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DARWIN REVIEW

Evolution under the sun: optimizing light harvesting in photosynthesis

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Abstract

The emergence and evolution of life on our planet was possible because the sun provides energy to our biosphere. Indeed, all life forms need energy for existence and proliferation in space and time. Light-energy conversion takes place in photosynthetic organisms that evolve in various environments featuring an impressive range of light intensities that span several orders of magnitude. This property is achieved by the evolution of mechanisms of efficient energy capture that involved development of antenna pigments and pigment–protein complexes as well as the emergence of various strategies on the organismal, cellular, and molecular levels to counteract the detrimental effects of high light intensity on the delicate photosynthetic apparatus. Darwin was one of the first to describe the behaviour of plants towards light. He noticed that some plants try to avoid full daylight and called this reaction paraheliotropism. However, it was only in the second half of the 20th century, when scientists began to discover the structure and molecular mechanisms of the photosynthetic machinery, that the reasons for paraheliotropisms became clear. This review explains the need for the evolution of light adaptations using the example of higher plants. The review focuses on short-term adaptation mechanisms that occur on the minute scale, showing that these processes are fast enough to track rapid fluctuations in light intensity and that they evolved to be effective, allowing for the expansion of plant habitats and promoting diversification and survival. Also introduced are the most recent developments in methods that enable quantification of the light intensities that can be tolerated by plants.

Key words: Evolution of light-harvesting antennae, light adaptation, photoinhibition, photoprotection, photosynthetic light harvesting, reaction centre turnover.

…leaves and cotyledons which when moderately illuminated are *diaheliotropic*; but which change their positions and present their edges to the light, when the sun shines brightly on them.

Charles Darwin, *The power of movement in plants* (1880)

….and this remarkable movement I have called *paraheliotropism*.

Charles Darwin, *Movements of plants* (1981)

Darwin on sun and plant: dia- and paraheliotropisms

Charles Darwin was a brilliant naturalist; an observer of nature, first of all. In his house, located in a quiet spot in the Kentish village Downe, surrounded by a meadow and dark woodland, he spent the later years of his life observing the behaviour of plants. His favourite subjects were climbing

Abbreviations: F_m, maximal chlorophyll fluorescence; F_v/F_m, ratio of variable to maximal chlorophyll fluorescence; LHCI/II, light-harvesting complex of PSI/II; NPQ, non-photochemical chlorophyll fluorescence quenching; PSI/PSII, photosystem I/photosystem II; RCI/RCII, reaction centre of photosystem I/II.

plants, insect-eating plants, and orchids. One possible explanation of his choice of these types is that they all either display features close to animal behaviour: climbing, eating insects, or interacting with animals, pollinators in the case of orchids. Since the majority of plants display few dynamics in their habits in comparison to animals, it can be curious to observe how some plants 'reach' for sunlight or climb walls and trees to considerable heights. Darwin wrote a fairly large treatise on these plants: 'On the movements and habits of climbing plants' (Darwin, 1865) in which he wrote: 'The object of all climbing plants is to reach the light and free air with as little expenditure of organic matter as possible.... We have also seen that the movement of a revolving shoot, and in some cases of a tendril, is retarded or accelerated in travelling from or to the light. In a few instances tendrils bend in a conspicuous manner towards the dark. Many authors speak as if the movement of a plant towards the light was as directly the result of the evaporation or of the oxygenation of the sap in the stem, as the elongation of a bar of iron from an increase in its temperature. But, seeing that tendrils are either attracted to or repelled by the light, it is more probable that their movements are only guided and stimulated by its action, in the same manner as they are guided by the force of attraction from or towards the centre of gravity.'

By observing the behaviour of climbing plants and performing very simple (but clever) experiments on them Darwin revealed the fundamental nature of the effect of light on plants that guides their development and growth, ensuring, first of all, the best light environment for the optimum energy input into the photosynthetic apparatus of the chloroplasts or, as he and his contemporaries called them, 'chlorophyll grains'. He went on observing various movements of plant leaves and discovered that apart from the heliotropic behaviour (Fig. 1A) that is necessary to increase the capture of light by a leaf, there were movements that were directed to decrease light absorption (Fig. 1B). These movements Darwin termed *paraheliotropisms*. He observed several types of such movements. On one occasion Darwin writes: 'With several species of *Hedychium*, a widely different paraheliotropic movement occurs, which may be compared with that of the leaflets of *Oxalis* and *Averrhoa*; for the lateral halves of the leaves, when exposed to bright sunshine, bend downwards, so that they meet beneath the leaf' (Darwin, 1881) (see Fig. 1B). However, as with all adaptations heliotropisms and paraheliotropisms have their limitations. Therefore, in the dark wood of his estate, where Darwin regularly walked, there are barely any plants growing under the dense canopies of hornbeam and beech trees while on the nearby meadow a variety of grasses and wild flowering plants thrive (Fig. 1E). But in Darwin's brightly sunlit greenhouse leaves of bromeliads look pale green, a sign of loss of chlorophyll and an adaptation on the cellular and molecular level to bright light (Fig. 1D). Interestingly, the other plant, a bush, *Actinidia kolomikta*, that grows in the orchard part of the Darwin gardens, possesses leaves that are partially covered with a white or pink wax deposit that is likely to be another protection strategy, a 'sunblock' response (Fig. 1F). Generally these plants prefer shady environments. However, the sunblocking deposit is a cautious measure preventing damage due to occasional exposure to bright light. Many other plants that live in arid environments possess a similar strategy that helps them to achieve sustained reduction in the absorption of energy in high light. With the respect to the detrimental effect of light on plants Darwin often mentioned the work of a prominent Austrian botanist Julius Wiesner, then a professor of plant anatomy and physiology at the University of Vienna, who observed the damaging effect of high light exposure upon chlorophyll in organic solvents and in intact leaves (Wiesner, 1875). Wiesner was one of the first who concluded that the presence of oxygen exacerbated this process and that plants evolved a number of 'natürliche Einrichtungen zum Schutze des Chlorophylls' (natural ways for protecting chlorophyll). Wiesner was speaking of such adaptations developed in a course of evolution as ability to grow in deep shade under canopy of other plants, particularly structure, anatomy, and positioning of leaves towards light. The latter was Darwinian paraheliotropism that in the experiments of Wiesner was often of a transient nature in young leaves containing in his definition 'immature chlorophyll grains' and absent in established leaves (Wiesner, 1875). For the three major primary survival strategies of plants, *competitive* (low stress, low disturbance), *stress-tolerant* (high stress, low disturbance), and *ruderal* [low stress, high disturbance; Darwin's 'growth under difficulties' (Darwin, 1877)] (Grime, 1977), heliotropisms and paraheliotropisms are directed to enable growth under either low or high light, ensuring better chances of survival in conditions of high stress. Later this review will show an example of a remarkable plasticity in the behaviour of a climbing plant *Hedera helix* (Fig. 1G) that possesses molecular mechanisms enabling its survival in both light-limiting and excess light environments, a phenomenon Darwin would have been curious to see explained. However, it took nearly a century for the plant sciences to uncover and understand the molecular mechanisms (invisible for Darwin and his contemporaries) taking place in the photosynthetic membrane that evolved to be vital for plant survival in a broad variety of light environments encountered on our planet.

Life and light: the key feature of photosynthesis

Light energy was the key element in the beginning of the evolution of life on our planet and the formation of the biosphere (Blankenship, 2002). Since all life forms are autonomic systems built of polymers, proteins, carbohydrates, lipids, and nucleic acids, they require an exchange of energy and substance with the environment in order to maintain their integrity and to develop, grow, adapt, and reproduce (Ruban, 2012). Hence, the essential prerequisite of life is the incidence of an unlimited amount of transferable light energy. This energy is constantly required to sustain the higher organizational order of life forms compared to the environment in which they exist. Therefore, it is expected that organisms that utilize primary light energy (autotrophs) evolved a variety of functions that sense, transform, and regulate light energy input into their systems to their advantage,

