REVIEW

Thylakoid membrane protein kinase activity as a signal transduction pathway in chloroplasts

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Abstract

In plants external stimuli are perceived through a cascade of signals and signal transduction pathways. Protein phosphorylation and de-phosphorylation is one of the most important transduction paths for the perception of signals in plants. The highest concentrations of plant phospho-proteins are located in chloroplasts. This facilitates the protection of thylakoid membranes from stress-induced damage and augments adaptive strategies in plants. In this review, the protein kinases associated with phosphorylation of thylakoid membrane protein, and the adaptive changes in thylakoid membrane architecture and developmental cues are given. The presence of membrane bound kinases in thylakoid membranes have evolutionary implications for the signal transduction pathways and the photosynthetic gene expression for thylakoid membrane protein dynamics.

Additional key words: D1 protein; evolution; gene expression; light-harvesting complex 2; phosphorylation; photosystems.

Introduction

Sensing a stimulus and communication to plant cells through inter- and intra-cellular chemical messenger systems are known as signal transduction. Living systems, especially plants and animals, share many common signal pathways. Cell signalling in plants involves G-proteins, protein kinases and phosphatases, secondary messengers such as calcium or phospholipid derived molecules (Redhed and Palme 1996) and plant hormones (Palme 1994). Some of the chemical messengers can pass through the plasma membrane and bind to the intercellular receptors, which either activate or inactivate specific genes. However, some chemical messengers do not pass through the plasma membrane but communicate through the chemicals called secondary messengers. The chemical messenger network in plants that perceive, amplify, and transmit the message in plant cells is a network of proteins. The signal transduction takes place through the phosphorylation and dephosphorylation of these proteins mediated by kinases and phosphatases (Blum *et al.* 1988, Dorbak *et al.* 1988, Halper *et al.* 1991).

Protein kinases in plants

More than 70 plant protein kinase genes have been identified. Protein kinases are classified by the amino acids, which are phosphorylated, into the following groups: (*1*) histidine kinases, (*2*) tyrosine kinases, and (*3*) serine and/or threonine kinases. Phospho-proteins were found in plant nuclei, plastids, mitochondria, and membranes (Trewavas and Blowers 1990).

Protein phosphorylation is one of the important signal transduction pathways affecting many aspects of prokaryotic and eukaryotic metabolism, gene expression, response to environmental stimuli, and plant growth and development (Bennett 1991). The highest concentration of plant phospho-proteins is found in the chloroplast (Bhalla and Bennett 1987). Phospho-proteins, first detected in thylakoid membranes (Bennett 1977) have also been located in other chloroplast compartments; namely

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Abbreviations: Chl – chlorophyll; CP – chlorophyll-protein; Cyt – cytochrome; G-protein – GTP binding protein; LHC – light-harvesting chlorophyll-protein complex; PAR – photosynthetically active radiation; PQ – plastoquinone; PS – photosystem; Q_A – primary electron acceptor in PS2 reaction centre; Q_B – secondary electron acceptor in PS2 reaction centre.

the soluble phase or stroma (Foyer 1985), chloroplast ribosomes (Guitton *et al.* 1984), the outer (Soll and Buchanan 1983) and inner envelope membranes, and the inter-envelope space (Soll and Bennett 1988).

Discovery of protein kinase in chloroplasts

Phosphorylation of thylakoid proteins was first observed in isolated irradiated pea chloroplasts supplied with ^{32}P-orthophosphate (Bennett 1977). In the dark-to-light adaptation of thylakoid membranes, some proteins are phosphorylated by a steadily active kinase, some proteins are phosphorylated only in darkness, and few in both light and darkness. Various classes of protein kinases are isolated from thylakoid membranes. Two kinases with molecular masses of 28 and 38 kDa (Lin *et al.* 1982, Lucero *et al.* 1982) and a third protein kinase (M_r 64 kDa) were identified in thylakoids (Coughlan and Hind 1986).

