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Review

Instantaneous switching between different modes of non-photochemical quenching in plants. Consequences for increasing biomass production

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ABSTRACT

Photosynthetic productivity usually saturates far below the maximum solar light intensity, meaning that in those conditions many absorbed photons and the resulting electronic excitations of the pigment molecules can no longer be utilized for photosynthesis. To avoid photodamage, various protection mechanisms are induced that dissipate excess excitations, which otherwise could lead to the formation of harmful molecular species like singlet oxygen. This **N**on-**P**hotochemical **Q**uenching (NPQ) of excitations can be monitored via a decrease of the chlorophyll fluorescence. There is consensus that in plants 1) there are at least two major NPQ (sub)processes and 2) NPQ (de)activation occurs on various time scales, ranging from (tens of) seconds to minutes. This relatively slow switching has a negative effect on photosynthetic efficiency, and Kromdijk et al. demonstrated in 2016 (Science 354, 857) that faster switching rates can lead to increased crop productivity. Very recently, we were involved in the discovery of a new NPQ process that switches off well within a millisecond (Farooq et al. (2018) Nat. Plants 4, 225). Here we describe the current level of knowledge regarding this process and discuss its implications.

1. Introduction

Leaves in plant canopies experience large fluctuations of the incoming sunlight during the day. Their photosynthetic apparatus must be optimized to effectively perform two apparently opposite functions: on the one hand, it should be organized in such a way that it ensures efficient light harvesting, thus supplying enough energy for the photochemical reactions that take place in the reaction centers (RCs). That is ensured by the structural organization of the pigment–protein complexes within the thylakoid membrane: each reaction center is surrounded by a relatively large peripheral antenna that absorbs photons and on a pico- to nanosecond time scale deliver the generated electronic excitations of the pigment molecules to a RC [1]. On the other hand, these highly efficient antenna systems can lead to over-excitation of the thylakoid membrane during strong sunlight. An increase of light intensity in itself is not dangerous but it raises the probability that a new photon is absorbed while the RC has not fully "recovered" from processing the previous excitation, in other words, when it is still closed. In that case there is an increased probability that a triplet state is formed on one of the chlorophylls (Chls). These Chl triplets can lead to the formation of singlet oxygen radicals, which are chemically very reactive and therefore dangerous (see e.g. [2]).

Therefore, to avoid photodamage, plants should also be able to regulate the amount of excitations that reach the RC. This self-regulation is a complicated physiological process occurring on multiple time and space scales. On the level of the whole plant it is e.g. manifested through the motion of leaves. On a microscopic level, the rate of the photosynthetic light reactions can exhibit both long-term acclimation through the regulation of the biochemical synthesis of the pigment–protein complexes or a short-term one via reorganization of the structural arrangement of the light-harvesting antenna within the thylakoid membrane. However, the most efficient regulation occurs at the molecular level and results in the harmless dissipation of excess excitation energy in the form of heat. This **N**on-**P**hotochemical **Q**uenching (NPQ, termed as such to contrast it with photochemical quenching that is caused by charge separation in the RCs) of excitations can be monitored via a reduction of the chlorophyll fluorescence, as will be shown below [3–6].

The dominant part of NPQ that is reversibly induced within minutes upon exposure to the high-light conditions is called energy-dependent

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quenching qE [7] and requires a low thylakoid lumenal pH for activation. The site and mechanism of quenching are still under debate but it is clear that the protein PsbS, a subunit of photosystem II (PSII), is involved [8,9]; it acts as a sensor of the lumenal pH [8,10,11] and might even be the site of quenching [12]. It is also known that under strong sunlight reversible de-epoxidation of the xanthophyll violaxanthin, found in the photosynthetic light-harvesting antenna, to antheraxanthin and zeaxanthin via the xanthophyll cycle takes place [13,14], and the resulting zeaxanthin molecule was proposed to either be a quencher itself [15,16] or play an allosteric role, inducing quenching capacity in the light-harvesting antenna [17,18].

Switching between the light-harvesting and photoprotective mode does not occur instantaneously. The time required to de-activate NPQ when incoming sunlight is reduced can lead to substantial energy losses in a dynamic crop canopy due to underperformance of the photosynthesis process during this transitional period. It has been demonstrated that overexpression of PsbS results in a higher rate of induction and relaxation of qE [19–21]. Upon increasing in addition the amount of xanthophyll cycle enzymes—violaxanthin de-epoxidase and zeaxanthin epoxidase—Kromdijk et al. managed to increase the NPQ switching rate in tobacco plants, leading to a 15% rise of biomass production [22]. This achievement holds great promise for improving productivity in other food crops: it has been estimated that increases as large as 30% may be feasible [23], which is one of the reasons why a detailed understanding of the NPQ phenomenon is very important.

A large number of studies, performed during the last three decades, has provided a substantial amount of information about NPQ. Based on the experimental observations, several models for the underlying molecular mechanism of NPQ have been proposed, most of them ascribing a leading role to the short-lived optically dark S_1 state of carotenoid (Car) molecules and attributing NPQ to either the formation of a Chl–Car charge-transfer state [15,16], coherent mixing between Chl and Car excited states [24,25] and incoherent energy transfer from Chl to the nearby Car pigment [18,26]. While formation of the Chl–Chl charge-transfer state in the antenna has also been proposed to be responsible for NPQ [27], recent studies have concluded that such a state is not related to NPQ [28,29].

