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Electron Transfer Routes in Oxygenic Photosynthesis: Regulatory Mechanisms and New Perspectives

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1. Introduction

In nature, the intensity of light changes rapidly, spanning from 2000 µmol $_{PHOTONS}$ m^{-2} s^{-1} during the brightest sunlight, down to 200-500 µmol $_{PHOTONS}$ m^{-2} s^{-1}, when shaded by clouds, followed by the period of dark, to encircle with the next sunrise of barely 10 µmol $_{PHOTONS}$ m^{-2} s^{-1}. Plants, capable of performing limited movements, like leaf and chloroplast movements, respectively, have been forced to evolve different means of coping with the unpredictable nature. To better understand the acclimatization mechanisms, the intensity of light has been roughly divided into low-light and high-light conditions, shaped by the laboratory conditions of plant growth and the strength of the measuring apparatus available. Moreover, looking at the time scale, it is possible to differentiate between short-term and long-term acclimatization processes in plants. These artificial divisions are needed to simplify the most important process in photo-synthesis in vascular plants: the rate and the distribution of excitation energy between two photosystems. Photosystem units are organized into large supercomplexes with peripherally attached antenna complexes, being further assembled into megacomplexes [1]. Two types of peripheral antenna proteins associated to photosystem II (PSII) are known: the major Light-Harvesting Complex II (LHCII), which occurs as a trimeric complex containing the proteins Lhcb1, Lhcb2 and Lhcb3, and three minor monomeric complexes, namely Lhcb4 (CP29), Lhcb5 (CP26), and Lhcb6 (CP24). These peripheral complexes bind variable number of molecules of chlorophyll *a* and *b*, and of some xanthophyll molecules (for physicochemical properties of chlorophylls see the Chapter Kobayashi et al.). In Arabidopsis PSII-LHCII supercomplexes, Lhcb are organized into two rings around the dimeric PSII core complexes, with Lhcb1, Lhcb2, Lhcb4 and Lhcb5 detected in the inner ring, where Lhcb1 and Lhcb2 participate in strongly bound LHCII trimer, while the outer ring consists of Lhcb6 and of moderately bound LHCII trimer (the Lhcb1 and Lhcb3 gene products). Photosystem I (PSI) is associated with the Light-

Harvesting Complex I (LHCI) that binds 10 molecules of chlorophyll *a* or chlorophyll *b* plus a few xanthophylls per one Lhca protein. In green plants, LHCI consists of four polypeptides (Lhca1-Lhca4) from the Lhc protein superfamily. In Arabidopsis, two additional proteins have been identified (Lhca5 and Lhca6), but their contribution to LHCI is still under debate [1]. More distantly related family members are the photoprotective early light-induced stress response proteins (ELIPS), and the component of PSII, PsbS [2].

2. The way the plant protects itself: Non-photochemical quenching

The non-photochemical quenching (NPQ) is a short-term response by which plants harmlessly dissipate excess excitation energy into heat under high-light conditions. NPQ is observed in all higher plants, in lower plants, green algae and diatoms [3]. NPQ is also present in oceanic picophytoplankton species (see Chapter Kulk et al.). Basically, during the absorption of sunlight by light-harvesting complexes (LHCs) associated with reaction centres, a chlorophyll *a* molecule shifts from its ground energetic state to its singlet excited state. It can return to its ground state *via* one of several pathways: re-emission of excitation energy in the form of chlorophyll fluorescence; transfer of excitation energy to reaction centres to be utilised in photochemistry reactions; de-excitation by dissipating heat (NPQ); production of triplet excited state, which would be a highly profitable valve for excess excitation, but it indirectly produces a very reactive oxygen species (ROS), singlet oxygen, by transferring energy to the ground-state oxygen [4]. In addition to the dissipation of excitation energy, non-photochemical processes also quench or diminish chlorophyll *a* fluorescence, therefore being mainly observed at PSII. The phenomenon of quenching of chlorophyll *a* fluorescence is usually analysed in terms of three components, based on their relaxation kinetics: state-transitions (qT), ΔpH-dependent quenching (qE) and photoinhibition (qI). The majority of NPQ is believed to occur through qE in the PSII antenna pigments bound to the LHCII proteins [5, 6]. State-transitions are considered to be the component of NPQ because the fluorescence yield of PSII diminishes due to the lateral redistribution of the phosphorylated LHCII proteins and their attachment to PSI [5]. Photoinhibition exibits the slowest relaxation and it is the least defined. qI quenching is proposed to be involved in long-term down-regulation of PSII [4, 7].

2.1. ΔpH-dependent quenching

The process of qE is triggered by acidification of the thylakoid lumen under light-saturating conditions, which activates the interconversion of specific xanthophyll pigments (oxygenated carotenoids), that are mostly bound to the LHC proteins. For comparison, in cyanobacteria, strong blue-green or white light activates the orange carotenoid protein (OCP) which interacts with phycobilisome and dissipates the excess energy in the form of heat [8]. The xanthophyll cycle in plants, green and brown algae consists of the pH-dependent conversion from viola-xanthin first to antheraxanthin and then to zeaxanthin. In plants, the reactions towards zeaxanthin are catalysed by the enzyme violaxanthin de-epoxidase, while the relatively slow reactions towards violaxanthin are catalysed by the enzyme zeaxanthin epoxidase [4]. The *npq1* mutants are unable to convert violaxanthin to zeaxanthin but still exhibit qE, demon-

strating that the xanthophyll cycle is not the prerequisite for qE formation [9]. Zeaxanthin was demonstrated to have an additional photoprotective function not connected with NPQ, but rather with thylakoid membrane lipids. It is hypothesised that the function of the non-protein bound zeaxanthin could be the removal of highly deleterious singlet oxygen, working together with the well-known antioxidant tocopherol [10]. The *npq4* mutant, which completely lacks the PsbS protein and qE, can survive under high-light conditions, thus implying that carotenoids may compensate to some extent the deficiency in qE formation [9].

