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Greening of intermittent-light-grown bean plants in continuous light: Thylakoid components in relation to photosynthetic performance and capacity for photoprotection

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Phaseolus vulgaris (cvv. Windsor longpod and snap bean) plants, etiolated during germination, were exposed to intermittent light (2 min light every 2 hr) for up to 68 hr and then transferred to continuous white light. On transfer of the plants to continuous light (100 photons μmol m^{-2} s^{-1}, 24°C), the quantum yield of oxygen evolution increased two-fold in about 30 hr. The chlorophyll content per unit leaf area or unit fresh weight increased dramatically, but the fresh weight per unit leaf area was relatively constant. The changes were expressed on the basis of fresh weight or leaf area. On this basis, the contents of photosystem (PS) I and II increased in continuous light, by a factor of 3 and 8, respectively. While the chlorophyll *b* content and the contents of apoproteins of light-harvesting chlorophyll-protein complexes (LHCIIb, CP29, CP26 and CP24) increased markedly, neither the total carotenoid content nor the de-epoxidation state of the xanthophylls [ratio of zeaxanthin(Z) + antheraxanthin(A) to (Z+ A + violaxanthin) was about 0.4)] responded significantly on transfer to continuous light. The fast rise of the flash-induced electrochromic signal (ΔA518) was well correlated with the increases in PS I and PS II reaction centres, and with chlorophyll *b* and total carotenoid contents. The increase in the quantum yield of oxygen evolution during greening in continuous light is attributed to a more balanced distribution of excitation energy between the two photosystems, facilitated by the increased number of PS II units, the increased antenna size of each unit and the enhancement of grana formation. The chloroplast in intermittent light was found to contain abundant xanthophyll cycle pigments and the *psbS* gene product, presumably adequate for photoprotection in continuous light as soon as chlorophyll *a/b*- protein complexes are synthesized. The results suggest that greening in continuous light is accompanied by adjustments that include enhanced quantum efficiency of photosynthesis and development of a capacity for harmless dissipation of excess excitation energy.

Introduction

Argyroudi-Akoyunoglou and Akoyunoglou[1] first showed that bean plants developed under intermittent light (cycles of 2 min light and 98 min dark) preferentially accumulated Chl *a* in the green plastids. Subsequently, Armond *et al.*[2] observed that pea plants grown under cycles of 2 min light and 118 min dark developed similarly. They further showed that the photochemical efficiency of light-limited whole-chain electron transport in isolated thylakoids was low, due to a lack of regulation of energy distribution between the two photosystems, but that continuous light induced grana formation and an increase in photosynthetic unit size. Intermittent-light-grown plants are thought to have fully active photosynthetic reaction centres which are served by the core antennae that are devoid of most of the peripheral light-harvesting antenna[2-6].

Early studies of intermittent-light-grown plants generally employed *in vitro* isolation techniques to investigate the relationships between the composition, structure and function of plastids. The results of such *in vitro* studies could be influenced by isolation artefacts in preparations of fragile plastids from intermittent-light-grown plants. With the availability of modern instrumentation, attention has shifted to *in vivo* measurements, mainly of chlorophyll *a* fluorescence[6-9] and P700 photo-oxidation[9] in

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Abbreviations used – A, antheraxanthin; Chl, chlorophyll; cyt, cytochrome; D1, product of the *psbA* gene; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; NPQ, non-photochemical quenching; P700, special chlorophyll pair in the PS I reaction centre; P_{max}, light- and CO_2-saturated rate of oxygen evolution; PS, photosystem; V, violaxanthin; Z, zeaxanthin.

intermittent-light-grown plants. Thus, it has been demonstrated *in vivo* that pea plants grown in intermittent light (cycles of 2 min light and 118 min dark) but not in flash light (cycles of 1 ms-flash every 15 min dark) have fully developed reaction centres with functional oxygen "clocks", and that upon transfer to continuous light, the number of PS II units and their connectivity increase[9].

In the present investigation of bean plants transferred from intermittent light to continuous light, we aimed to measure (1) the development of the quantum efficiency of oxygen evolution *in vivo*; (2) the changes in the contents of functional photosystem (PS) I and II reaction centres *in vivo*; (3) the functionality of PS I and PS II *in vivo* during greening, by measurement of the electrochromic signals associated with stable charge separation in the two photosystems in leaves; and (4) the capacity for non-photochemical quenching of chlorophyll fluorescence in relation to the pigments and the proteins to which the pigments are bound.

Materials and Methods

Plants

Seeds of *Phaseolus vulgaris* (cvv. Windsor longpod and snap bean) were germinated in darkness at 21°C in a seeding mix covered with vermiculite. Seven days after sowing, the seedlings were exposed to 2 min intermittent light (50 μmol photons m^{-2} s^{-1}) every 2 hr for 66 hr (sometimes 68 hr). At a time indicated by a vertical line in the figures, the seedlings were transferred to continuous light in a growth cabinet (100 μmol photons m^{-2} s^{-1}, 24°C). When seeds of cv. Windsor longpod became unavailable, we used *Phaseolus vulgaris* cv. snap bean which gave comparable responses during greening. Results obtained with both cultivars were pooled whenever appropriate.

