenching did not have the same varietal dependence.

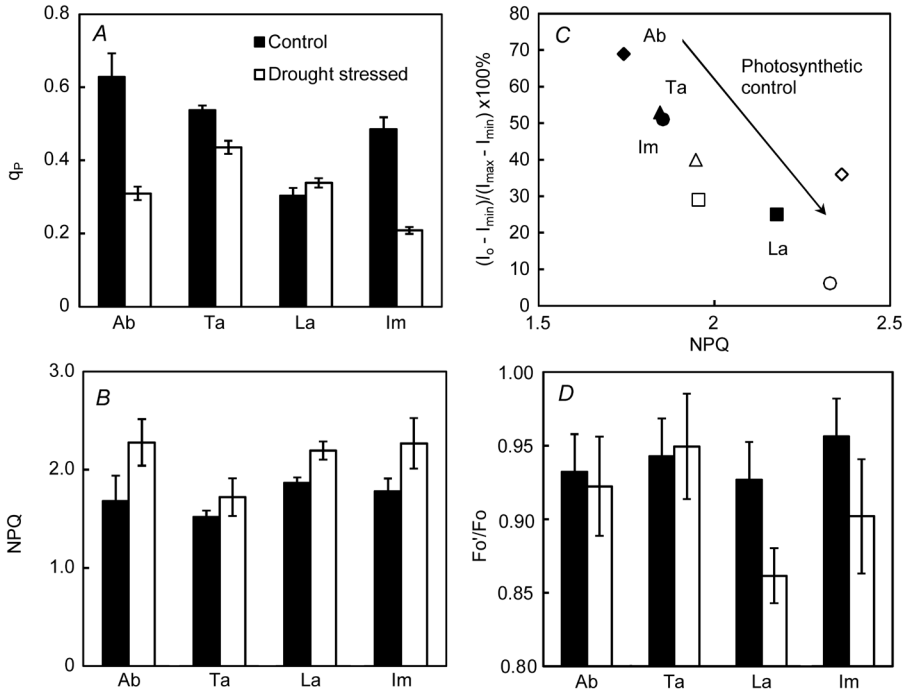

Fig. 6. Effect of 14 d of water deficit on several photosynthetic parameters of four barley varieties and landraces (Lannaceur, Aït Baha, Immouzer, and Tarodant, *see* Oukarroum *et al*. 2007), determined under steady state conditions [340×10^{-6} mol(photon) m^{-2} s^{-1}]. q_P (*A*); NPQ (*B*); Photosynthetic Control, which is reflected here by the relationship between the redox state of $PC+P_{700}$ $[(I_0 - I_{min})/(I_{max} - I_{min}) \times 100]$ in the steady state just before the actinic light was turned off and NPQ (*C*); F_0' quenching (*D*). *Closed symbols* refer to control plants and *open symbols* to drought stressed plants. See the text for a more detailed description of the determination of the $PC+P_{700}$ redox state.

Table 1. Schematic summary of the main conclusions that can be drawn from the presented data.

Schematic summary of the main points of the text	
1.	Mn-cluster + extrinsic proteins OEC in place → lumenal ascorbate cannot act as a donor to PSII Mn-cluster + extrinsic proteins OEC lost → lumenal ascorbate can donate electrons to Tyr_Z (halftime tens of ms)
2.	If stress leads to lower ATP-synthase activity → lower lumen pH → higher NPQ + more Photosynthetic Control → P_{700} more oxidized
3.	a. In untreated angiosperm leaves activation of linear electron flow beyond PSI is slow b. In untreated gymnosperm leaves/needles activation of linear electron flow beyond PSI is fast c. In angiosperm leaves infiltrated with valinomycin linear electron flow beyond PSI is instantaneous d. In angiosperm leaves infiltrated with nigericin activation of linear electron flow beyond PSI is slowed down/inhibited for hundreds of seconds

In Table 1, we have summarized the main results of the text discussed above.

Concluding remarks

The aim of this paper was to show that in addition to the application of the popular JIP-test, Reto Strasser made important contributions to the development of new fluorimeters that enabled the discovery of new mechanisms and allowed us to prove ideas regarding the functioning of the photosynthetic apparatus and, in addition, to study of phenomena underlying the OJIP transient. The experience we gained in the laboratory of Reto Strasser taught us that Chl *a* fluorescence, especially in combination with 820-nm measurements, offers many possibilities for the development of new assays and approaches for the study of the photosynthetic apparatus. We are also very grateful to Reto Strasser for the work environment in the laboratory, which inspired us and gave us the freedom to successfully do scientific research.

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