LHC phosphorylation and state transitions

The adaptation of thylakoid membranes by protein phosphorylation to changes in radiation is a complex process, but changes in irradiance and quality of radiation is accompanied by phosphorylation or dephosphorylation of several thylakoid proteins (Allen 1992, Gal *et al.* 1997). These proteins belong to photosystem 2 (PS2). Most of the thylakoid protein kinase reactions are redox dependent (Alfonso *et al.* 1999, 2000, Silverstein *et al.* 1993a,b, Vener *et al.* 1998). Green algae and higher plants can acclimate to changes in the spectral quality of radiation through a phenomenon known as the state I-to-state II transitions (Myers 1971, Barber 1982, Telfer *et al.* 1983, Fork and Satoh 1986). The irradiation conditions and kinetics of state transitions are comparable to those of reversible LHC2 phosphorylation (Telfer *et al.* 1983). When PS2 is overexcited relative to PS1 (under 645 nm radiation), chloroplasts are driven into state II. Then the PQ pool and the cytochrome complex(es) become reduced and the LHC2 kinase is activated. As a result the detachment of LHC2 units from PS2 α-centres and migration of some of these LHC2 units to PS1 occur. Thus state II corresponds to phosphorylation of LHC2. Reversibly, over-excitation of PS1 by 710 nm radiation drives chloroplast into state I. It leads to oxidation of PQ and cytochrome complex, inactivation of the kinase, and restoration of a large antenna to PS2 through de-phosphorylation of LHC2; thus state I correspond to de-phosphorylation of LHC2.

Signal transduction pathway(s) in LHC phosphorylation

Reversible changes in LHC2 phosphorylation in response to successive exposure to PS1 radiation and PS2 radiation were observed both *in vivo* and *in vitro* (Horton and Black 1980, Owens and Ohad 1982, Bennett 1983, Delepelaire and Wollman 1985, Bhalla and Bennett 1987, Bennett *et al.* 1988). But in *Chlamydomonas reinhardtii* the *in vivo* changes in LHC2 phosphorylation are more dramatic if the redox state of the pool is manipulated through changes in chlororespiration (Delepelaire and Wollman 1985, Vener *et al.* 1995, 1997, Alfonso *et al.* 2000). The energy transducing membrane systems in cyanobacteria contain both aerobic respiration and oxygenic photosynthesis components such as PQ pool, Cyt b_6f complex, and PC (or Cyt c_{553}) (Hirano *et al.* 1980, Peschek 1987). The redox states of both the respiratory and photosynthetic electron transport could be altered by changing irradiation and nutrient regimes of the microalga or by the addition of electron acceptors and inhibitors (Alfonso *et al.* 2000). These treatments also affect the phosphorylation state of LHC2. The occupancy of Q_0 site in the Cyt b_6f by a plastoquinol molecule is the signal for activation of light-dependent LHC2 kinase (Vener *et al.* 1995, 1997, Zito *et al.* 1999). The signal transduction pathway connecting light-driven electron flow with thylakoid protein kinase(s) activation involves the interaction of PQ oxidation of Cyt b_6f complex by the reduction of Rieske Fe-S centre and Cyt f (Vener *et al.* 1997, 1998, Zer *et al.* 1999).

Light-induced changes in LHC conformation lead to the phosphorylation

About 75-80 % of the LHC2 is found in stacked membranes, functioning as the peripheral antenna for PS2. A part of the LHC2 pool is mobile and able to migrate laterally from the stacked to the unstacked regions (Kyle *et al.* 1983, Larsson and Andersson 1985). The mobile action is enriched in phosphorylated LHC2, especially the 25-kDa form. The phosphorylated 25 and 27 kDa LHC2 units are detached from PS2 units and migrate from the stacked membranes to the unstacked membranes (Fig. 1). They then act as antennae for PS1 and decrease the energy transfer to PS2. So the distribution of LHC2 between the two membrane regions of the thylakoid would be determined by a combination of brownian motion and purely local protein-protein interactions that were modulated by phosphorylation of the N-terminus of LHC2. Using a partially purified