Oxygen measurements

The number of functional PS II complexes was determined by using a leaf disc oxygen electrode (Hansatech, King's Lynn, Norfolk, UK) and the method of Chow *et al.*[10]. A leaf segment was dark treated for about 10 min in moist air enriched with 1% CO_2 and illuminated with a train of single-turnover, saturating white light flashes (10 Hz, 2.5 μs full-width at half-peak height; xenon lamp FX 200, EG & G Electro Optics, USA) in the presence of background far-red light[10]. The flashes were delivered for 4 min, followed by 4 min darkness, and this alternation was repeated. A small heating artefact was taken into account, while any limitation to linear electron flow due to PS I was removed by the background far-red illumination. After determination of the functional PS II content, the leaf segments were illuminated with continuous white light from a projector (900 μmol photons m^{-2} s^{-1}) to estimate the maximum capacity for gross oxygen evolution (P_{max}). The gross rate of oxygen evolution was estimated after taking the dark drift (mainly due to dark respiration) into account. Functional PS II contents and P_{max} rates were expressed on the basis of either fresh weight or chlorophyll content; the latter was determined in 80% buffered acetone[11]. The results from up to four batches of seedlings were pooled.

The quantum yield of oxygen evolution on an absorbed light basis was determined by measuring rates of oxygen evolution in limiting light, the irradiance of which was varied by neutral density filters. Leaf absorbance was calculated from the transmittance and reflectance of a leaf segment obtained with an integrating sphere. In this experiment, only the cultivar snap bean was used.

Measurement of P700 absorbance changes at 820 nm

Photo-oxidizable P700 (in relative units on a leaf area basis) was measured with a modified pulse modulation fluorometer (PAM 101, 102, Walz, Effeltrich, Germany) fitted with an 820 nm light emitting diode to provide the modulated measuring light. The modulated signal was measured in the reflectance mode[12]. To photo-oxidize P700, leaves were first illuminated for 19 s with far-red light (170 μmol photons m^{-2} s^{-1}) from a projector (Schott KL 1500), transmitted through an RG 9 filter (Schott). To ensure complete oxidation of P700, it was necessary to superimpose strong actinic light which was admitted for 1 s (after 19 s of far-red light) by an electronic shutter[13]. The white actinic light (6500 μmol photons m^{-2} s^{-1}), filtered through a Calflex C heat reflection filter, momentarily oxidized P700 completely. Then both far-red light and white light were turned off, and P700 relaxed to a completely reduced state within about 1 s. The modulated signal from the PAM fluorometer was off-set, then amplified 20-fold and recorded by a digital storage oscilloscope (Gould Type 1421, Hainsault, England) before being plotted on a chart recorder.

The quantification of P700 content was performed essentially as described previously[14], by the measurement of photo-oxidisable P700 in isolated

thylakoids in the presence of a detergent and electron donors and electron acceptor, methyl viologen. To obtain consistent and maximal P700 content, a cocktail of protease inhibitors (leupeptin, 1 mg/ml in water; pepstatin A, 2 m*M* in methanol; phenylmethanesulfonyl fluoride, 200 m*M* in ethanol; aminocaproic acid, 100 m*M* in water; and 5'-*p*-fluorosulfonylbenzonyl adenosine, 1 mg/ml in water) consisting of 0.50 ml of each stock solution was included in 100 ml of isolation buffer[15].

Electrochromic signals

Laser-flash-induced absorbancy changes (electrochromic shifts) at 518 nm were measured by directing the measuring beam at 45° to the leaf surface; the actinic laser flash was also directed at 45° to the leaf, and at 90° to the measuring beam. Usually 16 flashes at 0.2 Hz were given to leaf segments that had been dark adapted for 20 min to stabilize the conductance of the ATP synthase complexes; otherwise, the relatively fast decay of the 518 nm signal, which depends on thylakoid ionic conductance, tended to obscure the slow rise in the signal. Data were digitised and stored in computer files, as previously described[16].

Measurement of non-photochemical quenching, NPQ

Non-photochemical quenching of chlorophyll fluorescence (NPQ) in leaves was measured using a PAM fluorometer (101 ED, Heinz Walz, Effeltrich, Germany). NPQ was determined (see eg., Gilmore *et al.*[17]) as (F_m/F_m' − 1), where F_m' was determined at the end of the five minute light period before the light was extinguished and F_m after a dark period of five minutes following the five minute light period. The irradiance used to drive NPQ was 1000 µmol photons m^{-2} s^{-1} of white light (passed through a heat absorbing Walz DT Cyan filter). The saturating (1 s) light pulse intensity used to close the PSII traps and determine F_m and F_m' was 10 000 µmol photons m^{-2} s^{-1} of white light, also passed through a Walz DT-Cyan filter. Prior to the light treatments, the leaf pieces were dark adapted for 30 min with background illumination by a weak far-red LED source (PAM 102, λ = 700 nm, irradiance = 15 µmol photons m^{-2} s^{-1}). The far-red background source remained actuated during and following the light period to ensure that all PSII traps were open. All dark adaptation and light treatments were performed in a humidified leaf disk chamber at approximately 20°C. Chloroplast pigments from matching leaf pieces, both before and following the light treatments, were extracted and analyzed by high performance liquid chromatography according to Gilmore and Yamamoto[18].

