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REVIEW

Photoinhibition of photosynthesis: Role of carotenoids in photoprotection of chloroplast constituents

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Abstract

Exposure of plants to irradiation, in excess to saturate photosynthesis, leads to reduction in photosynthetic capacity without any change in bulk pigment content. This effect is known as photoinhibition. Photoinhibition is followed by destruction of carotenoids (Cars), bleaching of chlorophylls (Chls), and increased lipid peroxidation due to formation of reactive oxygen species if the excess irradiance exposure continues. Photoinhibition of photosystem 2 (PS2) *in vivo* is often a photoprotective strategy rather than a damaging process. For sustainable maintenance of chloroplast function under high irradiance, the plants develop various photoprotective strategies. Cars perform essential photoprotective roles in chloroplasts by quenching the triplet Chl and scavenging singlet oxygen and other reactive oxygen species. Recently photoprotective role of xanthophylls (zeaxanthin) for dissipation of excess excitation energy under irradiance stress has been emphasised. The inter-conversion of violaxanthin (Vx) into zeaxanthin (Zx) in the light-harvesting complexes (LHC) serves to regulate photon harvesting and subsequent energy dissipation. De-epoxidation of Vx to Zx leads to changes in structure and properties of these xanthophylls which brings about significant structural changes in the LHC complex. This ultimately results in (*1*) direct quenching of Chl fluorescence by singlet-singlet energy transfer from Chl to Zx, (*2*) trans-thylakoid membrane mediated, ΔpH-dependent indirect quenching of Chl fluorescence. Apart from these, other processes such as early light-inducible proteins, D1 turnover, and several enzymatic defence mechanisms, operate in the chloroplasts, either for tolerance or to neutralise the harmful effect of high irradiance.

Additional key words: chlorophyll fluorescence; high irradiance; photosystem 1; photosystem 2; violaxanthin; xanthophyll cycle; zeaxanthin.

Introduction

The absorption of photons by chlorophyll (Chl) molecules is a physical process with no biological control over it. However, the biochemistry of photosynthesis and subsequently the utilisation of reducing power and chemical energy in CO_2 fixation are always under the control of various enzymes and metabolic intermediates in a highly co-ordinated manner. At irradiance limiting photosynthesis, photons are captured and utilised with the highest possible efficiency. Photosynthetic apparatus is capable of absorption of radiant energy over a wide range of photon flux density (PFD). However, with increasing PFD, the rate of photosynthesis initially increases linearly and above a certain PFD, the process is incapable of utilising all the absorbed energy (Demmig-Adams 1990) and hence declines.

The primary charge separation at PS2 reaction centre (RC) occurs much faster than the electron transport. When the rate of transfer of excitation energy from the antennae to RCs exceeds the rate of transfer from the RCs to the electron transport chain, photoinhibition is resulted. Typical manifestation of photoinhibition in leaves includes sustained decrease in photon yield and often a reduction in maximum photosynthetic capacity. Photoinhibition is often associated with damage to the photosynthetic apparatus under prolonged high irradiance (HI). One of the components most frequently suggested to be

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Abbreviations: Ax – antheraxanthin; Car – carotenoids; Chl – chlorophyll; HI – high irradiance; LHC – light-harvesting complex; OEC – oxygen evolving complex; PFD – photon flux density; PS – photosystem; RC – reaction centre; VDE – violaxanthin decpoxidase; Vx – violaxanthin; ZE – zeaxanthin epoxidase; Zx – zeaxanthin.
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damaged is the D1 protein of PS2 RC (Mattoo *et al.* 1984, Reisman and Ohad 1986, Jegerschöld *et al.* 1990, Aro *et al.* 1993, Critchley and Russell 1994, Anderson *et al.* 1997, Minkov *et al.* 1999).