While being different in their molecular details, all these models share the same basic idea: when NPQ is induced, it leads to the dissipation of excitations, thereby lowering the probability that an excitation arrives at a closed RC. For a long time, NPQ was thought to be non-selective, meaning that once it is induced, its quenching mechanism is independent of the oxidative state of the RC (open or closed). Later on, it was shown that after switching into a photoprotective mode under high-light conditions the structural reorganization of the light-harvesting antenna ensures that the effective absorption cross-section that is "sensed" by RC does not drop significantly compared to the dark-adapted NPQ-free conditions [30]. That observation led to the idea of "economic" photoprotection, when the presence of NPQ-traps does not prevent excitations to reach the RC and thus does not undermine the photosynthetic productivity. Recently, using ultrafast fluorescence experiments we have revealed together with our colleagues that natural photoprotection is even "more economic" in the sense that NPQ itself acts selectively and thus more efficiently than was previously thought [31]. In particular, the NPQ process is more active (the "rate of NPQ" is higher) when the RCs are closed as compared to the open ones. The corresponding switching time between these two NPQ regimes is much faster than 1 ms (see also below), in sharp contrast with the tens of seconds-to-minutes switching of the overall NPQ (de)activation times mentioned above. This allows plants to maintain a high photosynthetic efficiency and almost instantaneously react to the fluctuating light intensity, when NPQ has been activated in high-light conditions. At the moment, there is some evidence that this fast switching occurs only in plants and that PsbS is involved, whereas it is not present in green algae.

In this manuscript, we will first review the underlying concepts that determine the efficiency of photosynthesis at the level of the light-harvesting processes. We will start with a simplified picture that is often used in photosynthesis literature, and which corresponds to single-exponential processes. Processes like heat production, fluorescence, triplet formation, chemical and non-photochemical quenching will all be discussed. Then we will review an extended model that leads to a better description of the experimental data, while most of the underlying concepts remain the same. It will be explained why NPQ is needed to protect PSII and it will be pointed out that NPQ is in fact only necessary when the RCs are closed. It will then be described how recent experiments helped to measure the rate of NPQ in the presence of open vs. closed RCs, revealing that this rate is higher in the latter case, which obviously enhances the efficiency for photosynthesis. We then provide for the first time an upper limit for the switching time between the quenching states for open and closed RCs, which is extremely fast in comparison to the switching processes that were known before this discovery. Potential underlying mechanisms will be discussed and, finally, the possible consequences for crop productivity will be addressed as well as related differences between land plants and green algae.

2. Photosynthesis in a simple world

Before going into more detail about the dynamically changing rate of NPQ, we start with a simplified but commonly used description of the excitation dynamics in photosynthetic systems. First we consider a system without RC (or with closed RC), which contains a pool of Chl molecules. After absorption of a photon, the generated excitation energy can disappear when Chl undergoes internal conversion (heat formation), emission of a photon (fluorescence) or intersystem crossing (triplet formation) with rate constants k_{ic}, k_{rad} and k_{isc}, respectively [32]. Alternatively, excitation energy can be transferred to another nearby molecule with the rate constant k_{transf}, as is schematically shown in Fig. 1, or, in the case of the strongly coupled molecules, a collective excited state called exciton can be formed [33]. Since excitation energy transfer (EET) between the molecules does not reduce the overall excitation population in the system, the excited-state lifetime τ_{dis} is determined only by the sum of the first 3 rates: ${\tau_{dis}}^{-1} = k_{dis} = k_{ic} + k_{rad} + k_{isc}$ (here we neglect exciton–exciton annihilation that can appear under very high excitation intensities when more than one photon is simultaneously absorbed within the same Chl pool). This lifetime, also called fluorescence lifetime, can be obtained by measuring time-resolved fluorescence $F(t)$, which (assuming an instantaneous instrument response) decays exponentially according to:

$$F(t) = F_0 \exp(-k_{dis}\, t) = F_0 \exp(-t/\tau_{dis}), \quad (1)$$

where t reflects the time after excitation (with a short laser pulse) and F_0 is the initial fluorescence intensity at the time of excitation ($t = 0$). A typical Chl excited-state lifetime is of the order of 4–5 ns [34] whereas in the protein environment of light-harvesting complexes it usually drops to ~3.5 ns [35] and depends on temperature [28], the difference presumably arising from faster internal conversion, and in thylakoid membranes it is ~2 ns [36].

The quantum yield of intersystem crossing from the excited singlet state of free Chl (the ratio k_{isc}/k_{dis}) is about 0.64 [37]; a similar value of 0.61 was also obtained from singlet–triplet annihilation measurements in the plants' major light-harvesting complexes (LHCII) [35], meaning that there is a very high probability for the Chl triplet state to be formed if Chl excitation energy is not utilized in a different way. These Chl triplets are relatively long-lived (mean lifetime reaching several ms) [2] and can readily react with ground-state triplet oxygen to form dangerous excited singlet oxygen molecules, which are highly reactive and prone to cause severe damage to the whole photosynthetic apparatus and beyond. To protect themselves against this hazard, most photosynthetic organisms use carotenoids. These pigment molecules not only neutralize singlet oxygen by converting it to the ground-state triplet species but even more importantly, prevent singlet-oxygen formation