Due to the slower kinetics of formation and relaxation of qE compared to the proton gradient, and taking into account that the light causes changes in charge distribution, which consequently alter the aggregation state of thylakoids, it was proposed that such a conformational change might accompany the qE event [6, 11]. Indeed, reports from at least three independent laboratories confirmed the structural rearrangement of the PSII-LHCII macro-organization during qE [12-14]. Time-correlated single photon counting (TCSPC) measurements revealed an additional far-red fluorescence component in the leaves of the high-light-adapted wild-type, the mutant unable to accumulate zeaxanthin at high-light (*npq1*), and in the mutant overexpressing PsbS, the protein proposed to act as a luminal pH sensor that consequently determines the level of qE [12, 15], respectively. The same component was not observed in the mutant devoid of PsbS, *npq4*, or in the dark-adapted state of abovementioned plant lines [12]. It was concluded that this fluorescence originated from the major LHCII antenna complex detached specifically from PSII, with the required presence of the PsbS protein [12]. Furthermore, it was biochemically demonstrated that the supramolecular complex B4C (Lhcb4, Lhcb6, and moderately bound LHC trimer) dissociates during light exposure, and associates back during dark period [13]. However, *npq4* mutants did not show light-induced B4C dissociation, establishing the role of PsbS as the key player in thylakoid rearrangements [13]. Finally, Ruban group used freeze-fracture electron microscopy to demonstrate that the formation of qE was indeed associated with the reorganisation of PSII and LHCII within the thylakoids [14]. Their experimental data support and update the 20-years-old hypothetical model built to explain the mechanistics of qE, the LHCII aggregation model [16]. According to the model, LHCII antenna could be found in four different states: (i) dark-adapted, unquenched; (ii) dark-adapted, partially aggregated, quenched; (iii) illuminated, partially aggregated, quenched; and (iv) illuminated, aggregated, fully quenched. Illumination causes two events necessary for LHCII aggregation to occur: conversion of violaxanthin to zeaxanthin and the protonation of LHCII, respectively [6]. Precisely, the formation of ΔpH triggers a conformational change within the LHCII antenna, which leads to the partial dissociation of LHCII trimers from the PSII-LHCII supercomplexes, and, consequently, to their aggregation (Figure 1). In parallel, de-epoxidation of violaxanthin to zeaxanthin promotes the LHCII aggregation and the formation of NPQ [14]. The question for debate would be the number and the exact position of quenched complexes. According to Holzwarth group, two quenching centres are formed: detached and aggregated LHCII antenna measured within 1 to 5 minutes, and the minor components of LHCII; still attached to the PSII core, measured within 10 to 15 minutes [12]. As PSII core does not bind any zeaxanthin molecules, it is excluded as a quenching site; however, it is well-covered by formation of the second quenching centre.
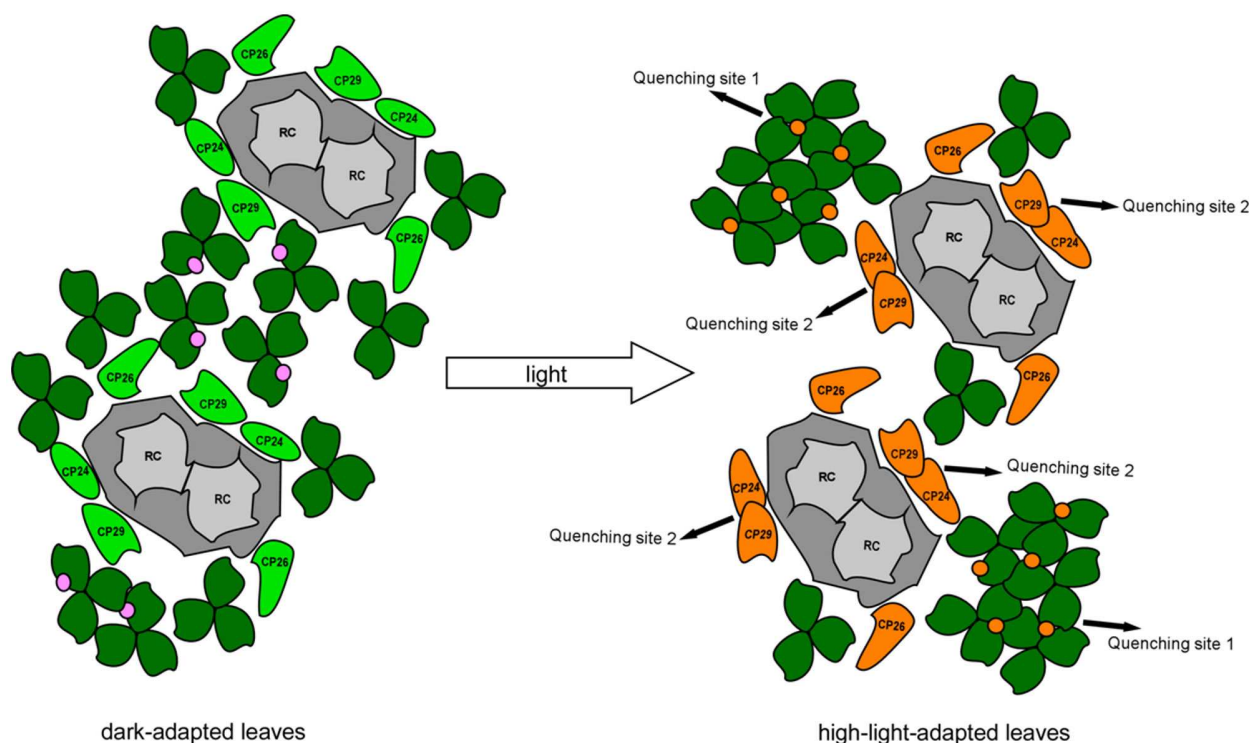
Figure 1. Structural rearrangement of the PSII-LHCII macro-organization during qE. Composition and arrangement of the LHCII trimers (dark green) and the minor antenna (light green) around the PSII RCs (light grey) in the wild-type plant during the dark period (left panel) and under the high-light conditions (right panel) were presented according to [1]. Pink circles on the left panel denote violaxanthin, while the orange circles on the right panel denote the process of conversion of violaxanthin to zeaxanthin. The change of colour of the minor antenna from light green (left panel) to orange (right panel), also denotes the conversion of violaxanthin to zeaxanthin, respectively. According to [14], in excess light, ΔpH triggers a conformational change within the LHCII antenna, which leads to the partial dissociation of LHCII trimers from the PSII LHCII supercomplexes, and, consequently, to their aggregation. In parallel, de-epoxidation of violaxanthin to zeaxanthin promotes the LHCII aggregation and the formation of NPQ (quenching site 1). Moreover, according to [12], quenching site 2 is also formed within the minor antenna still attached to the PSII.