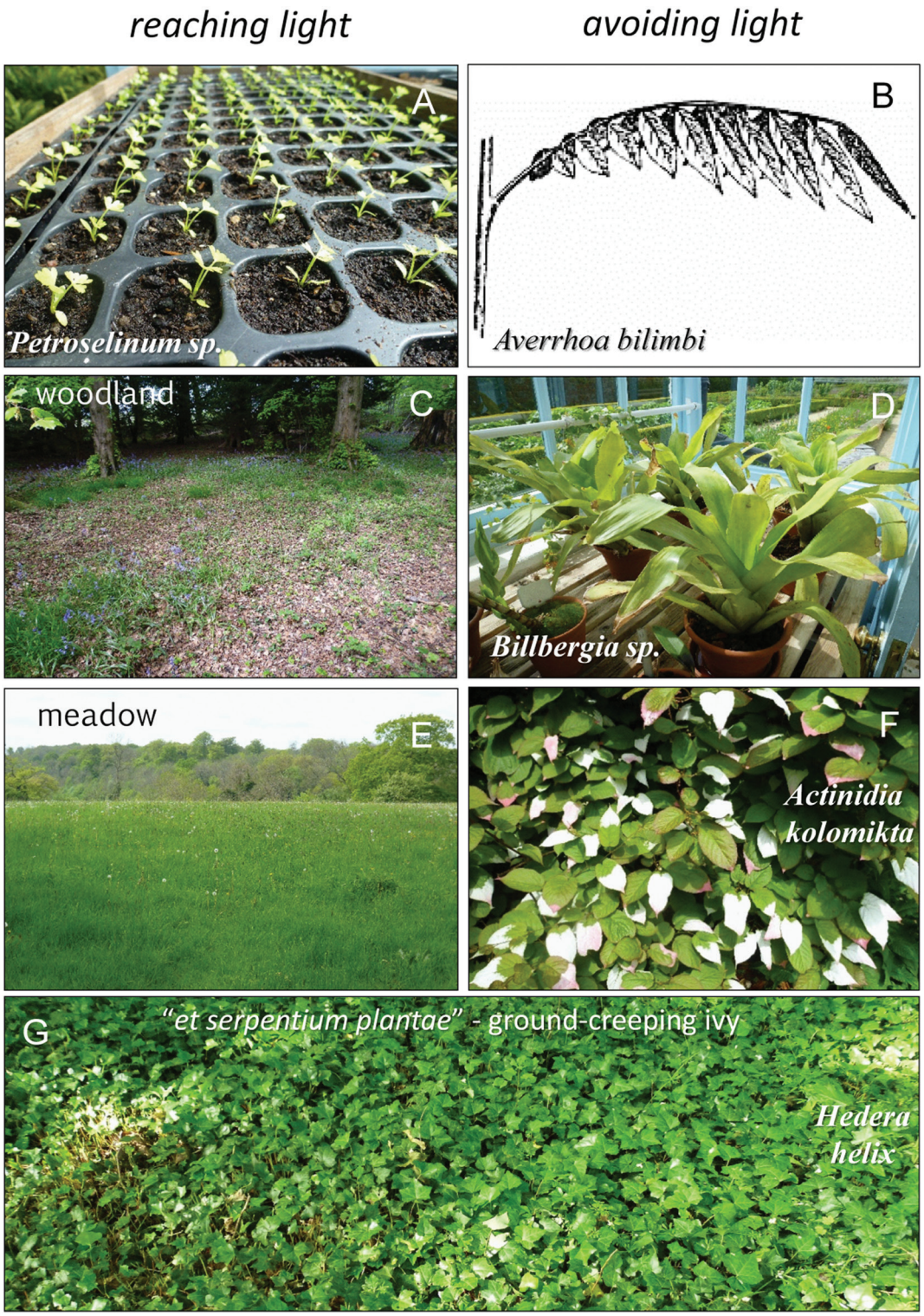

Fig. 1. Plants of the Darwin house in Downe. (A) Seedlings of parsley that are grown for the kitchen garden displaying heliotropic orientation. (B) *Averrhoa* leaves positioned as a result of paraheliotropic movement (from Darwin, 1980, Fig. 180; reproduced with permission from van Wyhe J, ed. 2002–. *The complete work of Charles Darwin online*; http://darwin-online.org.uk/). (C) Under the canopy of the dark wood in the Darwin estate. (D) Bromeliads in the Darwin greenhouse. (E) Meadow near the Darwin house. (F) Leaves of *Actinidia kolomikta* growing in the orchard of the Darwin estate. (G) Ivy (*Hedera helix*) crawling in the dark wood of the Château de Pougy, France. (A, C–G) Images by A. Ruban.

enabling survival and proliferation in time and space: the defining features of life.

Darwinian observations of the movements of climbing plants have clearly revealed that plants can use light for guiding their movements (heliotropisms) directed towards reaching the environment that provides optimum light energy absorption by leaves. Light absorption starts a number of light-energy-transformation events that constitute *photosynthesis*: a process that converts the energy of light into the chemical energy of organic compounds. Today, oxygenic

photosynthesis is the most common process of light-energy conversion in our biosphere. It transforms the energy of photons into the energy of glucose by reducing the carbon of CO_2 using electrons taken from the oxygen of water. Although it seems to be a simple chemical reaction, photosynthetic organisms evolved an extremely complex cascade of energy transformation events that actually make this reaction possible (Ruban, 2012). The sequential nature of photosynthesis is its key feature. Figure 2 illustrates the sheer complexity of this energy transformation in photosynthetic organisms. First, a photon is absorbed by a pigment, inducing an electronic excitation. Charged with photon energy the pigment passes it to another pigment and eventually to the one that can be oxidized, donating an electron to the sequence of redox reactions, enabling electron transport to the final acceptor—$NADP^+$—which is converted into NADPH, a first chemical form of light-energy storage. The energy of the proton gradient formed as a result of electron transport is used by ATPase to synthesize ATP, another form of photon energy storage in a chemical bond. This concludes the light phase of photosynthesis. However, the molecular machinery that is involved in this cascade of energy transformation is extremely complex and localized in the plane of the specialized membrane that forms chloroplast thylakoids (Fig. 2B). There are dozens of integral and peripheral membrane proteins that form the multisubunit macromolecular structures of photosystems I and II (PSI and PSII), cytochrome b_6f, and ATPase. ATP and NADPH enter the CO_2 reduction cycle (Calvin–Benson cycle) where glucose molecules are formed. Later it is polymerized into starch for storage in a compact form that does not affect the osmotic pressure of the organelle where the energy transformation takes place: the chloroplast.

The sequential nature of photosynthesis seems to be an inevitable feature. Indeed, photosynthetic energy-conversion stages tend to slow down the time scales of the reactions of energy transformation from the femtosecond scale of photon absorption, the micro- and millisecond scale of electron transfer, the second scale of the establishment of a proton

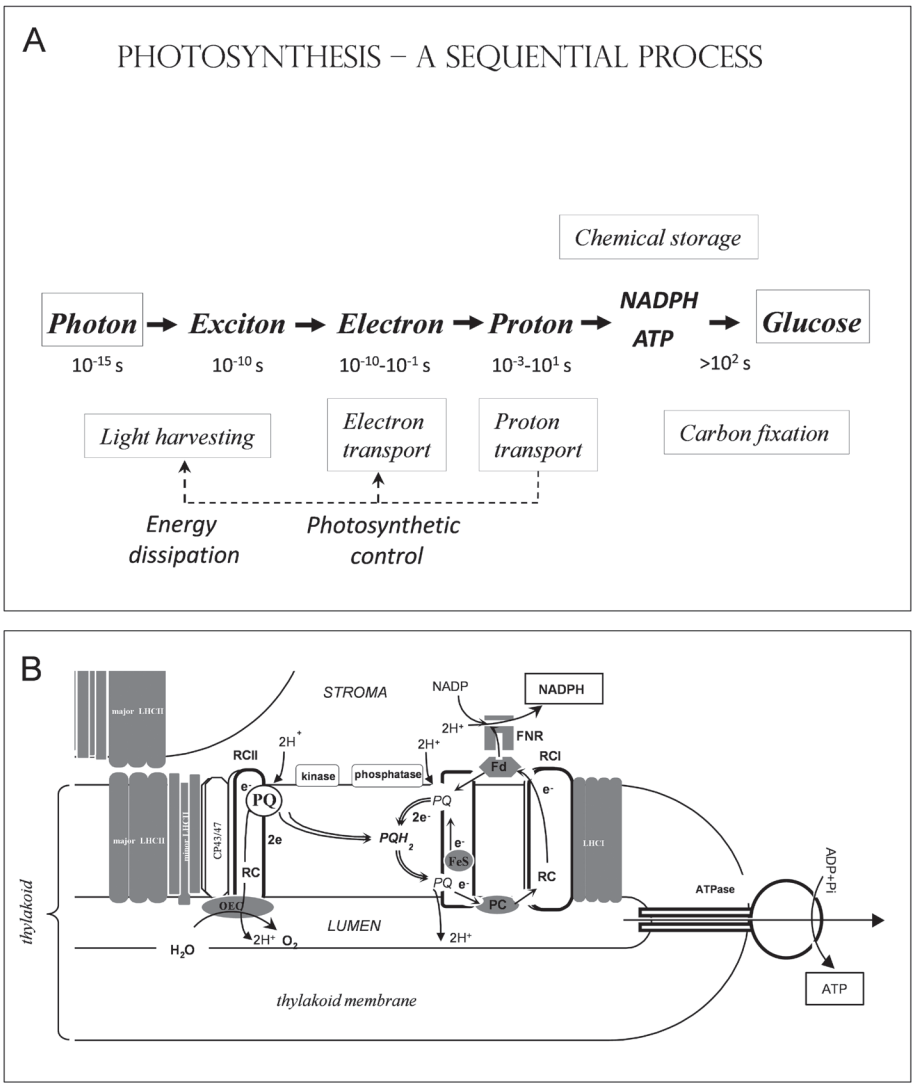

Fig. 2. (A) Sequential energy-transformation processes of photosynthesis. (B) A fragment of the photosynthetic membrane schematically depicting major events of the light phase of photosynthesis. LHCI, light-harvesting complex of PSI; LHCII, light-harvesting complex of PSII; CP43/47, core antenna complexes of PSII; RCII, reaction centre of PSII; OEC, oxygen-evolving complex; PQ, plastoquinone; e^-, electron; H^+, proton; FeS, iron-sulphur cluster; PC, plastocyanine; Fd, ferredoxin; FNR, ferredoxin-NADP-oxidoreductase.

gradient, to the minutes and hours of carbon reduction and the accumulation of glucose and starch. Indeed, small particles charged with energy move and change much faster than the large biomolecules of life (Fig. 2B). The cascade of photosynthetic reactions stabilizes the captured energy ensuring that it cannot easily escape. However, the downside of this is that each energy transformation step encounters losses: an inevitable price to pay. This makes photosynthesis an inefficient process. In the ideal scenario only about 10% of absorbed photon energy will end up stored in glucose (Ruban, 2012). Hence, light energy is precious for plants and heliotropisms are visible evidence for this. On the other hand, the phenomenon of paraheliotropic movements suggests that high light is not welcomed by the sequentially organized chain of photosynthetic reactions. This can be due to the likely occurrence of limiting steps or bottlenecks that can lead to accumulation of excessive amounts of intermediate products, charged with one form of energy or another, which can be disadvantageous for or even detrimental to the cell. Hence *craving* or *avoiding* light by plants suggests that the development of the processes that efficiently and promptly regulate light energy input into the photosynthetic apparatus should be an inevitable outcome of the molecular evolution of the photosynthetic function. Although being very effective light adaptations, heliotropisms have their limitations, as do all forms of adaptive strategy. They are relatively limited in regulating light absorption in very-low-light environments. Heliotropisms are also less effective when it comes to conditions of highly scattered, intense light that propagates in all directions. Moreover, the fact that many evolutionarily successful plants do not possess heliotropisms suggests that they may have developed alternative ways of coping with the light environment which are less visible to a classical naturalist like Darwin.