thylakoid membrane protein kinase and isolated native LHC2 as well as a recombinant LHC2, Zer *et al.* (1999) demonstrated that irradiation of Chl-protein substrate exposes the phosphorylation site to the kinase. PAR does not activate the phosphorylation of the LHC2 apoprotein nor the recombinant pigment-reconstituted complex lacking the N-terminal domain that contains the phosphothreonine site Thr-6 and/or Thr-7. The exposure of N-terminal domain of LHC2 due to PAR activation was conformed by the increased accessibility of this pigment-protein complex to tryptic cleavage after irradiation (Zer *et al.* 1999). Preferentially, the trimeric form of LHC2 is activated by PAR and is deactivated in darkness. PAR-activated exposure of the LHC2 N-terminal domain to endogenous protein kinase(s) and tryptic cleavage also occurs in thylakoid membranes. These results demonstrated that PAR might regulate thylakoid protein phosphorylation not only *via* the signal transduction loop connecting redox reactions to the protein kinase activation, but also by affecting the conformational changes in LHC2 (Fig. 2).

The mechanism of phosphorylation-induced dissociation of LHC2 from PS2 during state transition is still not fully understood. The conformational changes induced by phosphorylation of the N-terminal domain of LHC2 (Allen and Nillson 1997, Zer *et al.* 1999) and/or the charge repulsion between the phosphorylated LHC2 and PS2 cores (Barber 1982, Gal *et al.* 1997) may be involved in this process. The non-phosphorylated trimeric form of LHC2 is associated with PS2 (Zer *et al.* 1999). The PAR-induced conformational changes of the non-phosphorylated LHC2 may alter the interaction between PS2 and LHC2 trimers, concomitantly exposing the LHC2 N-terminal domain to the kinase. Subsequently, phosphorylation may stabilise the new conformation of the LHC2 and destabilise the PS2-LHC2 trimer interaction, leading to their dissociation (Zer *et al.* 1999).