Isolation and assay of proteins

Thylakoid membranes were isolated as described by Adamska *et al.*[19]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli[20] using the Hoeffer mini-gel system. Samples were loaded on an equal protein basis. Gels were either stained with silver nitrate according to Oakley *et al.*[21] or immunoblotted according to Towbin *et al.*[22] using polyvinylidene difluoride (PVDF) membrane. Blots were incubated with various antibodies, and the antigen-antibody complex detected by the enhanced chemiluminescence method (Amersham Corp.).

Results

During intermittent illumination, the Chl content per unit fresh weight remained low, but increased by an order of magnitude over the subsequent 100 hr of continuous illumination (Fig. 1A), whereas the Chl *a*/Chl *b* ratio decreased drastically in a few hours (Fig. 1B). In contrast, the fresh weight per unit leaf area was relatively constant in both intermittent and continuous light (Fig. 1C). Hence, expressing changes in photosynthetic parameters on the basis of either fresh weight or leaf area gives a more accurate measure of the increases in contents of photosynthetic components than on the basis of chlorophyll.

Photosynthetic activities

In plants grown under intermittent light, the quantum yield of O_2 evolution of leaf discs, in limiting light but saturating CO_2, was less than 0.04 mol O_2 per mol photons absorbed. Upon illumination with continuous light, it increased steadily to almost 0.10 after about 30 hr of continuous light (Fig. 2). That is, the quantum efficiency of light-limited photosynthesis improved with development of the photosynthetic apparatus during greening. The minimal quantum requirement reached a value of 10 per O_2 evolved (see a historical perspective[23]). Our observations *in vivo* agree with those of Armond *et al.*[2] who showed that the quantum efficiency of whole-chain electron transport in isolated thylakoids differed by a factor of 2 between intermittent-light-grown peas and those exposed to 24 hr of continuous light.

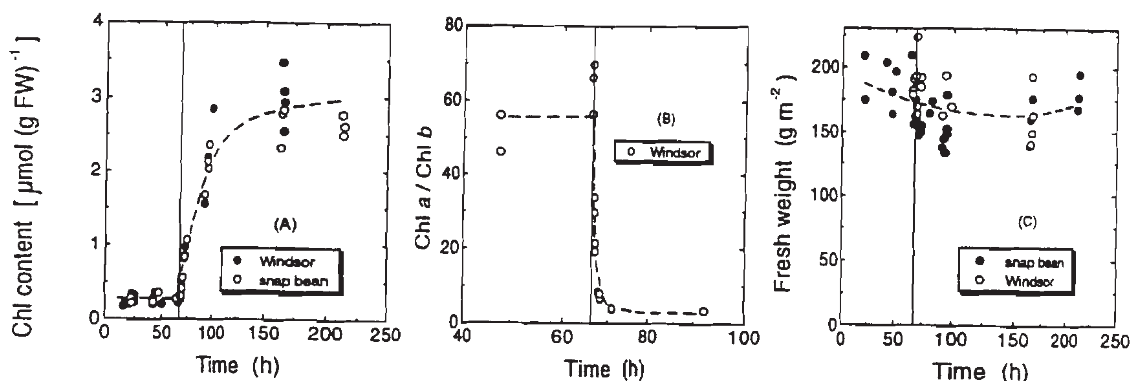

Fig. 1—Changes in chlorophyll content on a fresh weight basis (A); Chl *a*/Chl *b* (B); in fresh weight per unit leaf area (C) after transfer of intermittent-light-grown bean plants to continuous light. Plants were transferred after 66 hours of intermittent light (2 min every 2 hr), at the time indicated by a vertical line.

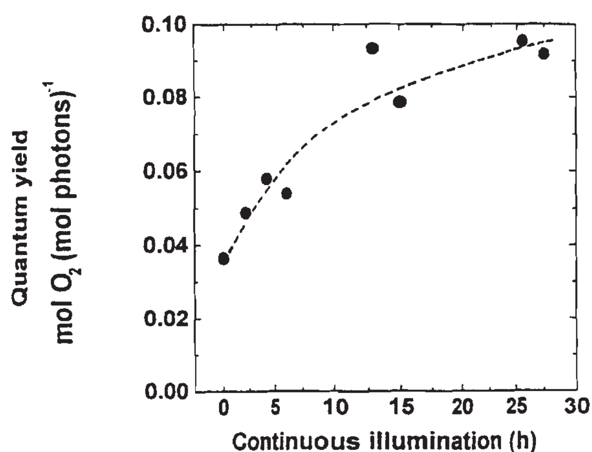

Fig. 2—Improvement in the quantum yield of O_2 evolution on an absorbed light basis following transfer of intermittent-light-grown bean plants to continuous light. The quantum yield was measured in leaf discs in limiting light and 1% CO_2.

The light- and CO_2-saturated rate of O_2 evolution (P_{max}, the photosynthetic capacity) on a fresh weight basis was also low in plants grown in intermittent light; however, it increased relatively abruptly by a factor of about 3 within about 30 hr in continuous light (Fig. 3A). When expressed on a Chl basis, P_{max} was high in plants grown under intermittent light; it decreased steadily in continuous light as the Chl content increased (Fig. 3B).