Acidification of the thylakoid lumen also causes a conformational change in thylakoids that can be monitored at 535 nm (ΔA_{535}). This change is most likely induced by protonation of the lumen-exposed carboxylate side chains in specific PSII proteins [15]. Interestingly, *npq4* mutants, which do not express the PSII PsbS protein, also lack ΔA_{535} [17]. It was proposed that PsbS is not necessary for efficient light harvesting and photosynthesis, but it is involved in NPQ by sensing luminal pH and consequently determining the level of qE [15]. Recently, it became clear that PsbS is indispensable for the physical state of the thylakoid membranes. In dark-adapted *npq4* mutants, the formation of ordered semi-crystalline arrays of PSII was increased, while in the plants over-expressing PsbS no arrays could be found, suggesting that PsbS disrupts the ordering and promotes the protein diffusion within the membrane, leading to the NPQ formation [18].

Although the main components for qE are known, the biophysical mechanism of de-excitation of the excited molecules of chlorophyll *a* is still unidentified. It is hypothesised that either xanthophylls act indirectly as allosteric regulators of the LHCs, causing the conformational change that facilitates the de-excitation, or xanthophylls directly de-excite

the excited molecules of chlorophyll *a* [4]. Models presented in Ruban and Holzwarth groups both permit the formation of internal dissipative pigment interactions, whether they occur between chlorophyll molecules or between carotenoid and chlorophyll molecules [19].

Variations in NPQ capacities and processes in different organisms suggest a strong evolutionary pressure to obtain optimized photoprotection [3]. For example, the LHCII proteins may have evolved from ancestors of contemporary stress-responsive proteins such as HLIP and ELIPS, which probably bind only carotenoids and are involved in photoprotection, while LHCII harvest light through high amounts of chlorophyll binding. In plants, PsbS binds pigments minimally and it is primarily involved in photoprotection, while the light-harvesting complex containing fucoxanthin (LHCF) in diatoms binds both chlorophyll and carotenoids in high amounts and contributes equally to the light harvesting and photoprotection. Under high-light conditions, the photosynthetic reaction centres can not accommodate all the electrons coming through electron transport chain, thus entering into the saturated, "closed" status. The already generated excitation energy becomes the burden for the photosynthetic membranes and has to be channeled safely, before destroying the reaction centres, especially the pigment core of PSII, the pair of the most potent oxidizers known to exist in nature, P680. If the vast energy is not diverted, P680 might rest in its prolonged oxidized state, $P680^+$, and oxidize the neighbour protein amino-acids and pigments, ultimately leading to the destruction of the PSII protein D1. However, if P680 can not submit the electron to the oxidized plastoquinone, due to the increased number of already reduced plastoqinones, P680 could go into triplet state, interacting with atmospheric triplet oxygen and producing deleterious singlet oxygen. Both of these processes lead to the photoinhibition, the state in which the decreasing of the electron transport is observed [6]. Photoinhibition has a specific signature that can be monitored by a lower oxygen production, analysis of the D1 protein level, formation of uncoupled chlorophyll and of triplet state of chlorophyll. However, photoinhibition provokes the formation of photoprotection processes, where most of the unwanted energy would be harmlessly dissipated as heat. This phenomenon leaves a palpable trace visible as a drop in the fluorescence intensity, measured by TCSPC. One of the approaches to study the origin of the quenching mechanism *in vivo* is the treatment of Arabidopsis with lincomycin, which blocks the synthesis of chloroplast-encoded proteins, such as the reaction centres of PSI and PSII (RCI and RCII, respectively). The thylakoids of treated plants contain diminished amounts of RCs, but are rich in antenna complexes. Although the maximum chlorophyll fluorescence lifetime in isolated PSII-LHCII supercomplex is 4 ns; when complex is an integral part of the thylakoid membranes, fluorescence lifetime decreases to 2 ns [20]. If RCIIs, when saturated, contribute to the quenching of excitation of LHCII antenna, in the system almost devoid of RCIIs, the fluorescence lifetimes should be higher than 2 ns. However, spectroscopic measurements of the long-term lincomycin-treated plants did not differ from the control measurements, *i.e.*, the fluorescence lifetime was still 2 ns, suggesting that the LHCII antenna, and not the closed RCs, are sufficient for the quenching to occur [20].