Photoinhibition may also result not only from some form of damage to PS2 but also from an increased thermal energy dissipation, which is a photoprotective process and does not reflect damage (Demmig-Adams 1990, Demmig-Adams and Adams 1992). It is now evident that photoinhibition, which results from the conjunction of high irradiance stress with other stress factors such as drought, chilling, or high temperature, has an important impact on plants under natural conditions. Plants have evolved photoadaptive and photoprotective mechanisms at levels ranging from the whole plant to leaves and thylakoid membrane of chloroplasts, to avoid excessive radiant energy interception, and have developed several mechanisms to minimise the energisation of the thylakoid under high irradiance.

Cars are a diverse group of lipophilic pigment molecules that are widely distributed in nature and present in all photosynthetic organisms. They act as light-harvesting antennae and also protect the photosynthetic apparatus from photodestruction in strong irradiance (Siefermann-Harms 1985, Bartley and Scolnik 1995). Cars protect photosynthetic apparatus through two important ways: (*1*) β-carotene (β-Car) directly quenches both triplet chlorophyll ($^3Chl^*$) and singlet oxygen ($^1O_2^*$). (*2*) The xanthophylls lower the $^3Chl^*$ formation by quenching excited singlet state of Chl ($^1Chl^*$).

The review focuses current views on photoinhibition and the primary photoprotective mechanism responsible for dissipation of excess absorbed photon energy in the light-harvesting antennae of PS2.

Photoinhibition of photosynthesis

Photoinhibition of photosynthesis generally denotes a decrease in photosynthetic activity when plants are exposed to HI that exceeds the ability of the light-harvesting system to dissipate the energy not used for photochemistry. Powles (1984) considered the photoinhibition as a first stage of HI-induced thylakoid damage leading to reduction of photosynthetic capacity. The second stage, pigment photooxidation, commences after a long-term exposure of the plants to strong irradiance and concerns the bleaching of antennae pigments. The later process requires oxygen.

Photoinhibition at PS2

The primary target of HI causing photoinhibition of photosynthesis is PS2. In this process, two mechanisms are involved which may affect either the acceptor side or the donor side of PS2. The two mechanisms are distinguished on the basis of differences in the primary site of electron transport malfunctioning, the subsequent D1 protein degradation, and the oxygen requirement of the process (Fig. 1).

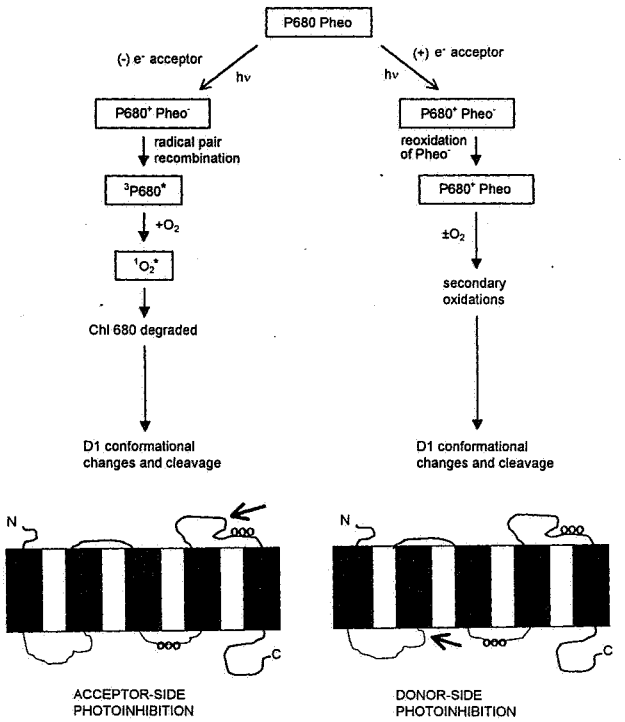


Fig. 1. Scheme showing the two routes of damage due to acceptor and donor side photoinhibition (Minkov *et al.* 1999). *Bold arrows* indicate site of photoinhibition.