The two photosystems

The number of functional PS II complexes on a fresh weight basis was low in plants grown under intermittent light. It increased by about 5-fold after 30 hr and about 8 fold after 90 hr of continuous light (Fig. 3C). Owing to the low Chl *a* content per unit fresh weight and the near-absence of Chl *b*, the functional PS II content per unit Chl was generally high (4 nmol per mol Chl) in plants grown under intermittent light. However, it decreased to a value of about 2.7 mmol per mol Chl (1 PS II per 370 Chl molecules) after 100 hr of continuous light (Fig. 3D).

The absorbance change at 820 nm, induced by a combination of continuous far-red light and a brief flash of strong white light, gives a measure of photo-oxidisable P700, the primary electron donor in PS I[13]. This signal is a relative measure of the P700 content in the leaf area (1.2 cm^2) defined by the combined end of the multifurcated light guide used in conjunction with the PAM fluorometer. Fig. 3E shows that the signal on a leaf area basis increased abruptly in the early period (4 hr) of continuous light, and relatively slowly for the next 50 hr or so. On a Chl basis, the P700 signal declined on illumination with continuous light (Fig. 3F).

In a separate batch of plants (cv. Windsor), the content of P700 in thylakoids, isolated from plants fully greened under 100 μmol photons m^{-2} s^{-1}, was determined to be 0.62 μmol m^{-2} [or 1.41 mmol P700 (mol Chl) $^{-1}$], while the functional PS II content was 1.34 μmol m^{-2}, i.e. PS II/PS I = 2.2, as measured by the methods used in this study.

The electrochromic shift

When a transmembrane electric field is established at PS II and PS I reaction centres following charge separation, pigments such as Chl *b* and carotenoids undergo an electrochromic shift in response to the subsequently delocalized electric field, giving the fast-rise phase of ΔA_{518} after a flash (applied at t = 0 in Fig. 4A). The magnitude of this fast phase depends on both the numbers of PS II and PS I capable of undergoing stable charge separation and the number of sensing pigments (Chl *b* and carotenoids). It is seen in Fig. 4A that the fast phase was very small (≤ 1 unit) at or before the beginning of continuous illumination (zero hr). After 5 hr of continuous illumination,

however, it had increased to about half the maximum value (Fig. 4A and B), which was reached after about 27 hr or more of continuous light.

After a few hours of continuous light, it was possible to see a slow rise of ΔA_{518} in addition to the fast phase. This slow rise phase also reached a maximum value after about 27 hr or more of continuous light. Significantly, the ratio of the slow to the fast phase increased with time in continuous light (triangles in Fig. 4B).