Emergence of the photosynthetic light-harvesting antenna

The first photosynthetic organisms evolved in aquatic, anaerobic environments which provided shelter from damaging ultraviolet radiation. In such an environment the light intensity can be as low as a few micromoles of photons per square metre per second. At this intensity the rate of photon absorption by reaction centre chlorophyll is one per several seconds. However, in normal circumstances reaction centre chlorophyll can undergo oxidation and re-reduction within about 10 ms, meaning that in such low-light conditions electron transport will run at less than 1% of its full capacity (Clayton, 1970; Clayton, 1980). In order to make reaction centres more effective the cheapest strategy is to synthesize light-harvesting antennae: a number of pigment-carrying proteins that could feed many more quanta per second into the reaction centres. In fact the photosynthetic antenna can increase the rate of energy delivery to the reaction centre by two or more orders of magnitude. The first evidence of the existence of the photosynthetic antenna was obtained by the experiments of Emerson and Arnold who used microsecond flashes of light in order to obtain one reaction centre turnover (oxidation–reduction cycle, described above) per flash. They discovered that the turnover rate was indeed about 100 per second. To their surprise they discovered that one absorbed photon of light can cause only one photochemical act (one PSII reaction centre turnover) among a population of 300 chlorophylls (Emerson and Arnold, 1932). This observation led to the conclusion that a single photosynthetic reaction centre must be somehow connected to about 300 molecules of chlorophyll that are capable of feeding it with excitation energy with high efficiency.

The conclusions of Emerson and Arnold have been confirmed in several independent experiments dealing with the estimation of the effective cross-section of the photosynthetic reaction centres as well as an analysis of the ratio between various electron transport chain components (cytochromes, reaction enter chlorophylls, etc.) and the total amount of chlorophyll in the membrane (Ruban, 2012). These observations led to the formulation of the concept of the photosynthetic unit, where the majority of pigments 'serve' one reaction centre with absorbed light energy. Close positioning of pigments relative to one another in antenna proteins is one of the major conditions that allow these pigments to exchange excitation energy and eventually deliver it to the reaction centre. The dominating mechanism that underlies this process is inductive resonance transfer described by the theory of Förster (1948). The rate of this transfer strongly depends upon the distance between interacting pigments, their mutual orientation, and the closeness of their excitation energies or, in other words, their spectral similarity. However, pigments that are too close to each other can lose absorbed energy via concentration quenching, preventing its delivery to the reaction enter (Beddard and Porter, 1976). To fulfill the requirements for the photosynthetic antenna nature evolved a family of genes that encode proteins that bind pigments in specific positions at optimal distances, orient them, tune their excitation energies, and direct energy transfer to the reaction centre (Blankenship, 2002; Ruban, 2012; Ruban *et al.*, 2012). Hence the *photosynthetic antenna* is a number of proteins that carry pigments and are organized in a specific way around the reaction centre in order to ensure the high efficiency of photon energy delivery to the photoactive pigments. Schematically the photosynthetic antenna can be depicted as a funnel, channeling pigment excitation energy to the reaction centre (Fig. 3). The proteins of antennae ensure that pigments do not lose much energy via triplet formation, fluorescence, and, most importantly, thermal dissipation. PSI and PSII possess their own antennae. While reaction centre of PSII (RCII) is served by inner (CP43/47 complexes) and peripheral antennae [minor light-harvesting complexes (LHCs) and major light-harvesting complex of PSII (LHCII)], the reaction centre of PSI (RCI) is served by the integral antenna pigments localized on the same polypeptides as RCI and peripheral antenna (LHCI) (Fig. 2B). The number of pigments serving RCII can reach 350, while for RCI this number is about 210 (Ruban, 2012).

It is important to note that the photosynthetic antenna was 'reinvented' a number of times in the course of evolution and

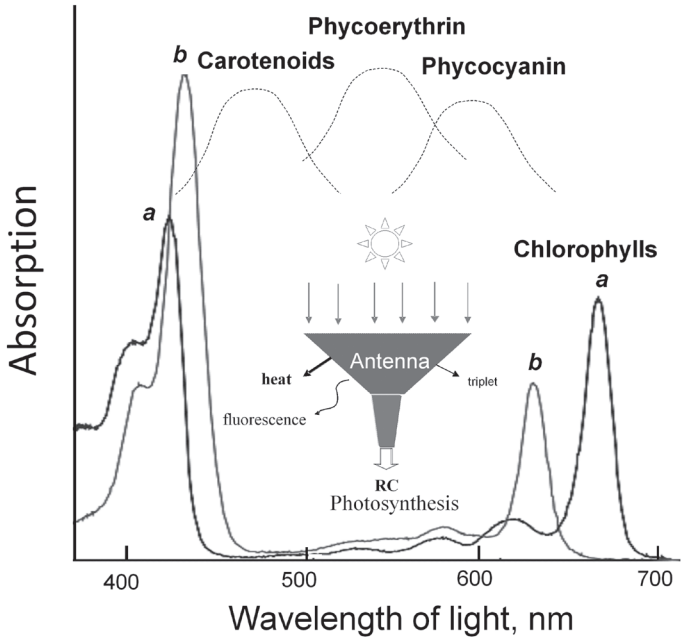

Fig. 3. A variety of photosynthetic antenna pigments are tuned to capture specific visible region of the light energy (light of various wavelengths). Centre: the light-harvesting antenna concept: the funnel of absorbed light energy that efficiently directs it to the reaction centre (RC) with some minor losses into heat, fluorescence or triplet formation (intersystem crossing).

hence originates from multiple ancestors. Indeed, the antennae of photosynthetic bacteria, various classes of algae, and higher plants are 'built' from very different types of protein that cannot often be related at all and different types of pigments too (Thornber, 1975; Cogdell and Isaacs, 1995; Green and Durnford, 1996; Blankenship, 2002; Camm and Green, 2004). It seems that antenna function was tried by evolution more intensively than the function of reaction centres, the engines of the light phase of photosynthesis (Blankenship, 2002). This is likely to be due to the changeable geological and solar environment that shaped our biosphere, inducing frequent alterations of habitats where the light environment differed in intensity and spectral composition. Hence, evolution improvised with different types of pigments, broadly tuning their spectral range of absorption from near ultraviolet (300–350 nm), through violet (400 nm) and blue (450–475 nm; chlorophylls and carotenoids) to green (500–530 nm; carotenoids), yellow and orange (570–600 nm; phycoerythrin and phycocyanin), and red (650–700 nm; chlorophylls) (Fig. 3). In purple antenna bacteriochlorophyll absorption went even further into the near infrared (Cogdell and Isaacs, 1995; Blankenship, 2002). On the other hand, nature varied the reaction centre pigments only a little: they are either bacteriochlorophylls or chlorophylls with absorption maxima of 680 (RCII), 700 (RCI), or 800–870 nm (bacterial RC). There are basically two classes of reaction centre that resemble either RCI or RCII and the possibility of a single ancestral origin is not excluded (Blankenship, 2002).

Multiple types of antenna pigments and proteins and macromolecular organizations can indicate that the photosynthetic antenna plays a crucial role in adaptations to the light environment. Not only can it vary *spectrally*, adapting to the colour of light, but it can change in *size* to supply more or less energy to the reaction centre, and it can *memorise* the history of light exposure so it may better respond to further alterations in light environment. And finally, the antenna of PSII can regulate its efficiency without compromising the electron transport rates of RCII, a form of 'economic protection' (discussed below). The mentioned adaptations of the photosynthetic antenna can be achieved in the course of long-term adaptations (acclimation, involving gene expression responses) or short-term molecular adaptations that take minutes or even tens of seconds, an unsuspected response from the point of view of a classical plant explorer like Darwin. Indeed, it appears that life's strategy of survival rests upon three fundamental and temporally different pillars: *evolution*, *acclimation*, and *adaptation*. While evolution drives towards improved adaptations and acclimations, eventually leading to development of new species, the latter two have a more conservative nature: they tend to preserve species to some extent.

Key determinants of antenna function

In order to fully understand the reasons for and types of photosynthetic antenna adaptations to light one has to define what actually makes the antenna efficient and adaptive. The first feature is that antennae consist of pigments that capture light but are bound to proteins that tune their function. Indeed protein is a 'programmed solvent' (Ruban, 2012) that prevents pigments clustering in a random way. It positions them fairly reproducibly, and orients them in order to ensure fast intermolecular energy transfer in directed fashion so that it quickly reaches the reaction centre chlorophyll. The latter is achieved by tuning the excitation energies of chlorophylls via interactions with its interior environment (hydrogen bonds; aromatic residues of tryptophan, phenylalanine, and tyrosine; proximity to other pigments). The antenna is normally built of a number of proteins or rather complexes of pigments, lipids, and protein (CP complexes), hence the *modular nature* of its organization. This enables an adjustment of antenna size that makes the RC energetic cross-section (*absorption cross-section*) larger or smaller in the process of acclimation to the light environment (shade versus sun plants).