Acceptor side-induced photoinhibition of PS2 occurs under HI when it exceeds the saturation of photosynthetic electron transport (Barber and Andersson 1992). Excess exposure causes non-physiological over-reduction of the first quinone electron acceptor in PS2. This brings about sequential modifications at the level of the Q_A and Q_B acceptors (Keren *et al.* 1997). These conditions lead to the recombination of the radical pair, $P680^+$ $Pheo^-$ (Vass *et al.* 1992) and the production of the triplet state of P680 ($^3P680^*$). Under aerobic conditions, this $^3Chl^*$ may be quenched by oxygen and $^1O_2^*$ is thus produced (Fig. 1). The addition of $^1O_2^*$ scavengers such as histidine (Mishra *et al.* 1994, Telfer *et al.* 1994), diazobicyclooctane (Barényi and Krause 1985, Miyao 1994), azid (Macpherson *et al.* 1993) as well as free radical scavengers such as uric acid or propylgallate (Sopory *et al.* 1990) provide partial photoprotection against the acceptor side-induced photoinhibition of PS2 in an isolated system. The $^1O_2^*$ initiates and also triggers degradation of the RC protein D1, probably by promoting a special conformational change which makes the protein susceptible to proteolytic cleavage (Fig. 1). One possibility is that, in complex *in vivo* systems, the D1 protein may be cleaved by the direct action of active oxygen. The possible cleavage site is on the stroma side of the thylakoid membrane and the characteristic degradation products of the D1 protein are 23 kDa

N-terminal and 10 kDa C-terminal fragments (Virgin *et al.* 1991, Barber 1992, De Las Rivas *et al.* 1993a). The D1 protein has the highest turnover rate among the proteins of PS2 complex that could be due to its requirement to repair the damaged PS2 by photoinhibitory irradiation (Minkov *et al.* 1999).

Donor side-induced photoinhibition of PS2 occurs when the capacity of water oxidising complex to donate electrons to the RC P680 is inactivated by high irradiance (Biswal and Biswal 1999, Minkov *et al.* 1999). Under such conditions the water oxidising complex is unable to keep up the rate at which electrons are transferred from P680 towards acceptor side components. This leads to an increase in the lifetime of $P680^+$ with a high oxidising potential (Thompson and Brudvig 1988). The $P680^+$ extracts electrons from the surrounding environment which subsequently leads to destruction of D1, Chl, and β-Car associated with the RC of PS2 (Barber 1994).

Irradiance absorbed by P680 leads to charge separation with stable charge pairs like $P680^+ Pheo^-$. The former possibly extracts an electron from Y_z resulting in the formation of Y_z^+. Both $P680^+$ and Y_z^+ are oxidants with high oxidising potential. The oxygen-evolving complex (OEC) has a very delicate structure and is highly susceptible even to a mild stress. This could initially trigger its damage and may result in its inability to donate electrons effectively to Y_z^+ and $P680^+$. These (Y_z^+ and $P680^+$) long-lived oxidants with strong oxidising capacity can oxidise pigments, lipids, and proteins in the vicinity of PS2. The inactivation of the Mn cluster in OEC and Y_z has been suggested as the major stress sensing signalling system operating at the donor side of PS2 (Andersson and Barber 1996, Biswal 1997). The events associated with the donor side route may bring about alteration in the D1 structure and its subsequent degradation. Jegerschöld and Styring (1996) have suggested damage of D1 protein by the strong oxidising $P680^+$. They have proposed that with an inactivated Mn cluster, but with effective functioning of the acceptor side components, $P680^+$ is the real oxidant that causes damage to the protein.