2.2. How does the plant repair the damaged D1 protein?

The D1 repair cycle is regulated *via* reversible protein phosphorylation in thylakoid membranes. When plants are exposed to the high-light stress, the PSII protein subunits become heavily phosphorylated and migrate from grana to stroma thylakoids, this process being facilitated by the actions of STN8 [21]. The migration is accompanied by sequential dephosphorylation of the CP43, D2 and D1 proteins, respectively. Turnover of the D1 protein includes degradation of the photo-damaged polypeptide and co-translational insertion of the newly synthesized protein [22]. The current model envisions that the D1 protein is proteolytically processed at both sides of the thylakoid membrane: from the N-terminal end on the stromal side by the FtsH, a member of the ATPases associated with various cellular activities-subfamily (AAA subfamily), and, on the lumen site by Deg, a member of serine proteases family that does not require ATP [23]. Recently, it was confirmed that FtsH and Deg act in a cooperative manner to efficiently cut the D1 protein under photoinhibitory conditions [24]. According to the D1 digestion model, under all light intensities D1 is processed by the FtsH complexes, to be additionally supported by the Deg proteases under photoinhibitory conditions, in the so-called escape pathway [24]. The requirement of D1 dephosphorylation prior to its proteolysis could be explained by low affinity of phosphorylated N-terminal end of D1 towards the FtsH [23].

3. Plastoquinone pool-inflicted plant responses

The most promising redox-active components are the pool of plastoquinone (PQ) and the PSI acceptor site (*e.g.* NADPH, thioredoxin, glutathione and glutaredoxin). The PQ pool regulates two temporally distant responses that acclimate the photosynthetic process to the prevailing environment: state-transitions that occur in the order of minutes (short-term response) [25, 26, 27], and photosystem stoichiometry adjustment that requires hours to days (long-term response) [28]. Both responses occur under low-light conditions, in contrast to the high-light provoked responses such as NPQ, D1 repair cycle or various stress-response programmes [29].

3.1. State-transitions

State-transitions re-distribute excitation energy between two photosystems, which are electrochemically connected in series, through the supramolecular reorganisation of the photosynthetic membranes. The molecular complexes that cause the reorganisation are light-harvesting proteins, which collect light excitation and channel it to the reaction centres. Already mentioned LHCII is the prototype of large and abundant class of chloroplast trans-membrane proteins that binds roughly half of the total chlorophyll in chloroplasts [2]. The absorption spectra of LHCI is in the far red region owing to the chlorophyll *a* enrichment, while LHCII is enriched in chlorophyll *b* and has the maximum absorption at shorter wavelengths in the red region, around 650 nm [26]. Under PQ reducing conditions, *i.e.* when PSII is

predominantly excited compared to PSI, a redox-sensitive protein kinase acts to phosphorylate the apoproteins of LHCII. Upon phosphorylation, LHCII partially dissociates from PSII and associates with PSI (state I). Under PQ oxidizing conditions, *i.e.* when PSI is predominantly excited compared to PSII, the kinase is inactive, but an activated phosphatase dephosphory-lates the mobile LHCII, which moves laterally, and associates with PSII (state II) [29]. Although state-transitions occur only under low-light conditions, they share the same mechanism of LHCII aggregation with the high-light response mechanism, at least in *Chlamydomonas reinhardtii* [30]. This opens an intriguing question of plant capacity to wisely use the same mechanism for different living conditions. In 2003, Jean-David Rochaix group proposed that the thylakoid-associated serine-threonine Stt7 kinase from the alga *C. reinhardtii* is involved in phosphorylation of LHCII and in state-transitions [31]. In 2005, the same group proposed that the homolog of the Stt7 in Arabidopsis, STN7, is involved in state-transitions [32]. Since LHCII phosphorylation is reversible process, an extensive search has been conducted to identify the protein phosphatase(s) that dephosphorylates LHCII. Recently, two groups described the product of the nuclear gene At4g27800 as a long-sought plastid protein phosphatase specifi-cally involved in dephosphorylation of the mobile pool of major LHCII proteins, titled PPH1 [33], and TAP38 [34], respectively. The specificity of PPH1/TAP38 for LHCII supports the hypothesis that several phosphatases must be involved in dephosphorylation of thylakoid phosphoproteins [33, 34]. Recently, a new phosphatase PBCP, capable of *in vivo* dephospory-lation of PSII core subunits CP43, D1, D2 and PsbH, respectively, was identified [35]. It seems that the PBCP phosphatase targets at proteins phosphorylated by the protein kinase STN8 [36], thus forming a pair with opposing effects on phosphorylation of the photosynthetic core proteins [35].

3.2. Stoichiometry adjustments

Photosystem stoichiometry adjustment is a long-term response that affects the relative amounts of the two photosystems by changing the expression of photosynthetic genes both in the chloroplast and in the nucleus [29]. It was shown that the redox state of the PQ pool serves as a major signal in the regulation of LHCII photo-acclimation [37]. In order to avoid excess excitation energy and to minimize oxidative damage, the LHCII protein level decreased by approximately 50% in *Lemna persusilla* upon transfer from low-light to high-light conditions [37]. In contrast to the state-transitions, where active redox-regulated thylakoid-associated STN7 kinase phosphorylates LHCII, thus leading to LHCII detachment from PSII and migra-tion towards stroma thylakoids and PSI, under high-light STN7 is inactive, hence the rate of the phosphorylated LHCII decreases and the excess LHCII undergoes proteolytic degradation. The same stands for the PSI and PSII genes: upon reduction of the PQ pool, the expression of the PSI genes is favoured, while upon oxidation of the PQ pool, the expression of the PSII is favoured [29]. In 2005, the coupling of the long-term response of adjusting photosystem stoichiometry and the short-term response of state-transitions by LHCII kinase STN7 was proposed [36].