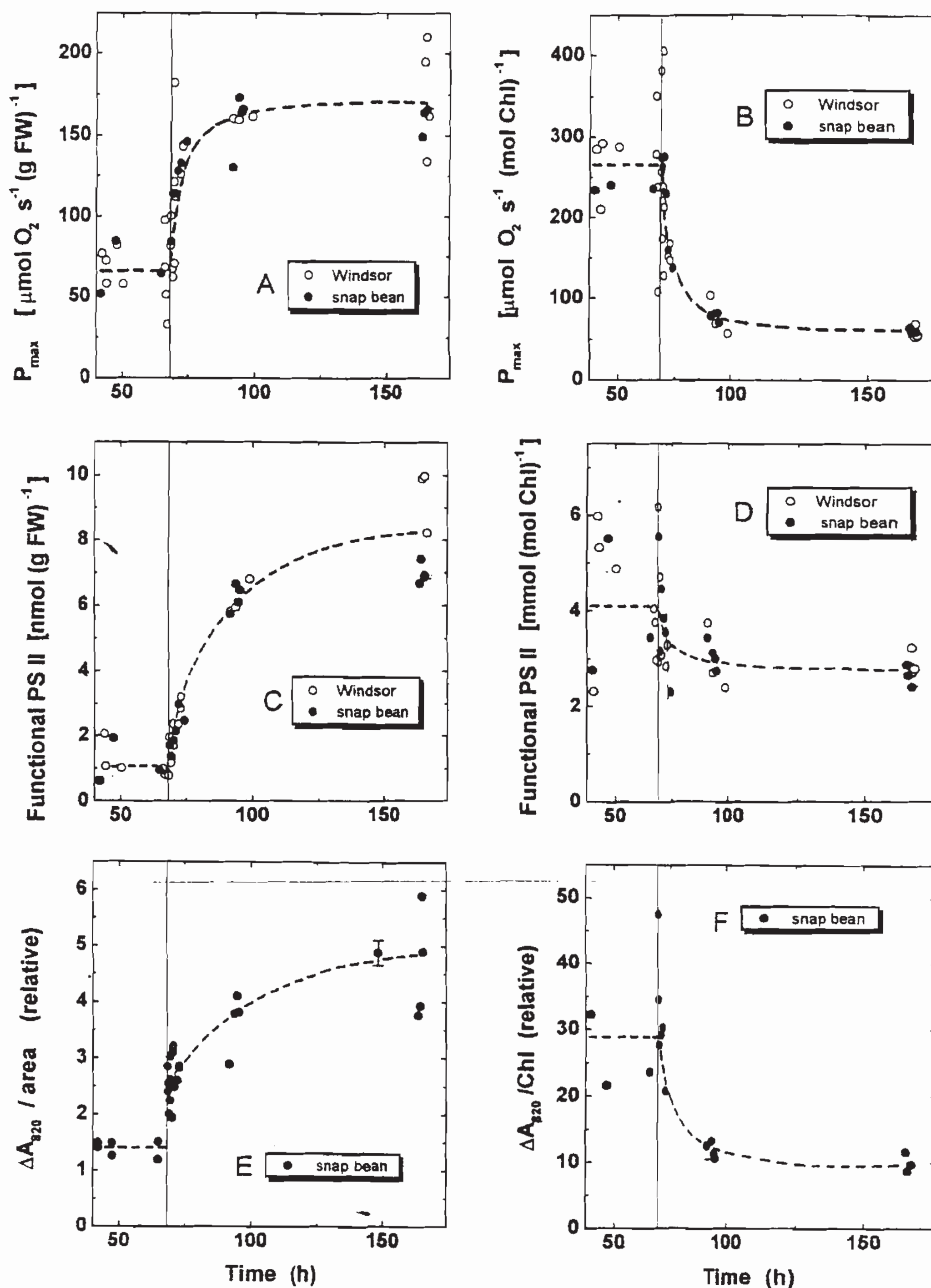

Fig. 3—Changes in light- and CO_2-saturated photosynthetic capacity (A, B), and contents of functional PS II (C, D) and PS I (E, F) after transfer of intermittent-light-grown bean plants to continuous light. Values of the parameters in the left panels are plotted on either a fresh weight or leaf area basis, while those in the right panels are expressed on a chlorophyll basis. The vertical lines indicate the time of transfer from intermittent to continuous light.

Nonphotochemical quenching and the pigments

A measure of the non-radiative dissipation of excess excitation energy is given by NPQ, the non-photochemical quenching of chlorophyll *a* fluorescence. As can be seen in Fig. 5A, NPQ (measured at 1000 µmol photons m^{-2} s^{-1}) was very low in plants grown under intermittent light; however, it increased rapidly by an order of magnitude upon transfer of plants to continuous light (100 µmol photons m^{-2} s^{-1}) for 10 hr or more. The increase in NPQ with greening time in continuous light was not accompanied by a corresponding change in the de-epoxidation status [i.e. (Z + A)/(Z + A + V)], which was measured after applying an actinic light (1000 µmol photons m^{-2} s^{-1}) for 5 min and which was already at a high value (~0.4) in plants from intermittent light (Fig. 5B). Rather, the rapid increase in NPQ on transfer to continuous light was matched by a rapid decline in the Chl *a*/Chl *b* ratio, shown by a correlation between NPQ and the Chl *a*/Chl *b* ratio in Fig. 5C.