Photoinhibition at PS1

Electron transport through PS1 is also inhibited by exposure of the chloroplasts to excess irradiance. However, the extent of inhibition is less than that of PS2 (Prášil *et al.* 1992, Choudhury *et al.* 1993, 1994). Moreover, the presence of oxygen is essential for photoinhibition of PS1 electron transport activity (Demmig-Adams 1990). The site of inhibition is close to the PS1 RCs. The mechanism of oxygen dependent light-induced inactiva-tion is similar to the acceptor side photoinhibition of PS2 (Fig. 1). The recombination of the primary charge sepa-ration products in PS1 results in the formation of P700 in triplet state ($^3P700^*$) and thus it leads to the production of the $^1O_2^*$ (Sonoike *et al.* 1994). Reactive oxygen species generated through $^3Chl^*$ subsequently damage the whole photosynthetic machinery as discussed under photoini-hibition at PS2.

Carotenoids and photoprotection

The primary functions of Cars in phototrophs is to act as chemical buffers against photooxidation of the cell constituents sensitised by Chl, thus conferring a high degree of immunity to endogenous photosensitisation. At HI, when photosynthesis is saturated, unusual photochemistry takes place that may lead to damage of the photosynthetic apparatus. This could be avoided if there is a mechanism to increase energy dissipation as heat or fluorescence. Based on the observations in a Car containing wild type *Rhodopseudomonas sphaeroides* and a blue green mutant that lacks Car, the essential photoprotective role of Car was initially proposed by Sistrom *et al.* (1956). Subsequently numerous researchers have confirmed the photoprotective role of Car in lower as well as higher plants (Siefermann-Harms 1987, Gilmore 1997, Biswal and Biswal 1999, Hong *et al.* 1999, Koblížek *et al.* 1999, Minkov *et al.* 1999, Ritz *et al.* 1999, Xu *et al.* 2000, Ye *et al.* 2000, Behera and Choudhury 2001, 2002, Muller *et al.* 2001, Tracewell *et al.* 2001).

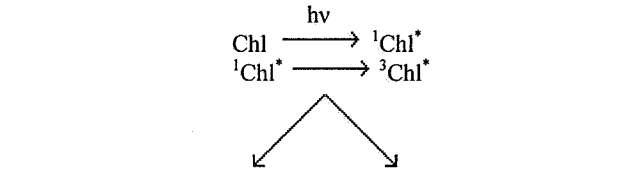
A majority of Cars possess one functional oxygen atom at least, while few are hydrocarbons. As there is heterogeneity in Car composition in the various pigment-protein complexes of photosystems, different functions are assigned to these yellow pigments in different protein/lipid environment of thylakoid membrane (Siefermann-Harms 1987, Govindjee 1999).

Role of β-carotene

Presence of two β-Car molecules in the RC of PS2 (Fig. 2) has been detected by fluorescence and linear dichroism spectroscopy (Van Dorssen *et al.* 1987, Newell *et al.* 1991, Telfer *et al.* 1991). The two molecules are spectrally different: one has absorption peak at 489 nm and the other has peaks at 507 and 467 nm. After absorption, they transfer the excitation energy to Chl *a*. These β-Car molecules also protect the RC Chl from HI damage (De Las Rivas *et al.* 1993b, Telfer *et al.* 1994). Trebst and Depka (1997) showed that β-Car is essential for the assembly of the D1 protein into functional PS2. Xiong *et al.* (1998) proposed a structural model of PS2 RC showing two β-Car molecules in parallel orientation whereas Mimuro *et al.* (1995) suggested perpendicular orientation of the molecules.

β-Car protects the PS2 RC against photo-oxidative damage *via* quenching of $^1O_2^*$ or $^3Chl^*$ that sensitise $^1O_2^*$ formation (Cogdell and Frank 1987). However, Telfer *et al.* (1994) showed that β-Car limits the destructive (oxidative) reactions by scavenging $^1O_2^*$ rather than trapping the $^3Chl^*$. Durrant *et al.* (1990) suggested that $^3Chl^*$ molecules are unable to give its spin to β-Car, as they are not situated sufficiently close to each other in the PS2

RC, thereby being unable to de-excite $^3Chl^*$. However, protec-tion of PS2 RC from HI stress by β-Car is undisputed, which may take place by either one or both ways as shown below.