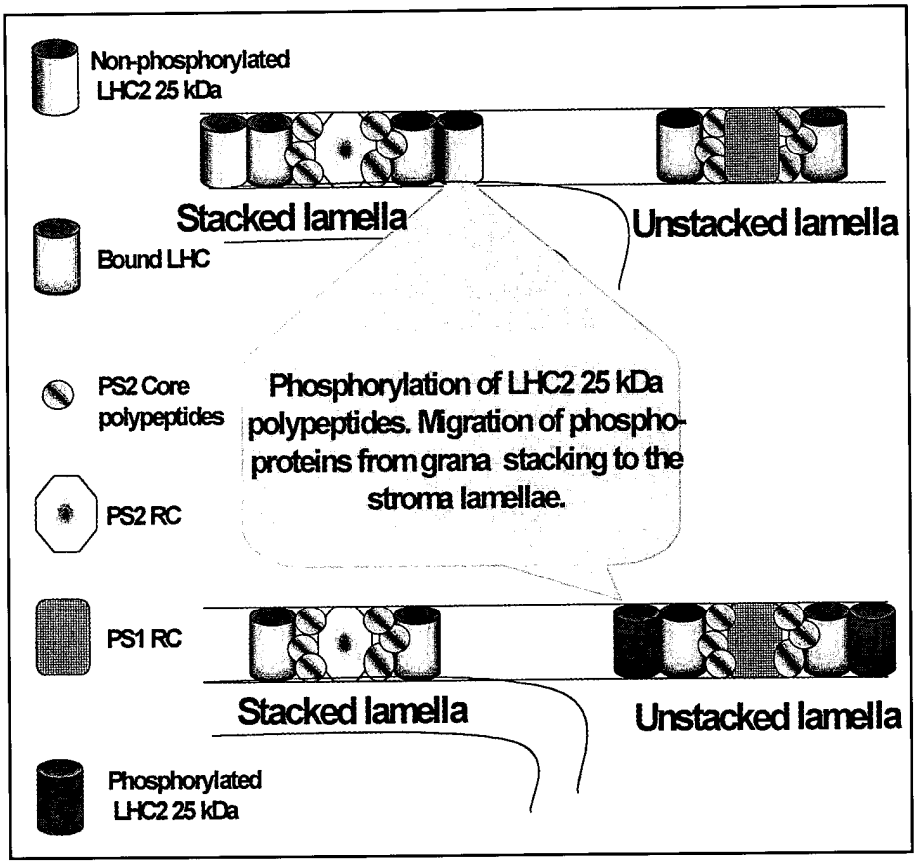

Fig. 1. Localisation of the non-phoshorylated LHC2 in the stacked (appressed or grana lamellae) regions of thylakoid membranes. Phosphorylation and migration of LHC2 to unstacked (stroma exposed) lamellae leading to state changes in photosystems (PS1 and PS2).

Phosphorylation of PS2 core polypeptides

Several polypeptides of the PS2 core complex can be phosphorylated in higher plants. They include the D1 and D2 proteins encoded by the *psb*A and *psb*D genes, as well as the products of the *psb*C and *psb*H genes. In

spinach the phosphorylated residue of all four proteins has been identified as an *o*-phospho-threonine near the N-terminus, which is exposed to the stromal face of the thylakoid membranes (Michel and Bennett 1987, Michel *et al.* 1988). In higher plants the PS2 complex may be assembled in the non-appressed region of the thylakoid membranes before it migrates to the grana region, where it accumulates (Schuster *et al.* 1988, de Vitry *et al.* 1989). The independence of phosphorylation of PS2 proteins from a functional Cyt b_6f complex was reported also in maize (Bennett *et al.* 1988). This feature distinguishes PS2 phosphorylation from the state transition, which relies on Cyt b_6f dependent LHC2 protein phosphorylation (Wollman and Lemaire 1988, Allen 1992). LHC2 and PS2 protein phosphorylations are dependent on the reduction of plastoquinone pool (Allen 1992, Ebbert and Godde 1996, Rintamaki *et al.* 1996). Many different roles have been suggested for PS2 phosphorylation, including spatial separation of PS2 complex between grana and stroma lamellae (Mattoo *et al.* 1989, Kruse *et al.* 1997, Summer *et al.* 1997).

Phosphorylated PS2 units are almost entirely restricted to stacked (grana) membranes. Their phosphoryl groups line the 5 nm "partition" gap between the stacked membranes. This partition forms part of the pathway for protons during photosynthesis (Polle and Junge 1986, Junge and McLaughlin 1987) with two protons entering PS2 for each PQ reduced. Many types of PS2 heterogeneity have been observed at the level of antenna and photochemistry, both at the donor and acceptor sides (Govindjee 1990, Hansson and Wydrzynski 1990, Krause and Weis 1991, Melis 1991). There are two types of heterogeneity in PS2 centres. The fast sigmoidal phase of Q_A reduction was related to PS2 α-centres and the subsequent slow phase to PS2 β-centre. PS2-α is located in grana partitions, whereas PS2-β in the stroma-exposed membrane regions (Melis 1991, Kruse *et al.* 1997). Analysis of experiments on stacking migration of PS2 polypeptides and LHC2 by PAR-regulated kinase activity *via* a redox control phenomena shows that the phosphorylated PS2 centres probably move enemas with the phosphorylated LHC2 acting as space shuttle between the stacked and un-stacked regions. The de-phosphorylation or reverse migration might also occur.