4. Immunophilins in photosynthesis

Immunophilins comprise a superfamily of conserved ubiquitous proteins consisting of two distinct subfamilies; cyclophilins and FKBPs (FK506/rapamycin-binding proteins), the targets of the immunosuppressive drugs cyclosporine A (CsA) and FK506/rapamycin, respectively [38]. Despite the lack of structural similarity, all cyclophilins and FKBPs share a common enzymatic, so-called PPIase or rotamase activity, catalizing *cis-trans* isomerization of prolinei-midic peptide bonds [39, 40]; (Rotation around peptide bonds is energetically disfavored due to their partial double-bond character. Delocalization of amide nitrogen electrons results in aprox. 22 kcal/mol energy barrier to rotation and restrains the peptide bond in either *cis* or *trans* configurations). A more recently discovered third group of proteins (parvulins), which is insensitive to immunosuppressive drugs, also possesses PPIase activity [41]. The molecular masses of cyclophilins, FKBPs, and parvulins range normally in the order of 18-21 kDa, 12-13 kDa, and 10-13 kDa, respectively, but several complex immunophilins of higher molecular weight have been detected recently [42, 43, 41]. The biological significance of this group of enzymes is a matter of intense research. Acceleration of protein folding processes by PPIase *in vitro* [44] and *in vivo* [45] has supported a physiological role as folding catalysts facilitating the slow and generally rate-limiting uncatalysed isomerisation around Xaa-Pro peptide bonds. Immunophilins can also perform chaperone functions [46], or cooperate with other chaperone proteins [47, 48, 49].

Complex immunophilins all contain additional protein-protein interaction domains, such as leucine-zipper motifs and/or tetratricopeptide repeat domains. They may be constituents of supramolecular structures, as shown for the human or avian steroid receptor complex [48] or chaperone supercomplexes [50], and may be involved in hsp90-dependent signal transduction [49, 51, 52]. Various members of the immunophilins are involved in phosphorylation processes *via* transient interaction with kinases [53, 51, 52] and phosphatases [54, 55]. It seems likely that, at least for the components of MAP kinase signaling system (Src, Raf, and Mek), hsp90-immunophilin interactions are essential for the kinase regulation [see 52]. The immunophilin FKBP65 together with hsp90 forms a regulatory association with the serine/threonine kinase c-Raf-1 [51]. In mammals, the Ca^{2+}/calmodulin-dependent heterodimeric protein phosphatase calcineurin can bind immunophilins with complex immunosupresive drugs. This interaction inhibits the protein phosphatase activity, resulting in interruption of the signal-transduction cascades required for T-cell activation [see 54]. A unique immunophilin-related protein is the PP5 protein phosphatase. This enzyme is a major constituent of the glucocorticoid receptor-hsp90 complex, with properties of an FK506-binding immunophilin with low affinity FK506 binding activity [56].

Plants possess a substantial number of immunophilins which are localized in cytosol, chloro-plasts, nucleus, mitochondria, or associated with secretory pathways. The majority of the cyclophilins are single-domain proteins, 23 isoforms in Arabidopsis [57]. AtCYP20-3, At-CYP20-2, AtCYP26-2, AtCYP28 and AtCYP37 can be found in chloroplasts, mostly in thylakoid lumen. The multidomain cyclophilin isoforms possess unique domain arrangements, as exemplified by AtCYP38. AtCYP40 contains a C-terminal tetratricopeptide repeat module

(TPR). Four additional multidomain cyclophilins contain RNA interaction domains, which implicates their involvement in nuclear RNA processing machinery.

Plant FKBPs encompass also 23 members in Arabidopsis and this family is one of the largest FKBP family identified to date. These FKBPs can also be divided into single and multidomain isoforms, consisting of 16 and 7 members, respectively [58]. Interestingly, 11 single-domain FKBPs appear to be targeted to the thylakoid lumen. Thus, this chloroplast sub-compartment appears to have very important role in immunophilin function, or the processes taking place in lumen require specific activity of this protein family.

The dephosphorylation of D1, D2 and CP43 in spinach is catalysed by a cyclophilin-regulated PP2A-like protein phosphatase, which was found to be associated with, and regulated by, a cyclophilin-like protein, TLP40 [59]. TLP40 is proposed to suppress phosphatase activity, when bound to the lumen-exposed epitope of the protein phosphatase, but to induce its activity, when released to the lumen [59]. However, it remains to be seen if the Arabidopsis PP2C phosphatase PBCP [35] is also under the control of TLP40. Vener group demonstrated that D1 was not only vulnerable to light, but also to high temperature [60]. Raising the temperature from 22 °C to 42 °C resulted in a very rapid dephosphorylation of the D1, D2 and CP43 proteins and in release of TLP40 from membrane into the thylakoid lumen. These events are proposed to trigger an accelerated repair of photodamaged PSII and to initiate other heat-shock responses in chloroplasts.

Higher plant thylakoid lumen prolineisomerases [38], or complex immunophilins, are found to be regulated by light and are responsive to various forms of environmental stress [61]. The structure of TLP40 and its association with the thylakoid membrane system implicates diverse functions and involvement in the intracellular signaling networks [62]. Binding of thylakoid membrane associated phosphatase involved in dephosphorylation of PSII core proteins might occur *via* two putative phosphatase-binding modules on the N-terminal side of TLP40 [59, 60]. In 2002, Baena-González and Aro [63] and in 2005 Aro et al. [64] further suggested that damaged D1 repair cycle includes TLP40 ortholog *At*CYP38, which lacks peptidyl–prolyl *cis/trans* isomerase (PPIase) activity [65, 66, 67]. It was shown that *At*CYP38 is involved in the assembly of oxygen evolving complex (OEC) [68] and maintenance of PSII [69]. Most recently, the interaction of the E-loop of chlorophyll protein 47 (CP47) with *At*CYP38 was demonstrated [67]. This interaction is mediated through putative cyclophilin domain [67]. Further, *in vivo* role of *At*CYP38 has been investigated in *cyp*38 mutant Arabidopsis plants [68, 69, 70], suggesting its primary role in PSII biogenesis and repair.