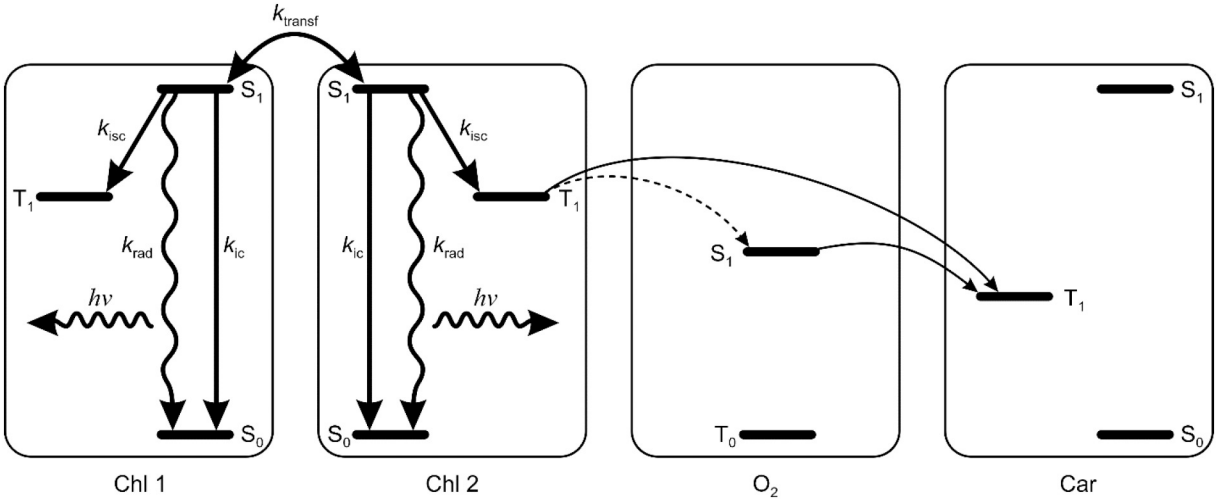

Fig. 1. Excited Chl molecule (S_1 state) can undergo relaxation to the ground (S_0) state via a radiative or non-radiative (internal conversion) transition, intersystem crossing to the triplet (T_1) state or excitation transfer to a nearby Chl pigment. Transition rates are indicated along the corresponding arrows, $h\nu$ denotes the emitted photon. While Chl is in a triplet state, its excitation energy can easily be transferred to an oxygen molecule, producing highly reactive singlet O_2 species. Both excited singlet O_2 and triplet Chl states can be neutralised by a nearby Car molecule.

by accepting triplets from Chl molecules [38] (see Fig. 1). Because the resulting lowest Car triplet state energy level in plants lies below that of singlet oxygen, no singlet oxygen can thus be produced [38].

The presence of Cars in all the peripheral antenna complexes and their close spatial association with the Chl *a* pigments ensure extremely efficient triplet transfer from Chls to Cars. For example, in LHCII two central luteins accept triplets from Chls [39,40] with almost 100% efficiency [2,41,42] at a rate of $(0.5\,\mathrm{ns})^{-1}$ [43], although a somewhat lower value (95%) has been reported for antenna complexes Lhcb4 and Lhcb5 [39]. On the other hand, the situation is completely different in the PSII reaction center, where Chls and Cars are spatially separated [44], meaning that Chl → Car triplet transfer is inhibited.

Chl triplet formation is not a problem in low-light conditions, when the rate of incoming solar radiation does not exceed the RC and downstream metabolic capacity, because Chl excitation energy is quickly transferred to the primary electron donor followed by even faster (several ps) initial charge separation [44]. Contrarily, under high-light conditions the capacity of the light-independent photosynthetic processes is too low to handle all the electrons produced in the photoreactions [45]. This means that the primary event of charge separation between the excited primary electron donor ($P680^*$) and the electron acceptor pheophytin (Phe) molecule cannot be followed by charge stabilization processes, and the charge-separated state $P680^+ \cdot Phe^-$ is eliminated by charge recombination. During recombination, spin conversion can occur, leading to formation of a triplet state on the primary donor Chl P680 [45]. Since there is no efficient triplet transfer to a nearby Car, singlet oxygen production is looming. The main strategy for plants to avoid this threat is to "sense" over-excitation of the light-harvesting antenna and to turn on the photoprotection mechanism known as NPQ. Generation of the NPQ-traps throughout the antenna lowers the excitation pressure on the RC, decreasing the probability that an excitation arrives at the RC while it is still in a closed state and triplet formation via recombination can occur.

When the RCs (either of photosystem I or II (PSI/PSII)) are open, a light-induced excitation will migrate through the antenna until it is dissipated via one of the channels described above or, preferentially, when it reaches the RC and disappears via trapping/charge separation (photochemical quenching) with the effective rate constant k_{photo}, where the subscript refers to "photosynthesis". The fluorescence lifetime τ_{open} in the presence of open RCs is then equal to

$$\tau_{open} = (k_{dis} + k_{photo})^{-1}, \tag{2}$$

so that the quantum yield of photochemical trapping ϕ_{photo}, is.

$$\phi_{photo} = k_{photo}/(k_{dis} + k_{photo}). \tag{3}$$

A typical value for ϕ_{photo} is around 0.83 for PSII [45], meaning that k_{photo} is roughly 5 times faster than k_{dis}. The numerical value of k_{photo} can be estimated by comparing the inverse lifetimes in the presence of open and closed RCs:

$$k_{photo} = \tau_{open}^{-1} - \tau_{closed}^{-1}, \tag{4}$$

where the lifetime τ_{closed} in the presence of closed RCs is very close to τ_{dis} in the thylakoid membrane [36].

After the induction of NPQ in high-light conditions, there will be an additional decay channel via non-photochemical quenching with the rate k_{NPQ}, which will further shorten the lifetimes in the presence of open and closed RC according to

$$\tau_{open/NPQ} = (k_{dis} + k_{photo} + k_{NPQ})^{-1} \tag{5}$$

and

$$\tau_{closed/NPQ} = (k_{dis} + k_{NPQ})^{-1}, \tag{6}$$

respectively. The rate k_{NPQ} can therefore be estimated as

$$k_{NPQ} = \tau_{open/NPQ}^{-1} - \tau_{open}^{-1} \tag{7}$$

or

$$k_{NPQ} = \tau_{closed/NPQ}^{-1} - \tau_{closed}^{-1}. \tag{8}$$

The high-light-induced non-photochemical quenching should on the one hand be effective when the reaction centers are closed, i.e. k_{NPQ} should be larger than k_{dis}. On the other hand, it should not be competing *too much* with k_{dis}, otherwise significant energy losses will occur in the intermediate regime when the incoming sunlight has decreased but NPQ has still not been deactivated. That means that ideally k_{NPQ} should be smaller than k_{photo}, which is indeed true in most cases and this was termed economic photoprotection [30].

However, we recently discovered that NPQ in fact behaves even more economically because its rate k_{NPQ} itself depends on the oxidative state of the RCs, i.e. whether the RCs are open or closed [31]. It turns out that k_{NPQ} decreases when the RCs are open and NPQ is in fact no longer needed and even unwanted, whereas its value again increases in case the RCs are closed, thereby leading to more efficient protection. The switching between both quenching regimes appears to occur on a sub-millisecond time scale. This was not explicitly deduced in our

previous work [31] and will be addressed in more detail below. We will also discuss potential physical mechanisms underlying this phenomenon and the possible implications of our finding on crop yields. However, before we can do this in a meaningful way, we need to switch from the simplified picture outlined above, where all the phenomena correspond to simple rates and therefore fluorescence signal should decay exponentially with time, to a more realistic one, where the excited-state kinetics are non-exponential.