$^3Chl^* + \beta\text{-Car} \rightarrow Chl + {}^3\beta\text{-Car}^*$
$^3\beta\text{-Car}^* \rightarrow \beta\text{-Car} + heat$

Quenching of triplet chlorophyll by β-Car

$^3Chl^* + O_2 \rightarrow Chl + {}^1O_2^*$
$^1O_2^* + \beta\text{-Car} \rightarrow O_2 + {}^3\beta\text{-Car}^*$
$^3\beta\text{-Car}^* \rightarrow \beta\text{-Car} + heat$

Quenching of singlet oxygen by β-Car

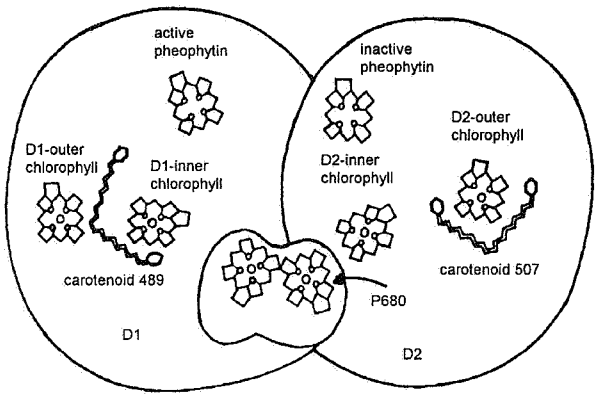


Fig. 2. A diagrammatic model of the arrangement of two carotenoids (carotenoid 489 and carotenoid 507) in the reaction centre of photosystem 2. P680 is the reaction centre chlorophyll *a* dimer whereas D1 and D2 are the two proteins where the chromophores are housed (adopted from Govindjee 1999).

Role of xanthophylls

The main function of the xanthophyll cycle pigments (Zx, Ax, and Vx) in chloroplasts is to increase non-radiative dissipation of excess excitation energy as heat in the pigment bed of antennae of PS2 and protect chloroplasts from HI stress (Demmig-Adams 1990, Gilmore and Govindjee 1999, Bukhov *et al.* 2001, Grudziński *et al.* 2001). Induction of an acid lumen pH is the basic requirement for the operation of xanthophyll cycle. The cycle basically involves the interconversion of Vx to Zx through the formation of an intermediate, Ax. The enzymes involved for the interconversion are violaxanthin deepoxidase (VDE) and zeaxanthin epoxidase (ZE).

The VDE enzyme facing the lumen side is responsible for converting Vx to Zx at low lumen pH (pH 5.2) induced by HI. On the other hand, the activity of ZE (pH 7.5), facing the stroma, enhances the formation of Vx from Zx. By the conversion of Vx to Zx, the excess absorbed energy in the PS2 antennae is ultimately dissipated as heat, generally referred to as energy dependent quenching (q_E) or ΔpH-dependent non-photochemical Chl fluorescence quenching (NPQ) (Demmig-Adams 1990). Therefore, NPQ decreases the efficiency of PS2 when the rates of electron transport and carbon metabolism reach saturation at high PFD and favours antennae based photoprotection (Osmond 1994).

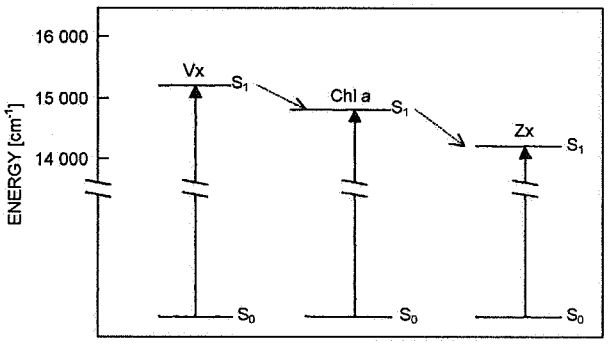


Fig. 3. The S_1 energy levels of violaxanthin (Vx) and zeaxanthin (Zx) relative to chlorophyll (Chl) *a* (Frank *et al.* 1996).