5. Flow and partitioning of photosynthetic electrons

Photosynthetic apparatus has to be able to efficiently convert energy at low light and to avoid over-reduction and damage at excess light. This requires switching between different regulatory mechanisms which keep the cellular ATP pool almost at the constant level. Exposure of plants to higher light intensities than required for efficient photosynthesis results in saturation of photosynthetic electron transport (PET). The over-reduction of PET chain can lead to the

formation of reactive oxygen species and may irreversibly damage the photosystems, as well as the cells and the whole organism [71, 72, 73]. To counteract and reduce the photoinhibitory damage, plants have developed several short- and long-term regulation mechanisms which include processes that modulate the structure and function of antenna complexes, including NPQ, alternative electron transport pathways and movement of chloroplasts, leaves and whole organisms away from intense light [74, 75]. Dissipation of excess light energy to heat in the antenna or in the reaction centre of PSII also counteracts the photoinhibitory damage. Retrograde signaling transduces information on the metabolic state of the organelle and induces many activities in the cytosol, nucleus and mitochondria, inducing alterations in nuclear gene expression of organelle-targeted proteins [76].

5.1. Linear, cyclic and pseudo-cyclic electron transfer routes

Various photosynthetic electron transfer routes become turned on and off, according to the need of the plant to adapt to wide-ranging quantities and qualities of light. Three major electron transfer pathways known are linear, cyclic and pseudo-cyclic electron transfer (LEF, CEF and PCEF, respectively). All three pathways are necessary for poised and sustained synthesis of ATP and NADPH and their interplay enables the flexibility of photosynthesis in meeting different metabolic demands [77].

During non-cyclic electron transport or LEF, light drives the conversion of water to oxygen at the level of oxygen-evolving complex of PSII and $NADP^+$ to NADPH on the stromal side of thylakoid membranes. The PET chain consists of PSII, the Cyt*b6f* complex, PSI, and the free electron carriers plastoquinone (PQ) and plastocyanin (PC). Electron transport includes the two quinine binding sites and two cytochromes b6 (the 'Q cycle') that give two protons translocated for each electron transferred from PSII to PSI. Hidrogen ions are transferred across chloroplast membrane and accumulated on the luminal side of the thylakoids, where they drive ATP synthesis through a membrane ATPase. This way electron transport helps to establish a proton gradient that powers ATP production and also stores energy in the reduced coenzyme NADPH to power the Calvin-Benson cycle to produce sugar and other carbohydrates.

Arnon, who discovered photophosphorylation in isolated chloroplasts in 1954, demonstrated that there is also CEF in the thylakoids, driven solely by PSI. CEF is a light-driven flow of electrons through a photosynthetic reaction centre with the electrons being transferred from PSI to Cyt*b6f* complex *via* ferredoxin (Fd), with associated formation of proton gradient. PQ is reduced by Fd or NADPH *via* one or more enzymes collectively called PQ reductase, rather than by PSII, as in LEF. From hidroplastoquinone (PQH2), electrons return to PSI *via* the Cyt*b6f* complex. Four possible routes of CEF that may operate in parallel have been proposed so far: NAD(P)H dehydrogenase (NDH)-dependent route, Fd-dependent route, Nda2, a type 2 NAD(P)H:PQ oxidoreductase route and Cyt*b6f* complex and FNR route [recently reviewed by 78].

CEF around PSI occurs under conditions when acceptor limitation or ATP shortage results in a highly reduced PQ pool and contributes to the formation and maintenance of a pH gradient

across a membrane but does not produce NADPH. The pH gradient generated may drive the production of ATP (cyclic photophosphorylation) or may regulate photosynthesis. When more light is absorbed than can be used for assimilation, the increased ΔpH is a switch to dissipate excess of the light absorbed by chlorophyll molecules of PSII [79]. CEF is diminished when its components are completely reduced. Also, there is no CEF when its components are completely oxidized because there are no electrons to cycle [80]. In an attempt to avoid these two extreme situations, kinetics, post-translational modifications [25, 26, 81] and redox control of reaction-centre gene expression [82] are all employed in maintaining a poised PQ pool. In spite of these control mechanisms, over-reduction easily occurs when the Calvin–Benson cycle is unable to use NADPH, usually due to the lack of ATP. The major physiological significance of CET most probably lies in additional availability of ATP.

LEF in chloroplasts produces a number of reduced components associated with PSI that may subsequently participate in reactions that reduce oxygen. When Fd transfers electrons to molecular oxygen instead of $NADP^+$, PCEF linked with phosphorylation arises. O_2 is directly reduced to superoxide radical in so-called Mehler reaction. Subsequently, two superoxides dismutate to form H_2O_2 and O_2, which are by further redox processes converted to water. A reduced state of the FeS pool (Fd and PSI centres) promotes the Mehler reaction. The Mehler reaction leading to PCEF restores the redox poise when the PET chain is over-reduced, thereby allowing CEF to function and to generate ATP for the Calvin-Benson cycle, which will in turn oxidize NADPH and restore LEF [83]. Under high light PCEF could also cover an increased energy demand, as long as the antioxidant systems for H_2O_2 removal is sufficiently active (ascorbate and GSH recycling).

A great number of contemporary research topics on photosynthesis aims at elucidating novel, alternative electron transfer routes as the safest pathways for channelling of unwanted electrons. Chlororespiration is a well-known respiratory process that can maintain a trans-thylakoid proton gradient, thus acting as an effective alternative electron sink in preventing over-reduction of the PQ pool and protecting RCIIs from photodamage under photoinhibitory light conditions [84]. Two enzymes are important for chlororespiratory function: NADH dehydrogenase complex and nucleus-encoded plastid-localized terminal oxidase (PTOX), through which electrons from plastoquinol are transferred to molecular oxygen, forming water in the stroma [85]. In oat leaves incubated at high temperature and under high-light intensities, the amounts of both enzymes were increased, suggesting that, under unfavourable conditions, chlororespiration can act as a protective mechanism [85].