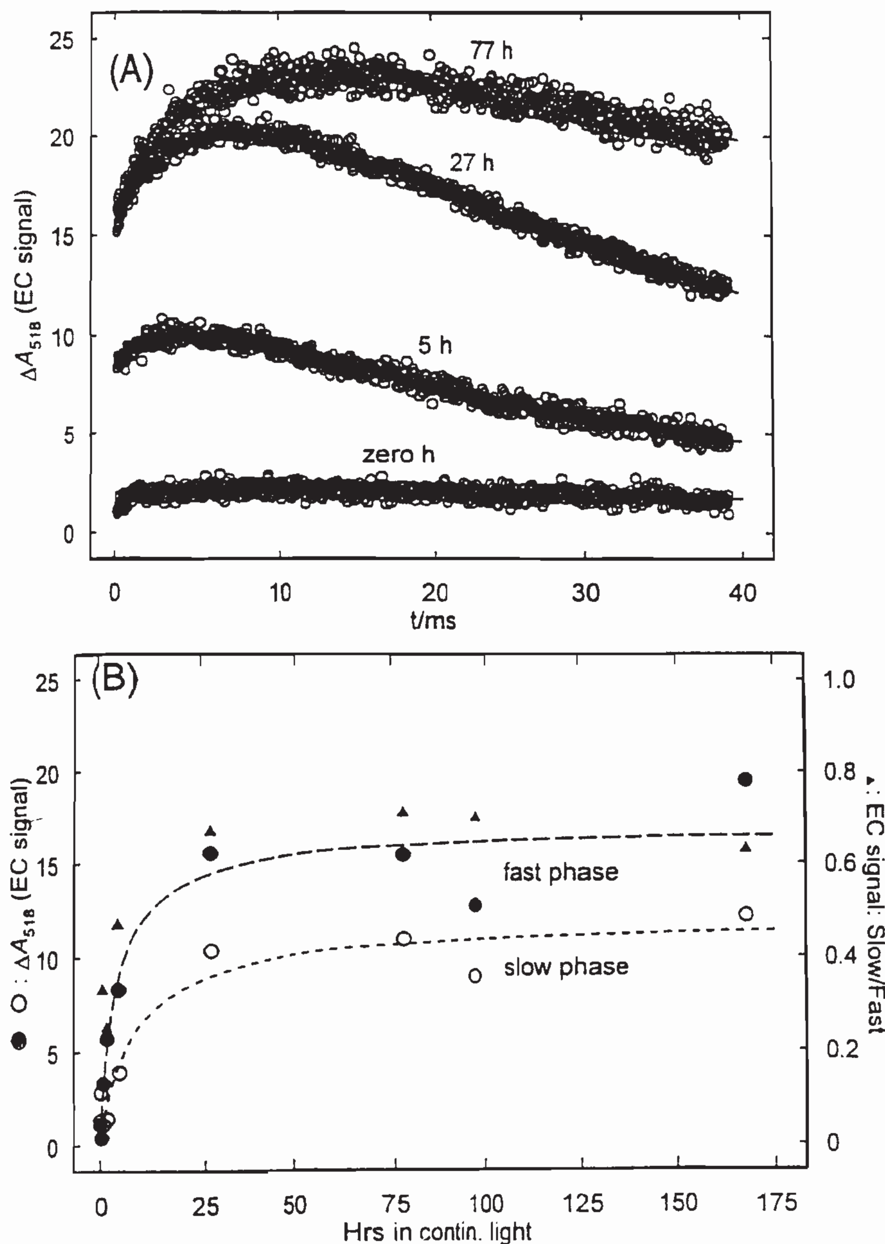

Fig. 4— (A) . Time courses (t in ms) of the flash-induced electrochromic signal after transfer of intermittent-light-grown bean plants to continuous light (transfer at zero hour). In (B), the rise of the electrochromic signal is separated into a fast phase (•) and a slow rise (o) as a function of time after transfer to continuous light. The ratio of the fast to the slow rise is also shown (solid triangles).

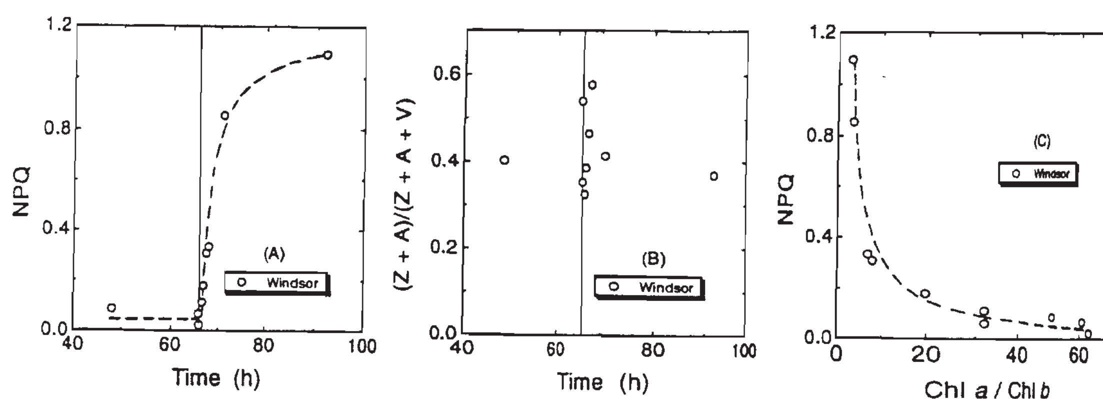

Fig. 5—Changes in (A) NPQ and (B) the de-epoxidation status after transfer of intermittent-light-grown bean plants to continuous light. Plants were transferred after 66 hours of intermittent light (2 min every 2 hr), at the time indicated by a vertical line. In (C), the correlation of NPQ with the Chl *a*/Chl *b* ratio is shown.

Table 1—Relative increases in the contents of some thylakoid proteins in PS II and carotenoids (Car) before and after transfer of bean plants from intermittent light (IML) to continuous light (CL). PSII-S is the *psbS* gene product, believed to play a direct role in ΔpH- and xanthophyll-dependent nonphotochemical quenching.

Apoprotein	Etiolated	24 hr IML	43 hr IML	66 hr IML	1 hr CL	2 hr CL	4 hr CL	6 hr CL	30 hr CL
LHC IIb	–	–	–	±	+	++	+++	+++	+++
CP29	–	+	+	++	+++	+++	+++	+++	+++
CP26	–	–	±	+	+++	+++	+++	+++	+++
CP24	–	–	–	–	–	+	++	+++	+++
PSII-S	+	+	++	+++	+++	+++	+++	+++	+++
Z + A + V (μmol m^{-2})	22.7	21.5	24.5	20.2	22.7	23.9	22.1	Nm	14.7
Total Car (μmol m^{-2})	67.6	74.2	88.6	74.4	82.4	89.8	114	Nm	114

Nm, not measured

Proteins

As shown in Table 1, the apoprotein of LHCIIb, the major light-harvesting chlorophyll *a*/*b*-protein complex associated with PS II, was scarcely detectable in leaves from plants grown under intermittent light. Upon transfer of plants to continuous light, it increased rapidly on a total thylakoid protein basis, reaching a maximum in approx. 4 hr. Of the three minor chlorophyll-protein complexes, the apoprotein of CP 29 and CP 26 increased even more rapidly, reaching a maximum at 1 to 2 hr of continuous light. The apoprotein of CP 24, in contrast, increased more slowly. Interestingly, PS II-S (*psbS* gene product), a pigment-binding protein essential for the regulation of photosynthetic light-harvesting[24], increased gradually during development in intermittent light, reaching a maximal amount by 66 hr of intermittent light; thereafter, its content seemed to increase little on transfer of the plants to continuous light (Table 1).