Absorption cross-section is determined also by the effectiveness of the *energy transfer* from the antenna to the RC. The latter is a function of the structure of individual antenna units, their arrangement around the RC protein(s), and the time excitation energy is kept in antenna. In general, the longer an excited state is kept by the pigments of antenna the higher the probability that it will reach the RC, hence the higher the efficiency of the charge separation (Ruban *et al.*, 2011; Valkunas *et al.*, 2011; Duffy *et al.*, 2013*a*). The chlorophyll excitation lifetime largely depends on the environment. The latter could influence pigment conformation, directly interfere with the optical π-electron configuration, or remove excitation via energy transfer, as an energy acceptor. Amino acids, carotenoids, lipids, and chlorophyll itself are the major modulators of the antenna chlorophyll lifetime. The excitation lifetime is proportional to the fluorescence yield, which

can be easily monitored in a steady state. Free chlorophyll in an appropriate solvent and at very low concentration has the highest fluorescence yield and therefore excitation lifetime. It was shown that at higher concentrations a so-called concentration quenching of chlorophyll fluorescence occurs due to either ground-state (Lee, 1975) or excited-state (excimer) dimer formation (Beddard and Porter, 1976). For the major peripheral antenna of PSII, LHCII trimers (Fig. 2B), the fluorescence yield was only slightly lower than that for diluted chlorophyll, despite a chlorophyll concentration almost 100 times greater than in solution (up to 1 M). The absence of concentration quenching is a remarkable achievement of nature and is likely to be due to the coordinated manner of the pigment arrangement (as mentioned previously), as can be seen from the LHCII model (Liu *et al.*, 2004). However, LHCII trimers are connected to each other to form higher oligomeric LHCs, as was suggested above. The fluorescence yield of these oligomers is much lower than that of trimers and is comparable to that of the thylakoids in the state when all reaction centres are closed (maximal chlorophyll fluorescence, F_m), which favours maximal excited-state lifetime and fluorescence yield (Belgio *et al.*, 2012; Ruban, 2012). This fact suggests that the energetic state of LHCII in the PSII antenna could be similar to that of the LHCII oligomer, not trimer.

The state with the shorter excitation lifetime has a lower probability of exciting the reaction centre; however, this is a fair price to pay for a more ordered, connected antenna and a high local concentration of chlorophyll. LHCII trimers can be aggregated artificially into two-dimensional arrays, which have even lower fluorescence yield than isolated oligomers (Ruban, 2012). However, even in this case the fluorescence yield is higher than that of chlorophyll aggregates *in vitro*. From this one can conclude that the fluorescence yield/lifetime of chlorophyll is an extremely flexible parameter, which can be and actually was exploited during the evolution of the light-adaptive processes via precise *in vivo* control of antenna architecture and dynamics (Ruban *et al.*, 2012; Kouril *et al.*, 2013; van Amerongen and Croce, 2013).

Living in shade

Plants that inhabit shaded environments tend to accumulate a rather large light-harvesting antenna, normally a peripheral type: trimeric LHCII complexes (Anderson and Osmond, 2001; Anderson *et al.*, 1988, 2008; Jia *et al.*, 2012). Since these complexes are the most enriched in chlorophyll *b* (the chlorophyll *a*/*b* ratio is 1.33; Liu *et al.*, 2004) simple determination of chlorophyll *a*/*b* ratio of leaf extracts can give an idea about the habitat of a plant. For example, leaves of *H. helix* depicted in Fig. 2G, which grow in the deep shade of the dark hornbeam and beech forests or in very shady pockets of gardens, possess chlorophyll *a*/*b* ratios ranging from 1.5 to 2.4 (Demmig *et al.*, 1987; Brugnoli *et al.*, 1998; Demmig-Adams, 1998). This is indicative of the presence of large numbers of LHCII trimers that significantly increase the PSII antenna in shade plants. When exposed to high light these plants can gradually reduce the amounts of LHCII. Some mechanistic details of the acclimative adjustment of the PSII antenna size have been reported (Lindahl and Andersson, 1992; Lindahl *et al.*, 1995; Andersson and Aro, 1997). It was discovered that the reduction in amounts of trimeric LHCII is caused by the proteolysis of its apoprotein. The protease responsible for the process has not been identified yet. It is suggested to be a serine- and/or cysteine-type of ATP-dependent enzyme, located extrinsically on the stromal side of the thylakoid membrane (see Fig. 2B). It takes up to 2 days for enzyme expression/post-translational activation to occur after exposing plants to a high-light environment. Once activated, it takes less than a day to complete proteolysis and reduce numbers of LHCII trimers by a half, which is approximately 30% of the total LHCII content.

Apart from acclimation to light, shade plants evolved a more rapid mechanism that compensates for an imbalance of light input into the two photosystems. This is not an acclimative but rather an adaptive response that takes place within minutes. It is called a 'state transition' and occurs at very low light intensity (Bonaventura and Mayers, 1969; Murata and Sugahara, 1969). State transitions are a purely LHCII antenna-based mechanism. It is needed when one of the photosystems starts to receive more energy than the other. And since they operate in series this imbalance causes limitations in the rate of electron transport, because the photosystem that receives less energy becomes a part of the electron transport chain of reactions that limits its rate (Fig. 2). The reason why such imbalance can occur originates from the fact that the PSI antenna absorbs far-red light and PSII just red light. The spectral quality of sunlight can quickly change for shade plants grown under a canopy that filters light or more slowly depending upon the time of day, causing differential alteration in the light energy absorbed by PSI and PSII.

The molecular mechanism of the state transition utilizes the redox state of the plastoquinone pool (Horton *et al.*, 1981) as a sensor/transducer, which can activate the membrane-bound protein kinase (Bennett, 1977; Bennett, 1983) (Fig. 2B and Fig. 4). When the energy imbalance is in favour of PSII, this kinase, activated by the reduced plastoquinone pool (Fig. 4), phosphorylates a part of trimeric LHCII, targeting threonine or serine residues of the stroma-exposed N-terminus of the apoprotein (Mullet, 1983). This so-called 'mobile' LHCII becomes dissociated from PSII under the influence of the negative charge introduced by phosphate (Kyle and Arntzen, 1983; Staehelin and Arntzen, 1983). As a consequence, the PSII antenna becomes reduced. Phosphorylation leads to a partial unstacking of the grana and the detached phospho-LHCII can migrate into those regions of the thylakoid membrane which are enriched in PSI (grana margins, stroma lamellae), and interact with the PSI complex. Early studies of the efficiency of energy transfer from chlorophyll *b* to P700 failed to prove the increase in PSI absorption cross-section (Haworth and Melis, 1983; Deng and Melis, 1986). However, according to low-temperature fluorescence experiments (fluorescence, excitation and fluorescence induction) phospho-LHCII brings an additional 20–35% of excitation energy to PSI (Kyle *et al.*, 1983; Telfer *et al.*, 1986; Ruban, 1991; Harrison and Allen, 1992). Assuming a decrease in

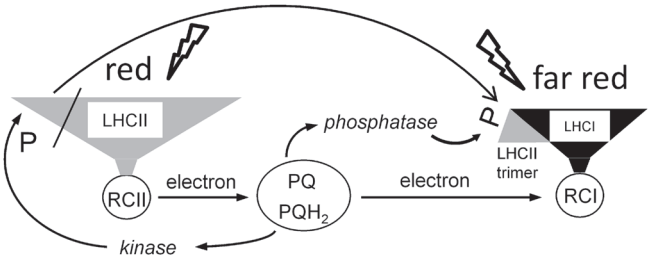

Fig. 4. The LHCII phosphorylation model of state transitions. Imbalance in the light energy input into the antennae of photosystems that leads to the preferential excitation of PSII causes the reduction of the plastoquinone pool (PQH_2). PQH_2 activates membrane-associated kinase (see Fig. 2B), which phosphorylates polypeptides of LHCII. The phosphorylated LHCII detaches from PSII, migrates towards PSI and incorporates into its antenna system. As a result of this incorporation PSI gains more excitation energy which leads to the increase in the reaction centre (RCI) turnover with the subsequent oxidation of PQH_2. When PSI gains relatively more excitation light than PSI it oxidizes PQ further. PQ activates phosphatase (see Fig. 2B) - an enzyme which dephosphorylates LHCII attached to the PSII. Dephosphorylated LHCII detaches from PSI and migrates and incorporates into PSII. The different colours of PSI (black) and PSII (light grey) antennae highlight their spectral difference.

the PSII antenna of approximately 30% and a corresponding increase in the PSI antenna, the total energy balance change between photosystems will reach 85%. This is large enough to be considered as significant for the adaptation to the changes in the light environment, particularly in shade-growing plants which have a larger pool of the peripheral LHCII. Since the increase in PSI cross-section occurs at the expense of chlorophyll *b* and short-wavelength forms of the chlorophyll *a* of phospho-LHCII, this makes the PSI antenna less spectrally distinguishable from PSII, facilitating further the balanced redistribution of the excitation energy in the thylakoid membrane. Oxidized plastoquinone activates phosphatase that removes the phosphate group from phospho-LHCII which subsequently migrates to PSII (Fig. 4). The size of the increase in cross-section that occurs as a consequence of LHCII phosphorylation is a function of cation concentration and temperature. It was found that elevated temperature on its own can cause detachment of LHCII from PSII and increase the direct energy transfer from PSII to PSI (spill-over) (Sundby and Andersson, 1985; Sundby *et al.*, 1985). Partial unstacking at low cation concentrations may enhance the effect of phosphorylation and also promote spill-over (Horton and Black, 1983). However, it was shown that at high cation concentration and room temperature the dominant process of the PSI cross-section increase occurs mainly due to the phospho-LHCII interaction with PSI (α-transfer) (Ruban, 1991; Ruban and Johnson, 2009). However, if under some conditions spill-over follows LHCII migration as well, as a result of significant unstacking, this should lead to even greater flexibility in the regulation of the electron transfer balance compared to the absorption cross-section change.