3. Photosynthesis in a more realistic world

The simplified framework given above is often used to describe the yield of primary charge separation and those of the competing loss processes. The same formalism can easily be applied to express all yields in terms of the steady-state fluorescence parameters, which is widely used in photosynthesis research. However, in real systems the situation becomes more complicated. First of all, in molecular complexes with some localized trapping center (e.g. RC or NPQ-trap) the time needed for excitation energy to transfer through the aggregate towards the traps usually cannot be neglected. Explicit calculation of this transfer time requires the precise knowledge of the structural organization of the photosynthetic system. The detailed crystal structure of distinct pigment–protein complexes [46,47] and whole photosynthetic units [44,48,49] revealed multi-scale packing of the pigment molecules within the light-harvesting antenna, resulting in different regimes of excitation energy transfer: very fast (sub-picosecond) coherent excitation energy equilibration within the various domains of closely-associated strongly-coupled Chls, slower (picoseconds) incoherent energy transfer between such domains (or between domains and more distant Chls) and even slower (tens of picoseconds) energy transfer between different pigment–protein complexes [50]. Therefore excitation dynamics in light-harvesting antenna is often described in terms of a coarse-grained model [28,51–53] by explicitly taking into account inter-complex excitation transfer rate while assuming instantaneous equilibration within the complex (see Fig. 2). Mathematically this is done by solving the system of Pauli Master equations:

$$\frac{\mathrm{d}p_i(t)}{\mathrm{d}t} = \sum_j k_{j\to i} p_j(t) - \sum_j k_{i\to j} p_i(t) - k_{\mathrm{loss}} p_i(t), \tag{9}$$

where $p_i(t)$ is time-dependent population of the i^{th} complex, $k_{j\to i}$ is the effective rate of excitation transfer from the j^{th} complex to the nearby i^{th} complex, and k_{loss} is the rate of the intrinsic population loss in the complex—either dissipation rate k_{dis} for the unquenched complexes or the trapping rate k_{trap} (rate of charge separation in the RC or quenching rate by the NPQ-traps) for the quenched ones. The experimentally measured fluorescence intensity $F(t)$ is proportional to the total excitation population in the system, $F(t) \propto \Sigma_j\, p_j(t)$.

As a result of such a description, in small molecular aggregates comprised of just several complexes the total excitation decay kinetics become multi-exponential. On the other hand, in the larger aggregates only the shortest lifetime dominates, which yields a single-exponential population decay. The mean excitation lifetime (that in the terms of the previous section should be attributed to either ${k_{\mathrm{photo}}}^{-1}$ or ${k_{\mathrm{NPQ}}}^{-1}$) in the absence of any dissipation in such system then can be written as follows [32]:

$$k_{\mathrm{photo/NPQ}}^{-1} = \tau_{mig} + \tau_{del} + \tau_{trap}, \tag{10}$$

here τ_{mig} is the mean excitation migration time through the molecular aggregate, τ_{del} is the delivery time from the pigments located around the trapping center to the trap, and τ_{trap} is the already mentioned trapping time by the trap. The presence of multiple quenchers as well as accounting for excitation energy loss due to intrinsic dissipation prevents one from obtaining simple analytical expression for the excitation lifetime, but qualitatively the outcome remains the same: the solution of Eq. (9) yields mostly single-exponential decay kinetics. This result, however, contradicts the experimental studies: the time-resolved fluorescence measurements on photosynthetic systems usually requires multi-exponential description [54], so that simple determination of the k_{photo} and k_{NPQ} rates from the experimental kinetics is no longer possible.

To account for these experimental observations, multi-step quenching mechanisms were often used, involving up to six subsequent intermediate state (“radical-pair” states in the RC) and allowing excitations to return to the antenna [55–57]. While mathematically

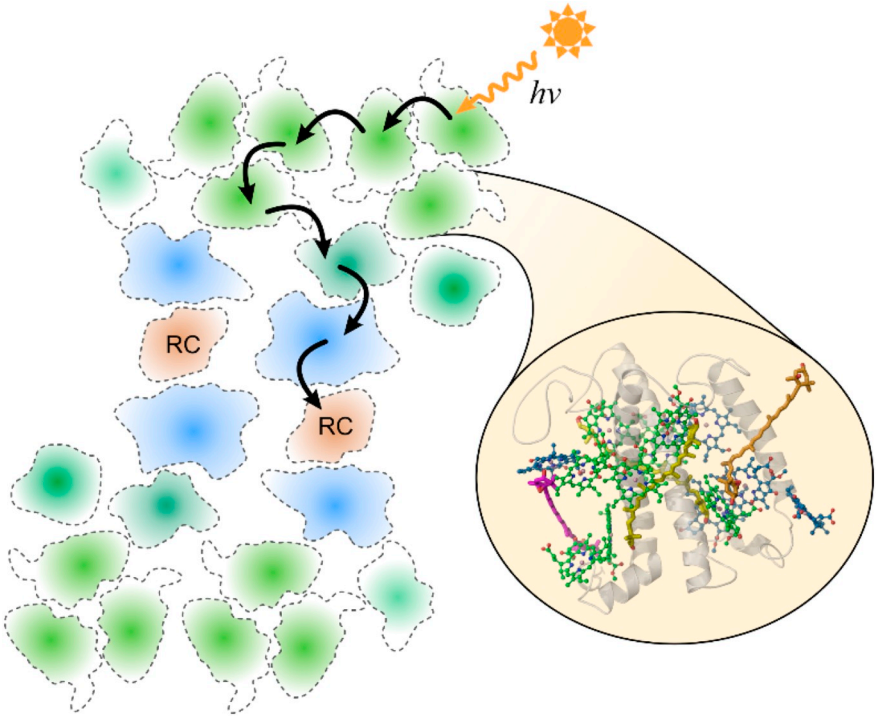

Fig. 2. Coarse-grained model of $C_2S_2M_2$ supercomplex of Photosystem II based on the structural arrangement of the pigment–proteins around the reaction centers. Light green denotes LHCII trimers, dark green—minor antenna complexes, blue–core antenna complexes, brown—RCs. Arrows denote random walk of the light-induced electronic excitation through the antenna until it reaches one of the RCs. No defined boundaries of the complexes are shown to emphasize that this structure is not fixed but rather exhibits small fluctuations. Inset shows the intrinsic structure of the LHCII monomer (protein helices are shown in grey, Chls *a*—in green, Chls *b*—in blue, Cars—in yellow, orange and magenta correspond to Luts, Neo and Zea, respectively).