Apart from the Mehler reaction and the chlororespiration, respectively, photorespiration is another efficient mechanism to adjust the ATP/NADPH ratio and to consume excess energy [86]. Although the major part of energy supplied in the form of ATP and NADPH by the light reaction is consumed in the Calvin-Benson cycle, it is also needed for multiple anabolic processes in chloroplasts, such as synthesis of lipids and proteins and of many secondary metabolites. ATP/NADPH ratio in chloroplasts could be increased by indirect export of reducing equivalents in the form of malate through the malate valve [87]. Malate can be used in numerous ways in cytoplasm and mitochondria, providing NADH for nitrate reduction and/or ATP for sucrose synthesis. Also, different stages of tissue growth require different ATP/

NADPH ratios. The ATP/NADPH ratio that is available in the light can vary substantially because of the many possibilities for electron pathways and regulatory mechanisms [88]. Therefore, the cooperation of different electron transport pathways enables optimization of ATP/NADPH stoichiometry [77]. In example, sudden dark-to-light transition induces over-reduction of the PET chain in the first minute, which is relieved by electron transport to O_2 [89], and by the rapid activation of the chloroplast NADP–MDH [90]. Calculating the photosynthetic stoichiometries, it was estimated that ATP/NADPH ratio arising from LEF is about 1.28, which is not sufficient for driving the Calvin-Benson cycle [91, 77]. It is therefore obvious that the optimal operation of the Calvin-Benson cycle requires both LEF and CEF, whose tuning enables adjustment of ATP/NADPH to meet the cellular demands. Green alga *C. reinhardtii* in which intracellular ATP depletion induces a switch from LEF to CEF is a good example for such regulation [92].

5.2. Electron partitioning at the ferredoxin hub

In photoautotrophic plants, ferredoxin (Fd) accepts one electron from the stromal side of PSI involving the subunits PsaC, PsaD and PsaE [93]. Fd acts simultaneously as bottleneck and as a hub which distributes high-energy electrons to a multitude of enzymes involved in chloroplast metabolism. The hierarchy of electron distribution and subsequent regulation of channeling of photosynthetically derived electrons into different areas of chloroplast metabolism is still not defined. Electrons are preferentially directed to carbon assimilation, which requires NADPH, and so the majority of Fd is immediately oxidized by the enzyme ferredoxin:NADPH reductase (FNR), which is associated with the thylakoid membrane.

Besides its crucial metabolic role in reducing NADP$^+$ and thioredoxin (TRX) *via* FNR and ferredoxin-thioredoxin-reductase (FTR) respectively, Fd-dependent enzymatic reactions are also linked to nitrite and sulfur metabolism by ferredoxin-nitrite-reductase and sulfite reductase. Furthermore, Fd is a electron donor for fatty acid desaturase and glutamine-2-oxoglutarate amino transferase. In some green algae, upon transition from dark to the light under anaerobic conditions, Fd transfers transiently electrons to chloroplast hydrogenases, which in turn catalyse the formation of hydrogen [94, 95] dissipating excess reducing power when the Calvin–Benson cycle is not yet fully activated. Fd and NADPH can also act in CEF, returning electrons to the PQ pool *via* the PGR5 (proton gradient regulation 5)-dependent [96] and NDH complex-dependent [97, 98, 99] pathways, respectively. FNR may also act as the direct Fd:PQ reductase, establishing redox regulation and antioxidant defense point. FNR activity seems to represent a critical point in photosynthetic electron partitioning, because it is integral to most of these electron cascades and can associate with several different membrane complexes.

At least four Fd isoforms occur in plants [100]. Fd1 and Fd2 are found in leaves, while Fd3 and Fd4 appear to play roles in non-photosynthetic metabolism [101, 100]. Fd as electron distributing hub is particularly suitable to provide information on the redox state of the system to be transmitted into the regulatory network. Fd contributes to the control of chloroplast energization by feeding electrons into the CEF pathway and thereby controls both phosphorylation potential and reductive power.

5.3. TROL-FNR interaction influences the energy conductance

The last step of the photosynthetic electron transfer from Fd to $NADP^+$ is catalyzed by FNR. There are two evolutionary conserved types of FNR in the chloroplasts of higher plants: predominantly, or exclusively, thylakoid membrane-bound isoproteins and 'soluble', non-tightly bound isoproteins [101, 102]. The membrane-bound FNR is supposed to be involved in electron transport, while the soluble enzyme provides protection against oxidative stress [103]. Two chloroplast-type *FNR* genes have been found in Arabidopsis genome. In 2008, Hanke et al. investigated knock-out mutant of Arabidopsis FNR isoprotein, *fnr1*. The loss of the strong thylakoid binding was observed, which affected the channeling of photosynthetic electrons into NADPH- and Fd-dependent metabolism. Also, these mutants had complex variation in CEF, dependent on light conditions [104]. In *fnr1*, thylakoid $NADP^+$ photoreduction was greatly reduced even on addition of soluble FNR to rate saturating concentrations, which is consistent with the lack of membrane-bound FNR [104].

$NADP^+$ photoreduction activity of FNR was shown to be greater when the enzyme is associated with the thylakoid membrane and it has been proposed that binding of FNR to the thylakoid membrane regulates the enzyme activity [105, 106]. Subsequently, interactions of FNR and several photosynthetic protein complexes, such as Cyt*b6f* [107, 108], PSI [109] or NDH complex [110] have been shown. However, the factors controlling relative localization of FNR to different membrane complexes have not yet been established. It was shown that maize contains three chloroplast FNR proteins with completely different membrane association and distribution between cells, conducting predominantly CEF in bundle sheath cells and LEF in mesophyll cells [111]. Expression of maize FNRs in Arabidopsis as chimeras and truncated proteins showed that N-terminus determines recruitment of FNR to different membrane complexes, which impacts the photosynthetic electron flow [111].