Phosphorylation of the PS2 core could be in part responsible for the reversible inactivation of 30 % electron transport under PAR. Under excess irradiance, the phosphorylation is a protective mechanism, the effect on each single PS2 polypeptide being different (Packham 1987, Giardi *et al.* 1994). Phosphorylation detaches the CP47 and CP43 from the PS2 core under strong irradiance (Giardi 1993), whereas under physiological irradiance these inner-antenna proteins were localised only in the appressed regions (Callahan *et al.* 1989). Re-synthesis and degradation of PS2 core proteins (particularly the D1 protein) may take place in the non-appressed stroma-exposed regions of the thylakoid membrane (Adir *et al.* 1990). Thus, the detachment of CP43 and CP47 observed under high irradiance could affect the cycle of degradation and re-synthesis of the PS2 core and movement from the appressed grana regions to the non-appressed stroma lamellae regions.

Under physiological and high PAR, D1 phosphorylation is a PAR-dependent step in the process of its degradation and turnover (Callahan *et al.* 1990, Elich *et al.* 1992, Misra 1993). The best-characterised function of the phosphorylation of D1 and D2 proteins is related to photoinhibition. In higher plants, phosphorylation protects these proteins against proteolytic degradation, and plays a role in the degradation repair cycle of D1 and D2 during photoinhibition (Schuster *et al.* 1988, Aro *et al.* 1993, Koivuniemi *et al.* 1995, Ebbert and Godde 1996).

Protein kinase and D1-protein turnover

D1 polypeptide, the 32 kDa protein of PS2 reaction centre, is characterised by extremely fast turnover under photoinhibitory conditions (Mattoo *et al.* 1984, Gong and Ohad 1995, Tyystjärvi *et al.* 1996). This degradation is believed to be due to proteolysis (Misra *et al.* 1991, Misra 1993) and/or to the radicals generated earlier under high irradiance (Sopory *et al.* 1990, Mishra and Ghanotakis 1994, Miyao 1994). The *in vivo* D1 degradation is efficient also in low irradiance (Sundby *et al.* 1993, Keren *et al.* 1995). In isolated pea thylakoids a stimulation of PS2 core protein degradation was found under conditions of thylakoid protein phosphorylation in low irradiance (Georgakopoulos and Argyroudi-Akoyunoglou 1997). The removal of LHC2 from the PS2 core proceeds *via* introduction of the repulsive negative charges of phosphate group in PS2 proteins, a process that renders the unprotected core proteins vulnerable to free radical attack (Georgakopoulos and Argyroudi-Akoyunoglou 1997). Extremely fast turn-over of the D1 protein of the PS2 heterodimer, observed in the light, is the result of monitoring the degradation of the D1. This degradation seems to be specific for the PS2 protein, and not for the P-subunits of the ATPase nor the LHC2 (Georgakopoulos and Argyroudi-Akoyunoglou 1997). Thylakoids pre-incubated in high irradiance and then incubated in the dark in the presence of ATP do not degrade D1 (Spetea *et al.* 1999), but in the presence of GTP this occurs, with accumulation of the 23-kDa degradation product. The GTP-activated Fts H protease may be involved in this process (Spetea *et al.* 1999). But protein phosphorylation seems to trigger the degradation, and this takes place in the light and not in the dark, unless the

kinase is activated. The ATP stimulation of PS2 core protein degradation may be in some way correlated with protein phosphorylation. D1 degradation is under redox control (Zer *et al.* 1994, Keren *et al.* 1995). On the other hand, kinase activity depends on the redox condition of PQ (Allen *et al.* 1981, Gal *et al.* 1997).

A close correlation observed between PS2 core protein degradation and kinase activity suggests that the two processes are under common control but not directly depend on each other. The kinase activation may lead to phosphorylation of PS2 proteins, which in turn result in disassembly of the PS2 unit and degradation of the heterodimer. Secondly, the PS2 core protein degradation may depend directly on kinase activation. The kinase activation may lead to phosphorylation of a degrading proteolytic system, active in its phosphorylated state (Georgakopoulos and Argyroudi-Akoyunoglou 1998). As to the first possibility, phosphorylation of PS2 proteins may introduce negative repulsive forces (phosphate groups) in PS2 units, leading to their disassembly; the unprotected core may then be attacked and replenished by newly synthesised proteins *via* activated transcription/ translation (Tyystjarvi *et al.* 1996, Georgakopoulos and Argyroudi-Akoyunoglou 1997). As to the second possibility, a mechanism that depends on the phosphorylation states of a degrading protease may be plausible. Whenever the thylakoid kinase activity is high, one might expect the protease to be also phosphorylated and to attack D1/D2. In the presence of kinase inhibitors, irrespective of their mode of action, the protease may be de-phosphorylated and inactive.