feasible, this assumption contradicts other studies revealing that charge separation in RCs is virtually irreversible [58]. Another possible, though mostly overlooked in previous studies, origin for the non-exponentiality of the excitation decay kinetics is the inherent inhomogeneity of all terms of Eq. (10). Indeed, even slight variations in the mutual arrangements of molecules within the pigment–protein complex or of the light-harvesting complexes themselves [59] will result in different excitation transfer rates and thus different excitation lifetimes. Moreover, fluorescence blinking observed in single light-harvesting complexes under continuous illumination [60–62] revealed an intrinsic ability of the antenna complexes to switch between the fluorescing and quenched states, meaning that in the photosynthetic antenna some sort of the dynamic equilibrium between particular complexes being in one or another conformational state is achieved [63]. NPQ observed under high-light conditions might then be the manifestation of this equilibrium being thermodynamically shifted towards the quenched state [28]. As a result, the exact number of the generated NPQ-traps as well as their location within the antenna might significantly fluctuate during the measurements, also resulting in non-exponential fluorescence decay kinetics. The effect of these thermodynamic fluctuations alone on the mean excitation lifetime in the variously sized aggregates of LHCII trimers is illustrated in Fig. 3. It can be seen that even a small increase of the probability of the quenched state to occur by just a few percent can drastically affect the fluorescence lifetime in larger antennae. Additional variations of the excitation transfer rates and light-intensity-sensitive variability in the antenna size [30] further complicate the analysis and interpretation of the raw experimental data.

These complications can at least partially be circumvented by making use of the so-called fluctuating antenna model [64], which will be introduced below. It allows not only describing multi-exponential excited-state kinetics with just a few parameters, but also comparing the efficiency of quenching by chemical and non-photochemical quenching in different situations. Such a detailed analysis is only possible by using ultrafast spectroscopic measurements and cannot be obtained with the use of, for instance, steady-state fluorescence techniques.

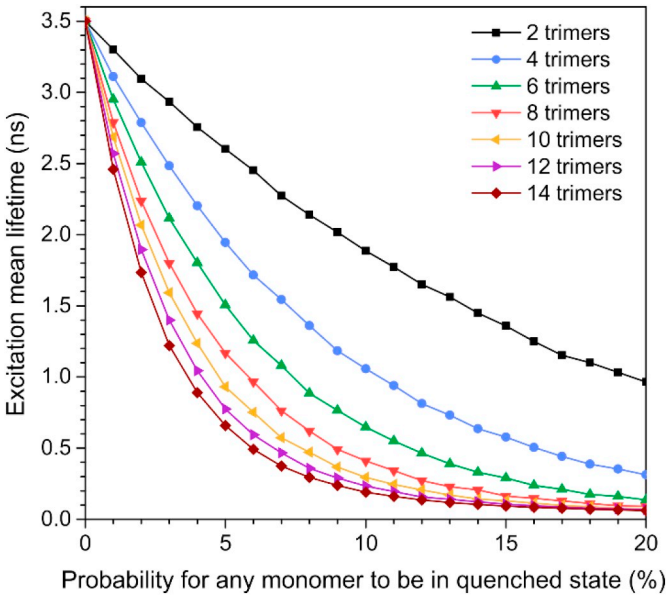

Fig. 3. The estimated relation between the mean excitation lifetime in variously-sized aggregates of LHCII trimers and the thermodynamic probability for any monomeric complex to be in the quenched conformational state. The lifetime was calculated from the excitation decay kinetics, obtained in terms of the coarse-grained model by solving a particular Pauli master Eq. (9) while assuming an inter-complex excitation hopping time of 25 ps, dissipation time of τ_{dis} = 3.5 ns, and trapping time by the complexes being in the quenched of τ_{trap} = 50 ps [28], and ensemble-averaging them over a random distribution of the traps (and their number) throughout the aggregate.

4. Fluctuating antenna model

As already mentioned above, constant fluctuating motion of the pigment–protein complexes and random spatial distribution of the generated NPQ-traps result in varying pathways of excitation migration through the light-harvesting antenna. These changes occur on a timescale from milliseconds to seconds, which usually is much shorter than the time needed to acquire the desired signal-to-noise ratio during the time-resolved fluorescence measurements. Moreover, in the standard bulk measurements one registers fluorescence signals that simultaneously come from spatially different points of the heterogenic thylakoid membranes. Therefore, experimental kinetics represent the time- and space-averaged kinetics originating from the ensemble of structurally slightly different "snapshots" of the light-harvesting antenna, which makes precise modelling of excitation dynamics a hardly accomplishable task. This fact is illustrated in Fig. 2, where no defined boundaries between the pigment–protein complexes are shown.