Although studied for a long time, some aspects of state transitions and LHCII phosphorylation are not well explored. An original hypothesis about the effect of phosphorylation on the N-terminus conformational state of the LHCII monomer (helix dynamics) was put forward by Allen and colleagues (Nilsson *et al.*, 1997; Allen and Nilsson, 1997). Although the concept requires firm experimental confirmation, it is a demonstration of exploring the conformational flexibility of LHCs, which could be a new functional feature of the antenna system. So far, there exists both spectral (Ruban and Johnson, 2009) and microscopy evidence (Kouril *et al.*, 2005) that phosphor-LHCII is a trimer, not a monomer. Other aspects of LHCII phosphorylation are the interaction with the long-term adaptation mechanisms (Allen and Nilsson, 1997), its role in cyclic electron transport (Fork and Herbert, 1993), and interactions with other short-term adaptation mechanisms. As mentioned above, state transitions should be particularly effective under low light, where the thylakoid membrane accumulates large PSII antenna due to the presence of LHCII. This population is also subject to phosphorylation; therefore, in low-light-grown plants the extent of the state transitions or phosphorylation-induced energy redistribution between photosystems will be highest (Harrison and Allen, 1993). Therefore, one can suggest that shade plants are better prepared for sudden fluctuations in the light quality, which can originate from sunflecks and changes in shading from the other plants (movements due to wind, diurnal changes in the angle of sun beams, etc.). On the other hand, in plants grown under high, saturating light where state transitions become obsolete, the outer antenna is reduced. Therefore, large outer-LHCII will constitute an adequate basis for more flexible adaptation to the sudden onset of energy imbalance between photosystems.

Combining ecophysiological experiments with some genetic manipulation approaches, designed to specifically under- or overexpress certain components of the thylakoid membrane, such as the LHC antenna etc., seem to be a promising approach to shed more light on the molecular mechanism and significance of 'state transitions' and protein phosphorylation *in vivo*. State-transition experiments, performed on plants lacking *lhca4* and *lhca1* gene products, have clearly demonstrated the vital importance of the LHCI antenna in linking phospho-LHCII to PSI (Horton and Ruban, unpublished work). Finally, it is important to note that enhancing light harvesting in plants that inhabit shaded environments cannot be done successfully if one does not take into account the synergistically related morphological and metabolic adjustments required for the plant's survival in these conditions.

Living in open air: the threat of photoinhibition

In high light, reaction centres become saturated with energy and the energy balance between them is no longer an issue. However, the build-up of the excess excitation energy in the antennae of photosystems is potentially dangerous because it can lead to irreversible damage to the reaction centres, resulting in a sustained decrease in their efficiency and electron transport rate, a phenomenon termed *photoinhibition*: Darwin called it 'injury of chlorophyll by high light' (Darwin, 1880; Powles, 1984; Ohad *et al.*, 1984; Barber, 1995). Wiesner

first observed that the 'injury' is enhanced by the presence of oxygen, as mentioned above. Hence, the photosynthetic pigments of oxygenic photosynthesis should be particularly vulnerable to photodamage. Indeed, PSII is particularly susceptible to photoinhibition because of the very strong oxidative potential of RCII, P680 (≈1.17 V), which is required to oxidize water (Fig. 2B). Under some conditions, when electron donation to P680 is less efficient than oxidation, an increase in the lifetime of $P680^+$ will occur. The powerful oxidant $P680^+$ will inevitably oxidize the nearest pigments and amino acids, causing their degradation and a subsequent degradation of D1 to follow (Barber, 1995). In other circumstances, when the acceptor side is less efficient, a radical pair can be formed. The recombination of this pair will lead to the formation of a P680 triplet state. In this state P680 can interact with *atmospheric triplet oxygen*, causing formation of highly reactive singlet oxygen, which in turn will bleach P680 (Telfer *et al.*, 1990), also leading to the degradation of D1 (Rivas *et al.*, 1993). Therefore, regardless of the damage scenario, the number of active PSII units will be decreased and because of the slow D1 repair cycle (hours; see Ohad *et al.*, 1984) and relatively fast onset of the damage (minutes; for the most recent kinetic study see Ruban and Belgio, 2014) the decline in electron transfer will last for some time even when excess light is no longer present. The accumulation of excess energy is very dangerous for wild and particularly non-shaded cultivated plants exposed to full sunlight. This is becoming an important problem for agriculture where the cost of photodamage can be high (Raven, 1994). Therefore the study of the mechanisms of photoprotection is crucial and the rest of this review will deal with them. These are fundamental, molecular mechanisms, developed in the course of evolution of oxygenic photosynthesis, that ensure flexible light energy utilization in a variety of plant habitats. Some of these mechanisms are very effective and prompt, occurring within minutes and even seconds.

Photoprotective measures: non-photochemical chlorophyll fluorescence quenching

Acclimative reduction in peripheral antenna size in plants grown in high light is a typical long-term response and was discussed above. This relieves RCII from excessive *excitation pressure* and therefore reduces the likelihood of the onset of photoinhibition. However, by far the most efficient and rapid responses to sudden high light exposure evolved to utilize feedback regulation principles in the long chain of photosynthetic energy transformation events. Dashed lines on the Fig. 2A display the two pathways of these feedback loops, both originating from the protons in the lumen generated as a result of electron transport. The first one is called *photosynthetic control* and the second *energy dissipation* in the PSII antenna, or *non-photochemical chlorophyll fluorescence quenching* (NPQ). In the light phase of photosynthesis, overproduction of ATP (and NADPH) causes an accumulation of protons in the thylakoid lumen (Fig. 2), which in turn leads to inhibition of a number of key electron transport enzymes (cytochrome *b/f* and the oxygen-evolving complex of PSII), causing reduction in the photosynthetic electron transport rate. Indeed, in analogy to respiratory control in mitochondria, the photosynthetic membrane possesses photosynthetic control as a mechanism for balancing ATP (and NADPH) production with electron transport. However, no matter how efficient photosynthetic control is, the path that starts from photon absorption to the primary charge separation in RCII requires an additional control loop. This feedback control exists in the form of the direct proton effect on PSII antenna efficiency.

Basically, nature explored the ability of LHCII complexes to undergo conformational changes that cause dramatic variations in the amount of heat dissipation in antennae, as shown in Fig. 3 (Ruban, 2012; Duffy *et al.*, 2013*a*). Hence this channel was explored by evolution as a prompt mechanism for regulating energy input into the photosynthetic membrane. The process can be monitored by measurement of chlorophyll fluorescence (a channel competitive with heat dissipation) (Fig. 3) and is called NPQ (Wright and Crofts, 1970; Briantais *et al.*, 1979; Schreiber, 1986; Genty *et al.*, 1989). Quenching or the decline in fluorescence intensity measures the increase in heat dissipation, hence the decrease in the detrimental excitation pressure under the conditions of high light exposure. NPQ is a very efficient process that protects PSII reaction centres from *photoinhibition* since it can develop promptly, within a few minutes, and can be of a reasonably large amplitude (which will be discussed below). Our recent studies using the method of millisecond fluorescence induction in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; Fig. 5A) revealed that NPQ is a form of *economic protection* that is effective only when RCII are closed due to saturation with high light. However, when the RCIIs are working under low light intensity with a low excitation pressure in the antenna, NPQ does not compete at all with the energy delivery. The reason for this conclusion came from the fact the NPQ had little effect on the millisecond fluorescence rise that followed the closure of RCIIs, meaning that this energy dissipation does not compete with the open reaction centre, or in other words NPQ is much slower than the energy trapping from the antenna by the reaction centre (Fig. 5B). Therefore, this form of protection prevents plants from losing light energy unnecessarily yet, in the long run, allows them to function safely under unpredictably and promptly variable illumination (Belgio *et al.*, 2014).

NPQ was discovered to be a heterogeneous process, both kinetically and mechanistically. For a long time a temporal criterion has been used to separate its components. The most slowly forming and relaxing NPQ component is called qI, a sustained quenching that was initially ascribed to the photoinhibitory damage of RCIIs that persists for many hours in the dark. The second slow component was called qT, the state-transitions-related fluorescence decline that forms and relaxes on a timescale of tens of minutes. It is predominantly observed in low light and is related to the balancing of excitation energy between PSII and PSI (as discussed above). The major component of NPQ develops and relaxes within tens