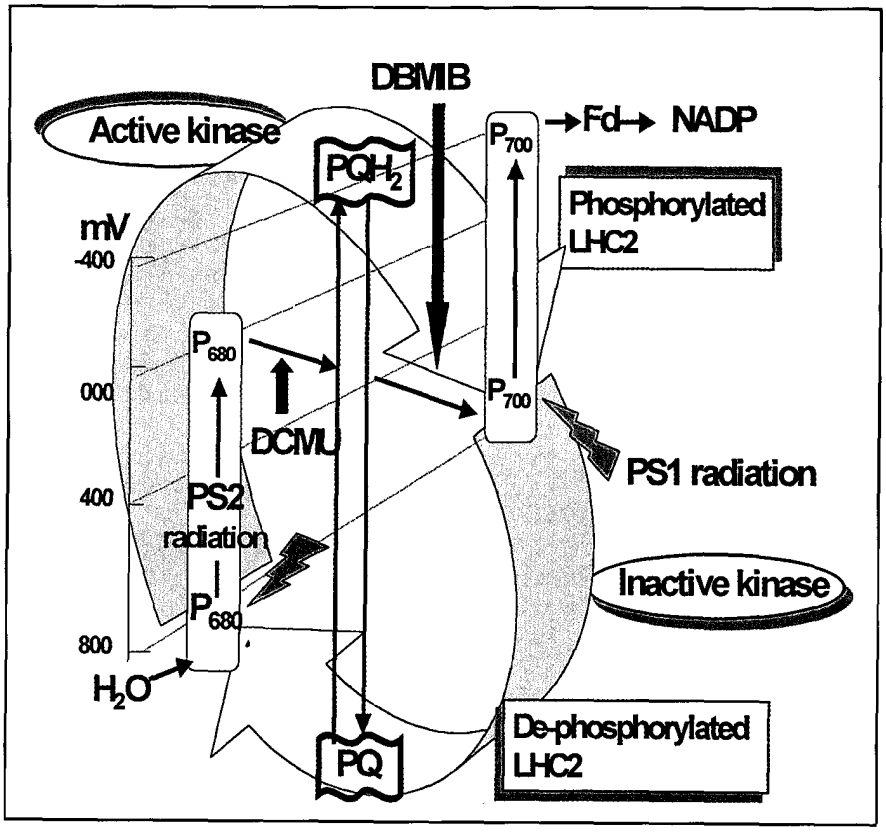

Fig. 2. Regulation of plastoquinone (PQ) redox reactions through photosystem (PS) 2 and PS1 electron transport in thylakoid membranes. The redox states of PQ regulate the active state of kinase and LHC2 phosphorylation. The LHC2 phosphorylation regulates the state transitions in thylakoid membranes.

Implications of D1 protein phosphorylation in chloroplasts

Under stress (water, irradiance) both the level of phosphorylation and the turnover rate of the D1 protein change. The phosphorylation and D1 turnover are strongly correlated so that phosphorylation could be responsible for the regulations of gene expression in higher plants (Allen 1993). The *psb*H protein participates in regulating and stabilising secondary electron transfer at the level of two PQ acceptors, Q_A and Q_B (Hodges *et al.* 1985, Packham 1987). Phosphorylation of PS2 core proteins reduces the ability of quinones and other artificial electron acceptors (as well as herbicides) to bind to the Q_B sites and as a result these PS2 centres become

artificial electron acceptors (as well as herbicides) to bind to the Q_B sites and as a result these PS2 centres become less efficient in electron transfer (Giardi *et al.* 1988, 1992). The decrease in quinone binding affinity in phosphorylated membranes also suggests that PAR energy causes the reduction in the PQ pool and activates the kinase, and the bound PQ may leave its site as a consequence of its reduction (Bennett 1991) and/or as a consequence of Q_B pocket phosphorylation. In this way phosphorylation of PS2 core proteins might be a mechanism of electron transfer regulation (Giardi *et al.* 1992). Some other effects of protein phosphorylation on PS2 core proteins are:

(*a*) increased stability (Jursinic and Kyle 1983) or reduced stability (Hodges *et al.* 1987) of Q_B^-, the anionic semi-quinone form of the secondary electron acceptor of PS2;

(*b*) increased negative surface charge density near the primary acceptor, Q_A;

(*c*) stimulation of a hydroxylamine sensitive cyclic flows of electrons around PS2 (Horton and Lee 1983);

(*d*) protection against photoinhibition (Horton and Lee 1985);

(*e*) decreased connectivity of PS2 units (Kyle *et al.* 1983); and

(*f*) inhibition of PAR-saturated electron transfer in PS2 (Horton and Lee 1984, Hodges *et al.* 1985, Packham 1987).

Evolutionary significance of signal transduction pathways in chloroplasts

The chloroplast genes *psb*A and *psb*D encode D1 and D2, the Chl binding proteins that comprise the reaction centre of PS2. Expression of these and other plastid and nuclear genes involved in photosynthesis increases co-ordinately with leaf and chloroplast development (Mullet 1988, 1993). PAR also regulates the initial accumulation of D1, P700, and CP43 during development in higher plants through the PAR-dependent accumulation of Chl, which is needed to stabilise Chl-binding proteins (Mullet *et al.* 1990, Kim *et al.* 1994). Once leaf and chloroplast biogenesis is complete, expression of most plastid genes decreases to levels needed for maintenance of the photosynthetic apparatus (Gamble *et al.* 1988). Synthesis of D1 and D2 is maintained at relatively high levels in mature chloroplasts of developed leaves (Gamble *et al.* 1988). Maintenance of high rates of synthesis of these proteins is needed to replace D1 and D2 sub-units that are damaged and turned over in irradiated plants (Mattoo *et al.* 1984, 1989, Ohad *et al.* 1985, Schuster *et al.* 1988).

Elevated D1 synthesis in mature chloroplasts is paralleled by high levels of *psb*A RNA (Mullet and Klein 1987, Gamble *et al.* 1988, Baumgartner *et al.* 1993). The relatively high abundance of *psb*A mRNA is due primarily to the unusual stability of these transcripts (Kim *et al.* 1993) and to a smaller extent to PAR-induced transcription (Klein and Mullet 1990). Even though *psb*A mRNA levels are relatively constant in dark and light in mature chloroplasts, D1 synthesis is light dependent and regulated at the levels of translation (Fromm *et al.* 1985, Malone *et al.* 1988). Irradiation regulates D1 polypeptide synthesis by ATP-dependent phosphorylation (Danon and Mayfield 1994a) and redox regulated association of an RNA-binding protein complex with the *psb*A RNA 5'-untranslated region (Danon and Mayfield 1994a,b, Constant *et al.* 1997). The stability of *psbA* mRNA is controlled directly or indirectly, more by the redox state of the inter-system electron carriers of the electron transport chain than by PAR (Mohamed and Jansson 1989, Alfonso *et al.* 2000). The decrease in the steady-state levels of *psa*E, *cpc*BA (Alfonso *et al.* 2000), *psb*D, and *rbc*L-S (Mohamed and Jansson 1989) mRNAs was faster than that of the *psb*A mRNA in darkness. This suggests that *psb*A transcript is more stable than that of other mRNAs, even when general transcription is not inhibited.