Instead of dealing with spatially undefined (anisotropic) connectivity between different complexes in an integer- (one-, two- or three-) dimensional space, we can consider excitation diffusion in an isotropic continuous medium of *fractional* dimensionality *d*. Using this approach, we have recently formulated the so-called fluctuating antenna model [64], which was then successfully applied to describe multi-exponential fluorescence decay kinetics in the variously sized PSII supercomplexes [64,65], LHCII aggregates [28,65], various PSI-containing supercomplexes [66], BBY particles [67], thylakoid membranes [65] and even leaves [31] using just 3 adjustable or fitting parameters. Briefly, instead of solving Pauli Master Eq. (9) where due to fluctuations excitation transfer rates $k_{i\to j}$ are undefined, we assume that the initially generated excitation diffuses freely through the system until it is trapped at some random distance *R*. Mathematically this process is described by the following diffusion equation:

$$\frac{\partial}{\partial t}p(\vec{r},t\mid R) = D\,\nabla_d^2\,p(\vec{r},t\mid R) - k_{\mathrm{dis}}p(\vec{r},t\mid R), \tag{11}$$

with the boundary condition $p(\vec{r},t\mid R)|_{\vec{r}|=R} = 0$ corresponding to infinitely fast trapping. Here $p(\vec{r},t\mid R)$ is the density of survived excitations at time point *t*, *D* is the diffusion constant, and k_{dis} is the already mentioned rate of intrinsic excitation dissipation. Due to isotropy, only the radial part of the *d*-dimensional Laplacian is relevant,

$$\nabla_d^2 = \frac{\partial^2}{\partial r^2} + \frac{d-1}{r}\frac{\partial}{\partial r}. \tag{12}$$

By solving Eq. (11) and averaging over different excitation diffusion path lengths *R*, we obtained inherently non-exponential excitation decay kinetics [64]. More importantly, instead of many parameters defining charge separation and recombination rates in a multi-step trapping scheme, here we have only three model parameters, all having a simple physical meaning: one of them is the already mentioned fractional dimensionality *d*, empirically reflecting structural organization of the light-harvesting antenna and presence or absence of links comprising the excitation transfer network. Another parameter k_{dis} defines the intrinsic rate of excitation dissipation, which generally can be fixed to $k_{\mathrm{dis}} = (3.5\,\mathrm{ns})^{-1}$, common to PSII antenna complexes [35]. The last model parameter $\eta = (Dc^{2/d})^{-1}$ determines the timescale of excitation decay kinetics and relates the diffusion coefficient, *D*, and the mean concentration of traps, *c*. In terms of the coarse-grained (or lattice) model, the diffusion coefficient can be expressed as $D \simeq a^2/(2d\tau_{\mathrm{hop}})$, where *a* is the mean spatial separation of the complexes and τ_{hop} is the mean excitation hopping time between the nearby complexes. Meanwhile, the concentration of traps is $c \simeq 1/(Na^d)$, where *N* is the mean number of the antenna complexes per trap. As a result, the

timescale parameter introduced above can be expressed as $\eta \simeq 2d\tau_{hop}N^{2/d}$. Although obviously oversimplified, this model nevertheless provides a useful approach to describe experimental data: by comparing model parameters d and η, obtained by fitting fluorescence decay kinetics measured under different conditions, one can extract information about the structural (re)organization of the light-harvesting antenna as well as the mean number of the present excitation traps. We therefore used this description to compare NPQ ability under different excitation conditions.

5. Discovery of the "instantaneous" switching of NPQ

5.1. Experiments

In order to compare NPQ in the presence of either open or closed RCs, non-invasive picosecond fluorescence measurements on intact spinach leaves were used [31]. The advantages of the time-resolved measurements as compared to quasi steady-state measurements were already discussed many times before: They are not sensitive to photobleaching or the movement/shielding of chloroplasts and they can provide additional information to test quantitative models of excitation dynamics in intact leaves [68–73].

The measurements were performed both in the presence and absence of NPQ. NPQ was induced by a strong actinic light source for tens of minutes and the fluorescence kinetics were measured for a period of 10 s during which the actinic light was temporarily shut off. After these 10 s the actinic illumination was applied again to keep a high level of NPQ, and after 1 min of illumination measurements were again performed for 10 s. This cycle was repeated until the signal-to-noise ratio was sufficient (typically 30 min). The crucial aspect of these measurements is the fact that the state of the RCs (open or closed) was determined by the intensity of the laser pulses that were used while performing the time-resolved fluorescence measurements (when the actinic light was switched off). To keep most of the RCs open, low-intensity laser pulses (100 nW) were used at a repetition rate of 3.8 MHz and a focal spot of 100 µm. To close most of the RCs, the laser power was increased to 1 mW, which is high enough to close most of the RCs but still low enough to avoid the occurrence of singlet–singlet annihilation.

5.2. Data analysis

A straightforward interpretation of the registered time-resolved fluorescence spectra was hindered by spectral and temporal overlap of signals originating from multiple sources in the collected data: the presence of both PSI and PSII as well as RCs and NPQ-traps. It is commonly known, however, that the mean excitation lifetime in PSI is around 50–100 ps [74], thus the fluorescence remaining after ~0.5 ns originates entirely from PSII. By looking at these asymptotic data, fluorescence spectra of PSII with open or closed RCs, with or without induced NPQ were obtained [31] (see Fig. 4a). To further disentangle time-resolved response of PSI and PSII, a multivariate curve resolution method was used [75] to decompose the experimental data $F(\lambda, t)$ into a sum of two components:

$$F(\lambda, t) = S_1(\lambda)K_1(t) + S_2(\lambda)K_2(t) + R(\lambda, t), \quad (13)$$

where $S_1(\lambda)$ and $S_2(\lambda)$ are steady-state fluorescence spectra and $K_1(t)$ and $K_2(t)$ are the corresponding excitation decay kinetics of photosystems I and II, respectively, while $R(\lambda, t)$ is the residual data which should be minimized during the optimization procedure. Such a decomposition is not unique in general, but the ambiguity could be reduced by introducing several physical constraints, like non-negativity of both spectra and kinetics, fixing the PSII spectrum to the one shown in Fig. 4a and requiring the ratio of the amplitudes of K_1 and K_2 kinetics to be close to the known PSI/PSII chlorophyll ratio of 0.8–1.0 [76]. This allowed determining the PSI fluorescence spectral lineshape and kinetics as well as PSII kinetics under different excitation conditions [31]. The PSII kinetics obtained in such a way as shown in Fig. 4b, clearly exhibit non-exponential decay behavior and can be further analysed in terms of the previously introduced fluctuating antenna model to evaluate the efficiency of NPQ under conditions of the open and closed RCs. These results are summarized in Table 1.