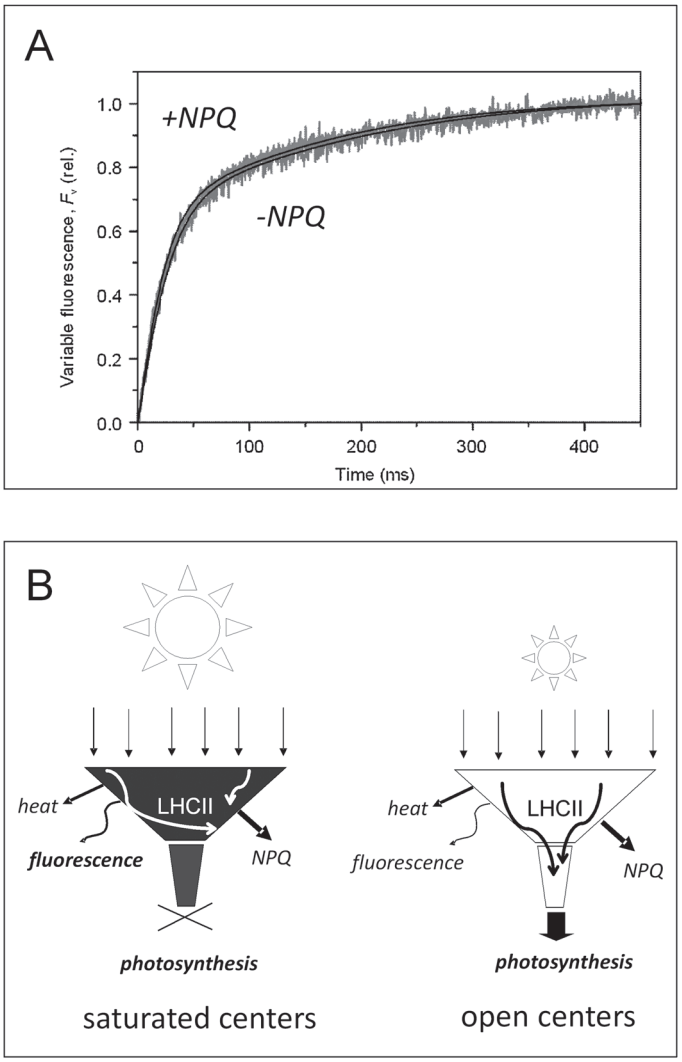

Fig. 5. (A) Millisecond fluorescence induction traces obtained on isolated spinach chloroplasts incubated with DCMU (30 μM) in the dark (open PSII reaction centres). Fluorescence was induced by 12 μmol photons·m^{-2}·s^{-1} of red light (650 nm) that triggered fluorescence rise from F_o (open RCII) to F_m (closed RCII) states. The presence of NPQ has a little effect of the fluorescence rise kinetics, indicating that the NPQ quencher does not compete well with RCII for captured light energy when reaction centres are open. (B) Bright light causes RCII closure/saturation and a rise in NPQ that damps the damaging excess energy into heat (left). When the light suddenly becomes very weak, RCII reopens and continues to effectively capture the excitation energy despite the presence of the NPQ quencher. Hence, NPQ only protects closed RCII and does not cause energy losses when RCII is open: evidence of an 'economic' type of protection.

of seconds to several minutes and is called qE or energy-dependent quenching (Wright and Crofts, 1970; Krause and Weis, 1991). qE was shown to be triggered by the gradient of protons across the thylakoid membrane, ΔpH, but followed somewhat slower kinetics of formation and, in particular, relaxation than the proton gradient (Wright and Crofts, 1970; Krause, 1974; Horton and Ruban, 1992; Horton *et al.*, 1996). This lack of a tight kinetic relationship between the proton gradient and qE led to the proposal that the thylakoid membrane (or specifically a part of PSII) should undergo some kind of conformational change in order to attain the photoprotective state (Krause, 1974). This was the first indication that protons must act upon the site where qE is formed to bring about some kind of change leading to formation of a quencher responsible for the fluorescence decrease in PSII.

Numerous experiments performed by Horton and colleagues at the beginning of 1990s produced evidence suggesting that qE originated from the peripheral light-harvesting antenna complexes, LHCII, and not in the RCII as was previously proposed (initially reviewed in Horton *et al.*, 1996). Most strikingly, it was observed that quenching in isolated LHCII and qE were found to be induced and modulated by the same agents: protonation (Wright and Crofts, 1970; Ruban *et al.*, 1994), xanthophyll-cycle carotenoids (violaxanthin and zeaxanthin; Demmig-Adams and Adams III, 1992; Ruban *et al.*, 1994), antimycin (Wright and Crofts, 1970; Horton *et al.*, 1991), magnesium (Noctor *et al.*, 1993; Ruban *et al.*, 1994), lutein (Pogson *et al.*, 1998; Johnson *et al.*, 2010), and *N*,*N*′-dicyclohexylcarbodiimide (DCCD; Ruban *et al.*, 1992, 1998). Recent observations convincingly suggesting that NPQ originates in LHCII are the discovery of the inhibitory effect of cross-linkers on NPQ formation and relaxation kinetics (Ilioaia *et al.*, 2008) and the persistence and even enhancement of qE in plants heavily depleted of RCII (Belgio *et al.*, 2012).

The discovery of zeaxanthin involvement in qE made by Barbara Demmig (Demmig-Adams, 1998) led to a detailed study on the relationship between qE and ΔpH in the isolated thylakoid membrane. Horton and colleagues performed a series of detailed titration studies revealing that it is possible to induce high levels of qE without zeaxanthin provided the lumen pH is lower than 4.5–5.0 (Rees *et al.*, 1989; Noctor *et al.*, 1991). Zeaxanthin was found to simply shift the relationship between qE and ΔpH so that the quenching could be activated at much lower ΔpH; that is, higher lumen pH. This shift was found to be about 1.3–1.7 pH units, which enabled significant quenching to be formed at a lumen pH of 5.7–6.2. A very similar effect of zeaxanthin on qE was also observed in the experiments on uncoupled thylakoids where the acidification of the buffer led to a pH-induced quenching in the dark (Rees *at al.*, 1992). It was therefore proposed that zeaxanthin may act as an allosteric modulator of qE by controlling the affinity of the LHCII antenna for protons rather than as a direct energy quencher (Bassi and Caffarri, 2000; Horton *et al.*, 2000). Furthermore, experiments on leaves confirmed the existence of significant quenching in the absence of zeaxanthin and highlighted not only the fact that zeaxanthin controlled the amplitude of qE but also its kinetics, accelerating qE formation and decelerating qE relaxation (Ruban and Horton, 1999). This observations have been used to formulate another feature of NPQ: the role of the xanthophyll cycle in controlling the *memory of illumination* in the photosynthetic membrane (Ruban and Horton, 1999; Ruban *et al.*, 2011; Ruban, 2012). Indeed, plants seem to react to light exposure—intensity, duration—by converting violaxanthin into zeaxanthin. The latter makes the LHCII antenna respond more quickly and efficiently to subsequent high light exposure. Moreover, when intense illumination stops, the antenna is reluctant to switch off NPQ as if it anticipates further periods of high light.

Another important factor, PsbS protein, was discovered by Niyogi and colleagues to actually enable qE, a rapidly forming and relaxing NPQ (Li *et al.*, 2000). Mutants lacking PsbS could form NPQ only very slowly. Initially this was considered as qI, a photoinhibition-related component of NPQ. Later it was found to be related to photoprotection (Johnson and Ruban, 2010) and it was concluded that without PsbS qE forms very slowly and therefore PsbS plays a role of a catalyst, a pH sensor for the quenching that occurs in the LHCII antenna (for review see Ruban *et al.*, 2012). Most importantly, plants lacking PsbS exhibited qE when they formed a large proton gradient enhanced by diaminodurene (DAD) (Johnson and Ruban, 2011). This observation reaffirmed the proton-sensing and catalytic roles of the PsbS protein in qE. Interestingly unlike zeaxanthin, which strongly decelerated qE recovery in the dark, the PsbS protein has an opposite effect: it accelerates this recovery (Crouchman *et al.*, 2006). Therefore, the sites in the LHCII antenna for zeaxanthin and PsbS action upon qE must be different. Indeed, while the xanthophyll-cycle carotenoids are intrinsically bound to LHCII trimers and monomers (Ruban *et al.*, 1999; Liu *et al.*, 2004) the PsbS protein seems to be localized around the LHCII complexes but obviously is a rather extrinsic factor (Bergantino *et al.*, 2003; Teardo *et al.*, 2007). It was observed that the absence of the protein increased the rigidity of the grana membrane and its resistance to detergent solubilization. PsbS was found to accelerate the grana stacking process induced by magnesium cations, suggesting that it may play a role as an enhancer of thylakoid membrane dynamics (Kiss *et al.*, 2008). Electron microscopy revealed an increase in the percentage of PSII units assembled into semicrystalline arrays in grana membranes lacking PsbS and fluorescence recovery after photobleaching indicated a reduction in the mobility of chlorophyll–protein complexes in grana (Goral *et al.*, 2012).

At the same time several new lines of evidence have emerged that provide further evidence for the change in PSII and LHCII organization brought about by ΔpH formation and NPQ. Firstly, Holzwarth's group provided spectroscopic data suggesting that upon formation of qE part of the major LHCII undergoes separation from the PSII supercomplex (Miloslavina *et al.*, 2008; Holzwarth *et al.*, 2009). This conclusion was based on the appearance of a new red-shifted emitting band in decay-associated fluorescence spectra in the qE state that was suggested to arise from LHCII aggregates. In addition, Bassi and colleagues obtained biochemical evidence suggesting that PsbS controlled the dissociation of a part of the PSII–LHCII supercomplex containing trimeric and monomeric LHCII under NPQ conditions (Betterle *et al.*, 2009). They found that the distance between PSII core complexes decreased under NPQ conditions in detergent solubilized grana membranes, providing further evidence that NPQ involved a reorganization of the PSII antenna. Finally my group obtained further structural information based upon freeze-fracture electron microscopy of intact chloroplast membranes displaying reversible clustering of PSII core units as well as LHCII antenna aggregation upon formation of qE (Johnson *et al.*, 2011). Importantly our findings support the original LHCII aggregation model by demonstrating that ΔpH and de-epoxidation of violaxanthin to zeaxanthin cooperatively drive LHCII aggregation (Fig. 6). Moreover the data confirm the common nature of qE and zeaxanthin-dependent qI as manifestations of the same LHCII aggregation phenomenon. Crucially the observed structural alterations induced by illumination occur on a timescale consistent with the formation and relaxation of qE (Horton *et al.*, 1996). These data therefore provide the first direct link between the structural change in the PSII antenna and qE in intact, unsolubilized thylakoid membranes. The model presented in Fig. 6 summarizes all the structural evidence regarding the change in PSII–LHCII macro-organization underlying qE. In the quenched state the LHCII complexes are reorganized around the RCII complexes in a more tightly oligomeric arrangement, leading to the aggregation. Our recent work provided evidence that this oligomeric LHCII is not energetically uncoupled from RCII (Belgio *et al.*, 2014). Unfortunately, current experimental resolution does not permit the exact location of PsbS to be known with certainty. It is also not known whether all complexes, only one, or a few in the locus are in the quenched state. However, together with the slow nature of the NPQ quencher proposed by my group (Belgio *et al.*, 2014) and calculations preformed on the energy transfer dynamics related to one of the proposed quenchers, lutein, in LHCII (Duffy *et al.*, 2013*b*), it is likely that the majority of LHCII complexes are in a quenched conformation.