It was also demonstrated that FNR interacts specifically with two chloroplast proteins, Tic62 (62 kDa component of the translocon at the inner envelope of cloroplasts) and TROL (thylakoid rhodanese-like protein), *via* a conserved Ser/Pro-rich motif [112, 113]. Both Tic62 and TROL seem to act as molecular anchors for FNR, because they form high molecular weight complexes with FNR at the thylakoid membranes. TROL possesses centrally positioned rhodanese-like domain, which is most probably involved in redox regulation of FNR binding and release [113]. We have proposed that such regulation could be important for balancing the redox status of stroma with the membrane electron transfer chain and therefore preventing the over-reduction of any of these two compartments and maintaining the redox poise [113]. TROL-FNR complex was clearly visible during the dark and it disappeared during light periods [112]. FNR-Tic62/TROL interaction is clearly pH-dependent. During high photosynthetic activity in the light, stroma becomes alkaline due to the transport of protons to the thylakoidal lumen. During the dark, stromal pH decreases again, and that is when FNR-Tic62/TROL complexes were found predominantly associated with the thylakoid membrane [112, 113]. The mechanism by which TROL influences the FNR activity could be that during the dark period, FNR is bound to the thylakoids *via* TROL and NADPH production does not occur. This stage could be sustained through the binding of small molecule, possibly oxidized PQ, to the RHO cavity. In conditions of growth-light, FNR is bound to the thylakoids *via* TROL and efficiently

produces NADPH. When the light is saturating, FNR is released from TROL by a signal molecule, possibly reduced PQ that competes for the RHO binding site. Once soluble, FNR acts as NADPH consumer and released protons are passed to an unknown scavenger [78, 113, 114]. It was reported that membrane attachment of FNR is influenced by the stromal redox state ($NADP^+$/NADPH ratio), which mimics variations in environmental conditions [115]. Therefore, reversible attachment of FNR to the thylakoid membrane *via* TROL and/or Tic62 provides an elegant way to store redundant molecules, not required when photosynthesis is less active or dormant.

We have already mentioned that reducing equivalents could be exported in the form of malate through the malate valve to increase the ATP/NADPH ratio in chloroplasts [87]. TROL-deficient plants grown under growth-light conditions show significant up-regulation of NADP-malic enzyme 2 that catalyses the oxidative decarboxylation of malate, producing pyruvate, carbon dioxide and NAD(P)H in cytosol [113]. Therefore, the TROL knock-out Arabidopsis mutant lines (*trol*) could act as efficient NADPH producers, fighting the possible hyper-reduction of the thylakoids by exporting the reducing energy in a form of malate to the cytosol.

The *trol* mutants show severely lowered relative electron transport rates at high-light intensities. Also, under high-light conditions, but in a short-term, NPQ amount was higher in the *trol* line, compared to the wild-type [113]. Moreover, TROL is important for NPQ, since the reversion of the plant without TROL to the plant that expresses TROL, even with certain alterations, leads to the restoration of wild-type levels of NPQ (Jurić, Fulgosi, Ruban, unpublished results). We are not dealing with extreme NPQ phenotype, but, the question remains, how is it possible that small changes in the TROL protein could modulate NPQ so effectively? Our unpublished data also suggest the possible enrolment of some LHCII subunits in the TROL-containing complexes, which, in the light of these results, should be thoroughly investigated. In addition, we observed that, after more than two weeks of exposure to the high-light conditions, *trol* plants exhibited NPQ almost at the wild-type levels, suggesting that some sort of long-term acclimation could also be involved.

6. Conclusions and perspectives

Photosynthesis is the crucial converter of sunlight into the chemical energy that is subsequently utilised to sustain life on Earth. However, without the layers of regulative pathways, it would be virtually impossible to discuss the efficient photosynthesis. Now, we are aware of the fact that more than 40-years-old "Z-scheme" has been constantly upgraded with many alternative routes allied with still not well-characterised electron sources and sinks. In addition, it seems that the thylakoid membranes are more flexible than anticipated, with PSII antenna involved directly in NPQ origin through detachment and aggregation.

One of the interesting domains in photosynthesis, but not enough explored, would be the involvement of lipids in a complex network of protein interactions. Lipids, especially phospholipids (phosphatidylglycerol; PG) and glycolipids (monogalactosyldiacylglycerol; MGDG,

digalactosyldiacylglycerol; DGDG, and sulfoquinovosyldiacylglycerol; SQDG) are major building blocks of thylakoid membranes, providing the safe docking sites for proteins and protein complexes, respectively. It has been proposed that DGDG and PG are especially involved in the assembly and repair of PSII [116]. Moreover, plants lacking almost 30% of the wild-type amount of PG, caused by the point mutation in one of the genes involved in PG synthesis pathway, displayed pale green leaves and somewhat reduced capacity for photosynthesis [117], while plants that accumulated only 10% of the wild-type PG amount were incapable of surviving on the growth medium without the addition of sucrose [118].

The other attractive domain would be retrograde signalling, *i.e.* the pathways flowing from chloroplasts to the nucleus. At least five classes of chloroplast-derived signals have been studied and discussed: tetrapyrrole biosynthesis, chloroplast gene expression-dependent pathway, redox state of chloroplasts, ROS, and sugar and hormone signalling. While some molecules look more promising than others (sugars and hormones for example), ROS could be more challenging to investigate, due to its non-specificity and a power to response quickly to different types of stress in different parts of the cell.

The rich past of exploring photosynthetic processes gave us a wealth of knowledge, but, it seems that we have just scratched the surface. In years to come, it would be interesting to fill the missing blanks and to develop some new, "out-of-the-box" perspectives.

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