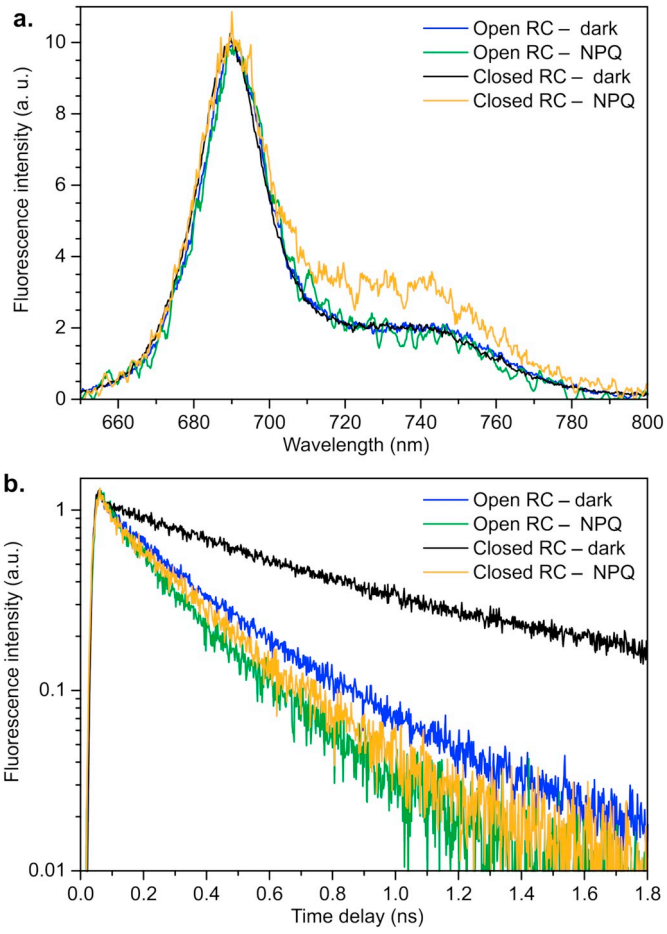

Fig. 4. Fluorescence spectra (a) and decay kinetics (b) of PSII, obtained from the time-resolved measurements performed on spinach leaves under different illumination conditions at room temperature. Fluorescence spectra were obtained by integrating experimental data over $t > 0.5$ ns, when contribution of PSI can be neglected, and fluorescence kinetics were determined by performing spectral decomposition according to Eq. (13). Data extracted from [31].

5.3. Revealed results

Based on the analysis of the registered time-resolved fluorescence, several conclusions were obtained [31]. First, as shown in Fig. 4a, PSIIs in three of the four samples (open RCs with or without induced NPQ as well as closed RCs without NPQ) exhibited absolutely the same steady-state fluorescence spectra with a strong peak at ~690 nm and extended sideband around 720–750 nm. However, after NPQ had been induced, a

Table 1
Fitted model parameter $\eta = (Dc^{2/d})^{-1}$ and extracted concentration of quenchers (total (i.e. NPQ + RC), c, and NPQ-traps only, $c^{(NPQ)}$) in PSIIs under different excitation conditions (relative to the dark-adapted PSII with open RCs) [31].

RC state & light condition	η (ns)	$c/c_{open,dark}$	$c^{(NPQ)}/c_{open,dark}$
Open RCs, dark	3.89	1	–
Open RCs, NPQ	2.53	1.50	0.50
Closed RCs, NPQ	3.27	1.18	1.18

significant increase in the fluorescence intensity of the mentioned sideband was observed, when the RCs were closed. Here we want to emphasize that this result was obtained from the raw experimental data by merely comparing the asymptotic time-resolved fluorescence spectra, without any additional sophisticated data analysis. Thus distinct spectral signatures of NPQ when RCs are either open or closed might reflect different quenching mechanisms in both cases.

Some more conclusions were obtained by fitting the decomposed PSII fluorescence decay kinetics (shown in Fig. 4b) using the fluctuating antenna model [31]. First, all the quenched (either due to open RCs, NPQ, or both) kinetics were described in terms of the same dimensionality $d = 1.9$, which reflects the well-known planar transfer of the excitation energy across the thylakoid membrane. By comparing the second model parameter η obtained for different samples, the quenching capacity in PSII under different conditions could be evaluated, assuming that the diffusion constant D remains unchanged and all the traps are ideal. As summarized in Table 1, induction of NPQ when RCs are open, increases the mean concentration of the quenchers by ~50%, i.e. NPQ is approximately half as strong as excitation quenching by open RCs. On the other hand, when RCs are closed, the only quenchers available are NPQ-traps, and their concentration increases more than two-fold.

We therefore conclude that the type of NPQ, that is induced when RCs are closed and an extra level of photoprotection is required, exhibits two distinct signatures: spectral (appearance of the red fluorescence band around 720–750 nm) and dynamic (increased excitation quenching rate due to faster k_{trap} or generation of additional NPQ-traps within the light-harvesting antenna). It was not addressed in [31] how fast the switching between these different quenching regimes was but this issue is discussed in more detail in the following paragraph.

5.4. Switching time for going from $k_{NPQ/open}$ to $k_{NPQ/closed}$

An upper limit for the time needed to switch from a state with a relatively low rate of NPQ with open RCs ($k_{NPQ/open}$) to a state with a high rate of NPQ for closed RCs ($k_{NPQ/closed}$) will now be estimated.