Table 1 also shows that the total content of the xanthophyll cycle pigments did not change significantly on transfer of plants from intermittent to continuous light; the total carotenoid pigments increased by about 45%.

Discussion

Quantification of the two photosystems in vivo

Since the chlorophyll content rapidly increased on a fresh weight basis on transfer from intermittent to continuous light, quantification of the two photosystems on the basis of chlorophyll does not reflect the increases in the reaction centres. On the other hand, expressing the contents of the two photosystems on either a leaf area basis or a fresh weight basis gives a good measure of the build up of reaction centres during illumination with continuous light. Thus, while PS II reaction centres in the case of intermittent growth light were capable of oxygen evolution in repetitive flashes, the number of such centres on a fresh weight basis was low. Only after transfer of plants to continuous light did the functional PS II content increase several fold (Fig. 3C). Similarly, PS I, as measured by the P700 photooxidation signal at 820 nm on an equal leaf area basis,

also increased by at least a factor of 3 in steady-state continuous illumination (Fig. 3E). Clearly, the near tripling of the light- and CO_2-saturated rate of oxygen evolution (Fig. 3A) must have been due to the massive increases in the two photosystems and other proteins, e.g. the content of cytochrome *f* matches that of PS II for samples grown in intermittent light separated by dark intervals of about 2 hr or shorter[3].

The low quantum yield of O_2 evolution in leaves developed under intermittent light

It is well known that naturally-grown C_3 plants of diverse origins in the absence of environmental stress have a similar optimal quantum yield of light-limited oxygen evolution, namely *ca.* 0.10 mol O_2 /(mol photons absorbed)[25,26]. In snap bean grown under intermittent light, the quantum yield of oxygen evolution was only about one-third of this value (Fig. 2). There are a number of reasons for the less-than-optimal quantum yield of light-limited oxygen evolution in intermittent-light-grown leaves. (1) Non-photosynthetic tissue and any free pigment unconnected to the reaction centres may absorb a significant fraction of the light when the photosynthetic pigment content is low. (2) Excessive spillover of excitation energy from PS II to PS I may occur due to poor granal stacking, leading to unbalanced excitation of the two photosystems. (3) The small antenna size of each PS II (LHCIIb being absent), as well as the low ratio of PS II to PS I reaction centres, also contributes to the imbalance of energy distribution. On an arbitrary scale, in continuous light at steady state, PS II/PSI ≈ 7/5 ≈ 1.4 (from Fig. 3C, 3E, snap bean), but in intermittent light, PS II/PSI ≈ 1/1.3 ≈ 0.8 arbitrary units (from Fig. 3C, 3E). That is, the PS II: PS I stoichiometry is lower in intermittent light, excessively favouring light absorption by PS I.

The small extent of the fast rise of the electrochromic signal in plants grown in intermittent light

The fast phase of the rise in the electrochromic signal is a good measure of the combined contributions of both photosystems to electrogenic charge transfer across the thylakoid membrane. Thus, if PS II is selectively inhibited, the remaining fast rise is due to the PS I reaction centres, and the relative contributions from the two photosystems agree well with the photosystem stoichiometry determined independently[27]. In intermittent-light-grown beans, the fast rise was scarcely detectable (solid circles, time 0 hr in Fig. 4B). Based on our quantification of the photosystems and measurements of the pigments which are likely to give the electrochromic response, it is possible to predict the size of the fast signal. as follows.

In continuous light, the functional PS II content reached approx. 8 nmol (g FW)$^{-1}$ (Fig. 3C). Taking the fresh weight to be 170 g m^{-2} (Fig. 1B), the PS II content is 1.35 μmol m^{-2}. By determining the P700 content of isolated thylakoids on a chlorophyll basis. and the chlorophyll content per unit leaf area in the same leaves used for thylakoid isolation, the P700 content of fully-greened leaves was measured to be 0.62 μmol m^{-2}. Therefore, the total content of reaction centres in continuous-light-grown plants was 1.35 + 0.62 = 1.97 μmol m^{-2}. In intermittent-light-grown plants, the functional PS II content was about 1 nmol (g FW)$^{-1}$ (Fig. 3C). Taking the fresh weight to be 180 g m^{-2} (Fig. 1C), the PS II content was 0.18 μmol m^{-2}. The PS I content in intermittent-light-grown plants can be estimated from Fig. 3E to be 0.62 × (1.3/5) = 0.16 μmol m^{-2} since it is smaller than that in continuous light by a factor of about 1.3/5. The total content of reaction centres in intermittent light was therefore 0.34 μmol m^{-2}. That is, there were 1.97/0.34 = 5.8 times as many reaction centres per unit leaf area in continuous light as in intermittent light. If the maximum size of the fast rise of ΔA_{518} is 16 units (Fig. 4B) in continuous light, then assuming for the moment that the sensing pigments (Chl *b* plus carotenoids) were equal in amount in both light regimes, we expect to observe 16 × (1/5.8) = 2.8 units in intermittent light. However, in intermittent light the total content of carotenoids ≈ 79 μmol m^{-2} (Table 1), and Chl *b* ≈ 0. On the other hand, in continuous light in the steady state, the content of carotenoids ≈ 114 μmol m^{-2} (Table 1) and Chl *b* ≈ 130 μmol m^{-2} (not shown), adding up to a total of 244 μmol m^{-2}. Assuming that each carotenoid molecule is as effective as each Chl *b* in the electrochromic response, we expect to measure 2.8 × (79/244) units for the fast rise phase in intermittent-light leaves, i.e. 0.9 units, approximately what was observed (Fig. 4B). Thus, we conclude that the fast rise phase of the flash-induced electrochromic signal is a good measure of the stable, electrogenic charge separation across the thylakoid membrane in PS I and PS II reaction centres, in both light regimes.