There are two main schools of thought concerning the nature and mechanism by which a simple alteration in the xanthophyll composition of LHC affects the balance between photon capture and energy dissipation at different irradiances (Young *et al.* 1997, Horton *et al.* 1999). The first describes a theoretical model based on direct singlet-singlet energy transfer from Chl to Zx (Fig. 3) resulting in quenching of Chl fluorescence and dissipation of excitation energy. The second elucidates the role of xanthophyll cycle pigments in controlling the spatial organisation of LHC2 (Fig. 4) leading to radiation-less energy dissipation.

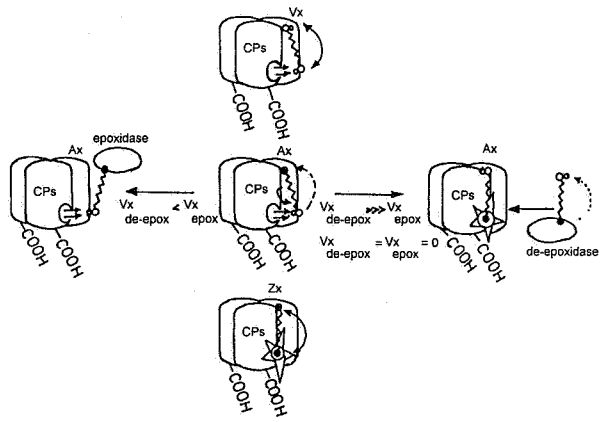


Fig. 4. The model shows how the structures of xanthophyll cycle pigments violaxanthin (Vx), antheraxanthin (Ax), and zeaxanthin (Zx) binding to protonated chlorophyll (Chl)-proteins (CPs) of the photosystem 2 (PS2) inner antennae cause non-photochemical quenching (NPQ) of PS2 Chl *a* fluorescence. *The sparks* indicate binding of Ax and Zx to CPs and inducing NPQ (Gilmore 1997).

Direct quenching: The possibility that differences in the S_1 energy levels of Vx and Zx might account for the operation of the xanthophyll mediated dissipation of excitation energy was first proposed by Demmig-Adams (1990) and later elucidated by Owens *et al.* (1992). They suggested that Zx has an S_1 energy identical to that of β-Car as these molecules are essentially iso-electronic. The S_1 state of Zx lies just below that of Chl *a* allowing the Zx molecule to act as a sink for excitation energy of Chl *a* (Fig. 3). The lowest energy content of the $^1Chl^*$ *a* in the LHC2 is in the region of 14 700-15 000 cm^{-1}. The value 14 700 cm^{-1} for Chl *a* is lower than the value determined for the S_1 state of Vx (15 290 cm^{-1}) but higher than that of Zx (14 170 cm^{-1}). This would suggest that it is energetically possible for the S_1 state of Zx to quench Chl fluorescence by deactivation of $^1Chl^*$. In contrast, the higher S_1 level of Vx would only permit it to function as a light-harvesting pigment, transferring its excitation energy to Chl *a*. Thus, at high PFD, when dissipation of excess excitation energy is required, Zx is formed within LHC2 and its formation serves to deactivate the $^1Chl^*$ *a* and dissipate excitation energy harmlessly as heat.