Alfonso *et al.* (2000) postulated the following hypotheses for the stabilty of *psb*A mRNA:

(*a*) binding of the factor that influences the stabilisation of mRNA depends directly on the redox state of the thylakoid electron transport chain;

(*b*) active degradation of the mRNA depends on D1 translation which progressively decreases in the dark. The *psb*A transcriptional stability increases if *psb*A mRNA translation is inhibited by PAR in the presence of lincomycin or after prolonged irradiation stress (Constant *et al.* 1997).

The formation of translation complexes could be a mechanism to initiate and/or facilitate the turnover of *psb*A mRNA in cyanobacteria or higher plants (Alfonso *et al.* 2000).

Fig. 3 depicts the redox control of photosynthetic genes that could support a novel mechanism for physiological and developmental adjustment of photosystem stoichiometry (Pfannschmidt *et al.* 1999). There is an evolutionary significance of these control systems and signal transduction pathways in chloroplasts. The ancestor of eukaryotic cells acquired many genes upon its merger with the eubacterial ancestors of chloroplasts. Most of the genes were subsequently transferred to the cell nucleus, but a small and relatively constant sub-set of genes remained *in situ*. The chloroplast genetic system permits a direct redox regulation of these genes within the organelle (Allen and Raven 1996). These are the reminiscence of transcriptional control in prokaryotic systems. Pfannschmidt *et al.* (1999) postulates that a rapid and direct regulatory coupling of redox control of photosynthetic gene expression may depend upon the genes concerned present in the same intracellular compartment

the persistence of the prokaryotically derived genes in the chloroplasts and ultimately the dynamics of photosynthetic membrane protein complexes.

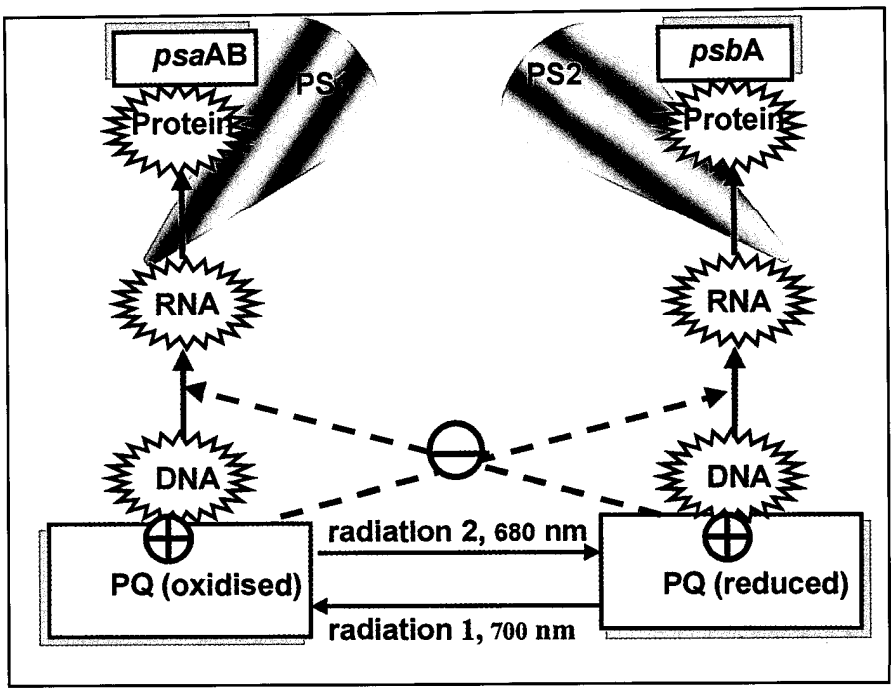

Fig. 3. Redox control of photosynthetic gene expression that regulates the photosystem stoichiometry in chloroplasts. The positive and negative regulation of gene expression is shown in the circles. The *dotted arrows* indicate the signal for negative control and the *continuous arrows* depict the positive regulation of photosynthetic gene expression in photosystem (PS) 1 and PS2.

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