The slow rise of the electrochromic signal

The slow rise in the electrochromic signal is most easily interpreted as being due to electrogenic electron transfer in the cytochrome *bf* complex as part of a Q-cycle or a similar cycle. Hope and Rich[28] have shown

in chloroplasts that both the slow electrochromic signal and cyt b_{563} reduction are accompanied by proton uptake sensitive to cytochrome *bf* inhibitors such as DBMIB. Fig. 4 shows that the slow rise phase increased gradually relative to the fast phase (see triangles), suggesting that activity of a Q-cycle or a similar cycle might not have been constant. After 25 hr or more in continuous light, the ratio of the slow phase to the fast phase reached *ca*. 0.7; this value is higher than the value of *ca*. 0.3 for control tobacco leaves (Fig. 1A in Chow and Hope[27]). Q-cycle activity alone does not seem to explain such a large ratio of 0.7. Assuming a maximum PS II/PS I ratio of 2.2 for fully greened beans (see Results) and that one electron reduces cyt b_{563} per pair of electrons originating from PS II, the slow phase should be 1.1 unit relative to the total fast phase of (2.2 + 1) units due to 2.2 PS II and 1 PS I, i.e. 1.1/(2.2 + 1) = 0.3. The higher ratio of slow phase/fast phase = 0.7 could have had a contribution from oxidation, at the cyt *bf* complex, of plastoquinol formed by electrons and protons on the acceptor side of PS I. That is, cyclic electron flow via PS I may result in more electron transfers to cyt b_{563} and an increased slow phase of the electrochromic signal. If so, given the variable ratio of slow phase/fast phase during greening, it seems that Q-cycle activity or cyclic PS I electron flow, or both, may vary according to growth conditions and developmental stages in plants.

Relationship of NPQ to the Chl b content

On transfer to continuous light, the increase in NPQ (Fig. 5A) was not well correlated with total carotenoid content or the xanthophyll cycle pigments (Table 1), or the de-expoxidation status (Fig. 5B). Rather, the increase in NPQ was well correlated with the Chl *a*/Chl *b* ratio (Fig. 5C) or the Chl *b* content itself (not shown). The correlation of NPQ with the Chl *a*/Chl *b* ratio is consistent with observations that maximum NPQ in sun and shade leaves is linearly correlated with photoconvertible violaxanthin which is in turn linearly correlated with the Chl *a*/Chl *b* ratio[29].

In plants grown only in intermittent light, however, the de-epoxidation state was already at a high value of 0.4, and was little changed upon transfer of plants to continuous light (Fig. 5B). Presumably, when the de-epoxidation state was at a high value, other factors were limiting NPQ in leaves developed under intermittent light. Indeed, Jahns and Schweig[7] have previously shown that plants grown in intermittent light are capable of forming large amounts of zeaxanthin, but energy-dependent quenching is substantially suppressed. Presumably, maximal NPQ is achieved when violaxanthin de-epoxidation, establishment of a ΔpH[17,30,31], the presence of the *psbS* gene product[24] and formation of inner and peripheral LHCII complexes (hence the dependence on Chl *b*) all occur together, these requirements being collectively achieved only in fully-greened tissue.

Significantly, the photosynthetic apparatus during growth in intermittent light was already equipped with abundant xanthophyll cycle pigments in a high de-epoxidation state. The *psbS* gene product, previously shown to be capable of binding chlorophyll[32], was found to be stable even in the absence of chlorophyll and carotenoids[33], and believed to be directly involved in ΔpH- and xanthophyll-dependent quenching of excitation energy[24], was also abundant (Table 1). The photosynthetic apparatus appeared fully equipped for photoprotection in continuous light as soon as LHCII pigment-protein complexes were synthesized, and as soon as an adequate ΔpH was achieved.

Concluding remarks

Greening represents a process whereby adjustments towards optimum photosynthetic performance take place; these adjustments include enhancement of quantum efficiency of photosynthesis and the development of a latent capacity for harmless dissipation of excess excitation energy.

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