The collective nature of the molecular events in PSII antenna leading to NPQ has evolved to ensure a prompt and substantial response of the photosynthetic membrane to sudden exposure to high light. This response possesses a *memory* element, is not wasteful (*economic*), and is subject to efficient regulation via acclimative adjustments in the amount of PsbS, LHCII, and xanthophyll-cycle carotenoids (for the most recent reviews see Jahns and Holzwarth, 2012; Niyogi and Truong, 2013; Ruban *et al.*, 2012; Zaks *et al.*, 2013).

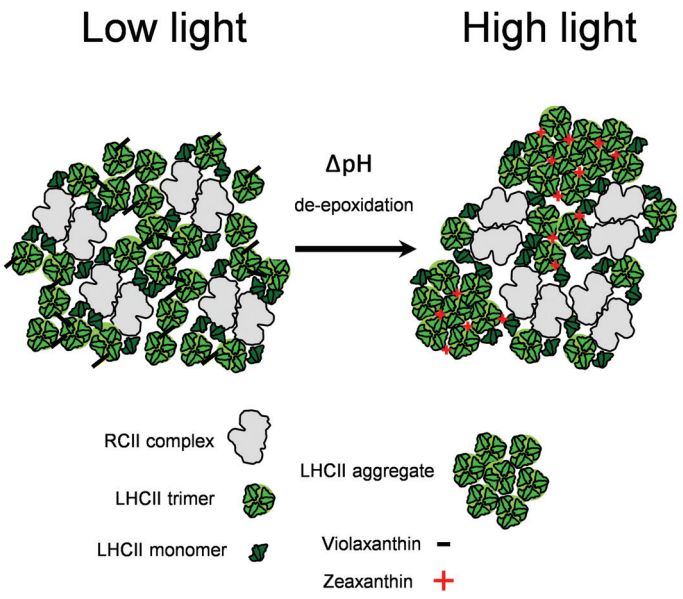

Fig. 6. A scheme illustrating PSII reorganization leading to LHCII aggregation and clustering of RCII complexes in the NPQ state induced by protonation and enhanced by the formation of zeaxanthin.

Effectiveness of NPQ and Darwinian fitness of plants

Despite our knowledge of the mechanistic elements and events that lead to NPQ, the efficacy of this process in protecting plants against high light is largely unknown. In other words the relationship between NPQ and the maximum light intensity that the photosynthetic apparatus can tolerate without being damaged is still to be unambiguously determined. Some early reports concerning isolated thylakoids even proposed that NPQ plays little or no role in the photoprotection of PSII (Santabarbara *et al.*, 2001). However, the common opinion on the subject is that the rapidly reversible NPQ component, qE, provides effective photoprotection (Jahns and Holzwarth, 2012; Ruban *et al.*, 2012; Niyogi and Truong, 2013). But how can this effectiveness can be assessed? Traditionally, the slowly reversible component of NPQ, qI, was assumed to result from the damage of RCII. However, qI was found to be highly heterogeneous and can be hard to resolve (Jahns and Holzwarth, 2012; Ruban *et al.*, 2012). It is believed that zeaxanthin, trapped protons, aggregated LHCII, and photodamage itself are all contributors to qI (Baker and Horton, 1987; Demmig-Adams, 1990; Ruban *et al.*, 2012). It seems that the temporal criterion for distinguishing between the photoprotective and photoinhibitory components of NPQ is often ambiguous and therefore other independent approaches are needed to verify the amount of protective NPQ. The process is clearly an effective adaptation to excessive light but the common occurrence of photoinhibition in nature shows that it may be limited in its protective power under some conditions in the same way as helio- and paraheliotropisms described by Darwin (see above).

Common measurements for photoinhibition include dark-adapted F_v/F_m, oxygen evolution, or D1 degradation. While these have been effective for assessing the threshold for damage these methods have drawbacks for physiological analyses especially where lab-based analysis is required (oxygen evolution and D1 turnover). In addition they require disruption of the light treatment, either by destructive sampling or imposition of a sustained dark period. The length of the dark period used for F_v/F_m measurements itself can lead to ambiguity. The required approach to this problem is a simple, rapid, and non-disrupting method that could test the *in vivo* photoprotective function of NPQ regardless of how quickly or slowly it recovers. My laboratory designed a pulse-amplitude-modulated (PAM) fluorescence analysis-based approach (tested on several plant species) that we believe could radically change our understanding of the NPQ process by quantifying its photoprotective potential in addition to the classic chlorophyll fluorescence induction analysis (Ruban and Murchie, 2012; Ruban and Belgio, 2014). The novel technique has a protocol similar to the one used in the measurements of light saturation curves of PSII fluorescence. Leaves of plants are exposed to periods of increasing actinic light intensity during which NPQ is monitored by applying saturating pulses and after which brief period of darkness are applied in order to assess the state of RCII (photochemical quenching). Figure 7 displays the typical course of this procedure. The level of F_o' fluorescence conditions when RCIIs are open after switching off actinic illumination is measured as well as calculated using the F_m' (closed RCIIs + NPQ) and initial F_o (all open RCIIs) levels (Oxborough and Baker, 1997). The light intensity at which the measured and calculated F_o' start to deviate (measured F_o' becomes higher than calculated, encircled on the Figure 7) is treated as critical or the highest tolerated

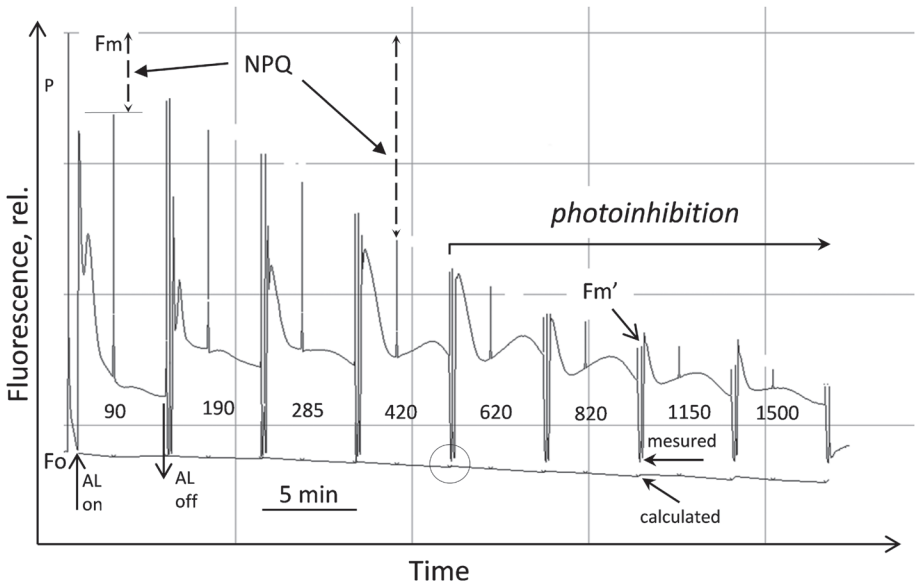

Fig. 7. Typical induction of chlorophyll fluorescence quenching in an *Arabidopsis* leaf using eight stepwise increasing actinic light levels of 90, 190, 285, 420, 620, 820, 1150, and 1500 μmol·m^{-2}·s^{-1}. Vertical solid arrows on the left of the figure indicate application (AL on) and removal (AL off) of the actinic light treatment. P is saturating pulse applied in order to close all RCII either when they are all open (F_m) or partially closed by application of AL (F_m'). Short arrows on the right of the figure indicate levels of calculated and actual F_o, fluorescence in the dark when all RCII are open. The AL intensity at which the two levels become different indicates the start of the onset of photoinhibition (thick horizontal arrow). NPQ rises with the AL intensity as indicated by the two vertical dashed arrows.

intensity and the corresponding level of NPQ is treated as the maximum protective NPQ. That point of the measurement indicates the onset of photoinhibition and the level of qP, photochemical fluorescence quenching, measured in the dark starts to decline below 1. This is because of a fraction of RCII becoming permanently closed and therefore no longer contributing to qP, which is termed qP_d. The described routine allows gradual development of the maximum attainable level of NPQ (Fig. 7, indicated by dashed vertical arrows) for each applied light intensity while avoiding the early onset of photoinhibition and allowing for the relation of this intensity to NPQ to qP_d. Measurements on *Arabidopsis* plants possessing different levels of NPQ revealed the relationship between protective NPQ and the actinic light intensity used in this routine. Figure 8A shows that plants possessing NPQ of 1 can tolerate a light intensity of approximately 400 μmol·m^{-2}·s^{-1} and that this relationship is approximately linear (Ruban and Belgio, 2014). The gradient of this line will depend first of all upon the LHCII antenna size and photostability of RCII itself.