First of all, the measurements on open RCs were done with low laser power (100 nW at repetition rate of 3.8 MHz and spot size of ~100 μm). It is important to realize that closing of the RCs was achieved by using a relatively high laser power in combination with a high repetition rate (1 mW at 3.8 MHz). That leads to approximately ~0.035 excitations per LHCII trimer per pulse [31] and thus ~0.15–0.20 excitations per pulse arriving at a PSII RC in the thylakoid membrane [54]. This implies that roughly speaking, 10 pulses were needed to close most of the RCs that were illuminated by the laser spot. Because the spinach leaves were fixed in a measuring cuvette that was simultaneously rotated and translated, the RCs only stayed in the laser spot for a finite time. The sample was moving at a speed of 1 ms^{-1} at the position of the 100 μm laser spot and therefore each RC was illuminated for around 100 μs. This time can be considered as an absolute upper limit for the time that is required to reach the observed rate of quenching $k_{NPQ/closed}$, starting from open RCs. However, it is probably much shorter and we cannot rule out that the process even occurs instantaneously upon closing the RC. It should be kept in mind that it took around 10 pulses to close the RCs whereas in total the RCs experienced ~380 pulses while passing the 100 μm spot with a repetition rate of 3.8 MHz. If the switching time was indeed as "slow" as 100 μs, then we might expect a substantial amount of variation of the amplitude of the red fluorescence shoulder upon variation of the cuvette rotation speed or the laser intensity. However, variation of either the laser intensity or the rotation speed with a factor of 2 did not lead to any detectable differences, implying that during most of the 380 pulses the NPQ state for closed RCs was already present.

It can therefore be concluded that the switching time from a regime with relatively low NPQ (open RCs) to a regime with high NPQ (closed RCs) is at most 100 μs but probably even much faster. No precise experimental data are currently available for the reverse process, but we presume that it is similarly fast (see below).

6. Origin and consequences of the fast switching process

It is important to realize that the fast switching process between the two quenching modes is only observed after strong actinic light has been provided for many minutes and qE/NPQ is completely activated. However, once NPQ has been induced, a substantial fraction of the quenching can be switched on/off almost instantaneously, depending on the fact whether the RCs are open or closed. When the relative rate of quenching is expressed as a relative number of perfect quenchers that irreversibly and instantaneously quench excitations once they reach the quenchers, then the ratio of the number of quenchers (or, alternatively, the effective quenching rate) for closed and open RCs is more than a factor of two. Taking into account the mean excitation lifetime of 240 ps under NPQ conditions with closed RCs, according to Fig. 3 that would correspond to ~ 14% and 6% concentrations of the quenched complexes in both cases, respectively. The induction of NPQ usually occurs on a time scale of tens of seconds (largely related to protonation of the PsbS protein) to minutes (largely related to xanthophyll cycle). An interesting aspect of the "instantaneous" quenching mechanism is that it is accompanied by the appearance of a red shoulder in the fluorescence spectrum. So when the RCs are closed and the additional quencher is active, a red fluorescence shoulder around 720 nm is observed, which then disappears upon opening of RCs [31]. While this result was originally obtained for spinach leaves, we have recently observed a similar effect for Arabidopsis leaves (unpublished results). Interestingly, Holzwarth and coworkers [68] performed time-resolved fluorescence measurements on Arabidopsis leaves in the presence and absence of NPQ (only with closed RCs, not with open RCs). Based on their multi-parameter global target analysis fitting results, they concluded that a red fluorescence shoulder was present in wild-type plants but it was absent in *npq4* mutants that lack PsbS. On the other hand, in *npq1* mutants, which are deficient in the violaxanthin deepoxidase and thus are unable to accumulate zeaxanthin in high light, the red shoulder was still present. Therefore, it is tempting to conclude that the "instantaneous" quenching is related to the PsbS protein. In this respect, it is interesting to note that Tian et al. did not observe a difference in the rate of NPQ for open and closed RCs in the green alga *Chlamydomonas reinhardtii* [77]. Although this alga has a gene for PsbS, it is known that its PsbS is not involved in NPQ. Therefore, this observation is consistent with the results on the *npq4* plants.

A similar red shoulder was also observed before for aggregated LHCII trimers at 77 K and was assigned to a Chl–Chl charge-transfer (CT) state by Miloslavina et al. [27]. However, this band disappears in the LHCII aggregates at higher ($T > 200$ K) temperatures [28], and although such a CT state is the most likely explanation for the observed red shoulder, it is probably not directly involved in NPQ [28,29]. That means that our observed red fluorescence band may just be a spectroscopic marker of the presence of strong NPQ when the RCs are closed, possibly as a consequence of the charge-separated state and the corresponding electric field. The fact that the switching time between two quenching modes is so much faster (at least by a factor of 10^5) than the known switching times involving PsbS protonation and the xanthophyll cycle, indicates that it is not biological or chemical but rather physical in nature.

6.1. Consequences of microsecond switching for (the improvement of) crop yields

In 2004, Zhu et al. [23] published a theoretical analysis of the losses that may occur in crop yield due to slow reversibility of NPQ, arriving at a number of ~30%. With this in mind, Kromdijk et al. [22] mutated tobacco plants and obtained faster switching rates which led to ~15% increase in biomass production. To which extent can this number still

be improved? The theoretical value of 30% is based on the amount of NPQ that is standardly measured in the presence of closed RCs, assuming that the quenching rates remain unaltered when the RCs reopen. However, if around 50% of the non-photochemical quenching disappears immediately upon opening the RCs, yield improvement can only be achieved by speeding up the disappearance of the remaining part of NPQ. This means that the potential yield increase is most likely lower than was predicted before. However, determining the exact percentages will depend on the relative amount of quenching that can be assigned to the various quenching processes that disappear on different time scales. It may be anticipated that speeding up the slowest process will have the largest consequences. On the other hand, there may also be substantial differences in the relative contribution of the various NPQ mechanisms in different crops, and increasing the relative amount of instantaneous switching will most likely lead to increases in crop yield. Therefore, it is extremely important not only to quantify the relative contributions and time scales of the various quenching mechanisms in different crops, but also to understand the underlying mechanisms. In addition, it may well be that (green) algae, in which PsbS does not play a role in NPQ, may become more efficient for biomass production if an "instantaneous" quenching mechanism, like the one in plants, can be engineered into their photosynthetic apparatus.

In conclusion, it will be highly important to determine the exact mechanism behind instantaneous NPQ switching and to determine to which extent instantaneous NPQ contributes to the protection of different crops (including also algal species), in order to further optimize photosynthesis and increase biomass production.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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