Indirect quenching: The structural differences between Vx and Zx may provide an explanation as to how the xanthophyll cycle pigments could indirectly control q_E. It is suggested that Zx induces structural changes of the light-harvesting system of thylakoid membrane (Fig. 4) that favour the radiation-less dissipation process (Gilmore 1997, Minkov *et al.* 1999). The irradiance-induced lowering of the lumen pH has two important roles: (*1*) it activates the VDE enzyme, and (*2*) it helps protonation of CP protein. These two events ultimately bring about specific structural changes of LHC and conversion of Vx to Zx. The changes in structure of LHC probably favour their aggregation. This aggregation is possible because of the interactions between the LHC trimers. This mechanism couples the changes in the aggregation of LHC2 with the increase in NPQ (Horton *et al.* 1991). In the light-harvesting Chl *a/b* binding protein, the Chl molecules are situated at close proximity, but are separated from each other by xanthophyll molecules. These strong anti-quenchers (xanthophylls) prevent close Chl-Chl interaction and quenching (Searle *et al.* 1991) but do not interfere with optimal energy transfer. The key process of energy dissipation by this mechanism is protonation-promoted changes in the protein structure leading to a Chl/xanthophyll aggregation and thereby allowing direct quenching of $^1Chl^*$ by Zx and energy dissipation (Eskling *et al.* 1997, Gilmore 1997).

When the lumen pH decreases, protonation rate of specific amino acids (glutamic acid) on the lumen side of LHC2, in the vicinity of Zx, increases. This makes energy transfer possible between Chl *a* and Zx by modulating the Chl *a*-Zx spectral overlap. Fig. 4 explains how Vx, Ax, and Zx with different molecular structure interact with the protonated CP complex (Gilmore 1997). Vx has no binding site affinity with the protonated CP complex, possibly because of a steric hindrance or hydrophobic repulsion from the NPQ active binding site for both of its epoxide cyclic end groups. When the rate of de-epoxidation is faster than the rate of epoxidation, the de-epoxidised end group of Ax is oriented towards the lumen side of thylakoid membrane. The capacity for Ax to bind to the protonated CP is strongly affected by the relative rates of de-epoxidation and epoxidation. The de-epoxidised end group of Ax tightly binding with protonated CP induces NPQ, whereas when it is bound to epoxidase active site, it may not efficiently bind to the CP to cause NPQ. The ability of Zx with two de-epoxide end groups to bind with CP initiating NPQ is not affected by the relative rate of de-epoxidation and epoxidation processes.

Recently, Demmig-Adams and Adams (2000) isolated a mutant of *Arabidopsis thaliana* that contained normal amounts of Zx but lacked the process of NPQ. Li *et al.* (2000) showed that this mutant lacks the CP22/PsbS protein (the product of PS2 gene *PsbS*). They used molecular and genetic markers to pinpoint the *PsbS* gene and reintroduced a normal copy of this gene into the mutant, which regained the ability to dissipate energy as NPQ after the reintegration of the gene. Based on these observations, they for the first time suggested that CP22 is essential for thermal energy dissipation in the chloroplasts. They further suggested that CP22 is required for conformational change in the photosynthetic membrane that engages thermal energy dissipation. At the same time, the conformational change in LHC2 also requires Zx and a pH gradient across the photosynthetic membrane. The proton-dependent structural changes in CP22 facilitate downhill energy transfer from surplus $^1Chl^*$ to Zx followed by rapid loss of the excitation energy from Zx in the form of heat (Li *et al.* 2000).

Conclusion

The exposure of leaves to HI results in photoinhibition, a process that brings down the photosynthetic efficiency of plants. One of the main reasons of the reduction is conformational change of the RC protein D1 under stress. This is considered a strategy of the plants to protect severe damage of the photosynthetic machinery when they are exposed to HI for prolonged period. Zx binding with CP complex in the antennae induces NPQ of Chl fluorescence thereby providing protection against irradiance stress. Only recently a specific protein (CP22) has been identified as an essential component of LHC2 responsible for NPQ. However, details of the interactions of CP22 and Zx inducing NPQ in the chloroplasts are still unclear.

The differential rate of photoinhibition of PS2 and

PS1 under similar HI has also not been explained yet. Hence, much work now has to be done to explain the precise role of CP22 *vis-à-vis* Zx to have a clear understanding of the mechanism of photoinhibition and photoprotection of chloroplasts under irradiance stress.

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