And now we return to *H. helix* from Fig. 1G, a climber turned to a crawling plant that grows in such a dark forest that it simply cannot live on extremely tall trees since it cannot catch enough light to grow all the way to the top of the canopy to reach brighter areas. Instead it crawls and covers the whole forest floor in order to intercept flecks of bright light, spending much less energy in comparison to that involved in climbing. But is it prepared to be exposed to bright light after

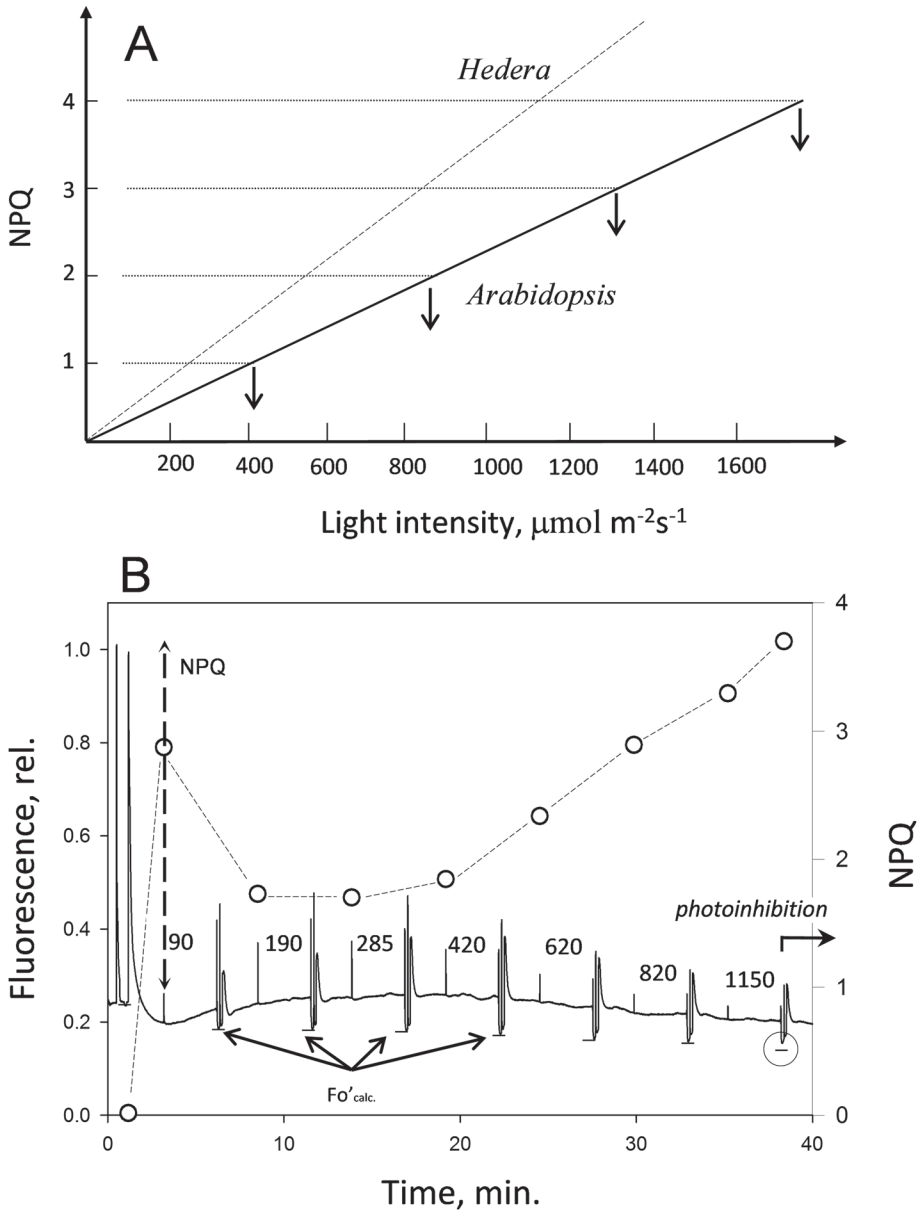

Fig. 8. (A) The relationship between the minimum level of NPQ and the light intensity at which it can protect all PSII reaction centres against photoinhibition. Solid line, *Arabidopsis*; dashed line, ivy (*H. helix*). (B) Fluorescence induction obtained using the routine described in the Fig. 7 for *H. helix* leaf. A remarkably large NPQ (open symbols) was induced by relatively low actinic light (AL) intensity (90 μmol·m^{-2}·s^{-1}) during the first 2 min of illumination. Owing to a fast NPQ formation with a large amplitude (up to 4) *Hedera* is well protected against sudden intense illumination (like sunfleck). Photoinhibition is only triggered by the AL intensity above 1000 μmol·m^{-2}·s^{-1} (compare with *Arabidopsis* fluorescence induction shown on the Fig. 7).

being acclimated to deep shade (only a few $\mu mol \cdot m^{-2} \cdot s^{-1}$)? Figure 8 shows the new NPQ induction routine performed on *Hedera*. Interestingly, after 2.5 min of only 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$ exposure the plant developed a NPQ of nearly 3 in comparison to 0.15 for *Arabidopsis* leaves exposed to the same light level for the same amount of time (Fig. 7)! Such a dramatic response to even moderate or rather low light intensities in *Hedera* means that it is well prepared to defend its RCIIs against light. Further, NPQ apparently dropped because the first burst of NPQ up to 3 over-downregulated the yield and electron transport, causing a decrease in ΔpH; hence there was a decline in NPQ. Only when light intensity was increased to 820 $\mu mol \cdot m^{-2} \cdot s^{-1}$ did NPQ go back to 3 and increase further. As a result, the onset of photoinhibition started at the light intensity of 1150 $\mu mol \cdot m^{-2} \cdot s^{-1}$, almost three times higher than that required to initiate photoinhibition in *Arabidopsis* grown under 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light. Therefore the evolution of the LHCII antenna in *Hedera* resulted in the development of exceptionally high NPQ that could be triggered by relatively low light. This suggests that the sensitivity of the LHCII/PsbS protein and violaxanthin de-epoxidase could be much higher than in *Arabidopsis*. Interestingly, the gradient of the relationship between NPQ and light intensity is higher in *Hedera* in comparison to *Arabidopsis* (Fig. 8A, dashed line). This is most likely the result of a significant acclimative increase in LHCII antenna size as shown by an abnormally low chlorophyll *a*/*b* ratio (was mentioned above). Increased antenna size would cause higher excitation pressure so that RCII of *Hedera* would require larger NPQ in comparison to plants that possess smaller antennae and endure less excitation pressure and will have less detrimental excess energy when illuminated by bright light. Therefore *H. helix* seems to be a champion plant that evolved its light-harvesting antenna to deal with both extremely low light as well as very high light spanning from only a few $\mu mol \cdot m^{-2} \cdot s^{-1}$ to almost 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. This is an exceptional example of light adaptation that makes *H. helix* one of a very few species that can live in extremely dark forests when other plants simply cannot survive (Fig. 1C).

An example of the importance of NPQ in plant fitness was also reported for *Arabidopsis* mutants lacking zeaxanthin or the PsbS protein (Külheim *et al.*, 2002). Interestingly, plants grown in laboratory conditions did not reveal significant differences in growth rate and seed production, parameters that are normally considered as contributing to Darwinian fitness (Demetrius and Ziehe, 2007). However, the mutants that grew outdoors and experienced frequent fluctuations in light intensity produced 30–50% fewer seeds than the wild type. The difference in seed output was explained by the fact that mutants produced fewer fruits and fewer seeds per fruit compared with the wild type. Thus, the fitness of plants lacking NPQ was greatly reduced under field conditions. The authors argued that the major cause of the poorer fitness of mutants was a larger extent of photoinhibition measured as a sustained decline in the PSII yield (Külheim *et al.*, 2002). They concluded that it is because the studied plants differed in the amplitude of the protective, rapidly relaxing qE component of NPQ that they displayed differences in fitness and therefore that qE confers an adaptive advantage because it provides fast tracking protection against exposure to high light. Rapid irregular fluctuations in light caused variations in excitation pressure in the field that are potentially detrimental to the photosynthetic apparatus and, owing to the rapid onset of energy dissipation, qE, plants are unable to quickly damp this excess energy into heat and save RCIIs from photodamage.

Conclusions

What did we learn from the research on how plants evolved their photosynthetic apparatus to deal with the only source of energy for life: light? It is hard not to notice the striking fact that photosynthesis is an *inefficient* process for conversion of light energy in the biosphere. The latter is a *spatially closed* but *energetically open* system that carefully maintains source/resource balance and perfectly *recycles biological matter*, a property that we must develop if we wish to survive in the future. Can we actually improve photosynthetic efficiency in order to enhance crop yields? Can we make plants better prepared for the coming global climate changes? Can we learn better ways of producing fuels or electricity from the photosynthetic energy-conversion processes? I think the only realistic way of answering these questions is to address them as intricately related issues. Indeed, what we should learn is how to produce energy without producing pollutants that damage the environment and cause changes in the global climate. We need to find ways to reduce the concentration of CO_2 in the atmosphere, make hydrogen molecules from water, and utilize our knowledge of the principles of highly efficient photon energy capture and trapping by the reaction centres—the primary processes of photosynthesis—in technical designs of photovoltaic devices. In addition, we need to assess the feasibility of improving plant resistance to various abiotic factors—light, in particular—and in combination with temperature and water stresses. This can be achieved by designing new technologies for assessing of plant tolerance to low, high, and fluctuating light intensity and colour. In addition, one must try to understand whether the electron turnover rate around the PSII reaction centre and the other limiting stages of the light phase of photosynthesis can be regulated and manipulated in order to achieve higher productivity of electron and proton transports. It took a few billion years for evolution to achieve photosynthesis as we know it today and it will take some time for us to explore it, use its principles, or improve it. A plethora of plant species that evolved to cope with the extraordinarily variable light environment of our planet can be a rich source for explorations by plant physiologists, biochemists, biophysicists, and molecular biologists in order to reveal key genetic, biochemical, and structural factors in the photosynthetic apparatus that enables Darwinian fitness.

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