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Photoinhibition and loss of photosystem II reaction centre proteins during senescence of soybean leaves. Enhancement of photoinhibition by the 'stay-green' mutation *cytG*

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The 'stay-green' mutation *cytG* in soybean (*Glycine max*) partially inhibits the degradation of the light-harvesting complex II (LHCII) and the associated chlorophyll during monocarpic senescence. *cytG* did not alter the breakdown of the cytochrome *b*6/*f* complex, thylakoid ATP synthase or components of Photosystem I. In contrast, *cytG* accelerated the loss of oxygen evolution activity and PSII reaction-centre proteins. These data suggest that LHCII and other thylakoid components are degraded by separate pathways. In leaves induced to senesce by darkness, *cytG* inhibited the breakdown of LHCII and chlorophyll, but it did not enhance the loss of PSII-core components, indicating that the accelerated degradation of PSII reaction centre proteins in *cytG* was light dependent. Illumination of mature and senescent leaves of wild-type soybean in the presence of an inhibitor (lincomycin) of chloroplast protein synthesis revealed that senescence per se did not affect the rate of photoinhibition in leaves. Likewise, mature leaves of the *cytG* mutant did not show more photoinhibition than wild-type leaves. However, in senescent *cytG* leaves, photoinhibition proceeded more rapidly than in the wild-type. We conclude that the *cytG* mutation enhances photoinhibition in senescing leaves, and photoinhibition causes the rapid loss of PSII reaction-centre proteins during senescence in *cytG*.

Introduction

The breakdown of thylakoid membranes and stromal proteins of the chloroplasts during senescence causes the photosynthetic activity of senescing leaves to decline to very low levels (Gepstein 1988, Noodén 1988). The dismantling of thylakoid membranes is characterized by chlorophyll (Chl) degradation and loss of photosynthetic electron transport activity (Holloway et al. 1983, Gepstein 1988). Initially, the decline in photosynthetic electron transport during senescence is due to the loss of the cyt *b*6/*f* complex, which is followed by a decrease in Photosystem I (PSI) and Photosystem II (PSII) activities and, later, ATP synthase (Holloway et al. 1983, Gepstein 1988). The decrease in the activity of the photosynthetic light reactions in the thylakoids correlates with the degradation of the protein components of each complex, but the mechanism and regulation of thylakoid protein catabolism are poorly understood.

The decline of photosystem activity during senescence may be caused in part by photodamage. Susceptibility to photodamage increases in excised rye leaves during low-light-induced senescence (Kar et al. 1993). Photoprotective mechanisms might decline during sen-

Abbreviations – Chl, chlorophyll; CP26, 26 kDa Chl *a*/*b*-binding protein; CP29, 29 kDa Chl *a*/*b*-binding protein; CP47, 47 kDa Chl *a*-binding protein; cyt *b*6/*f*, cytochrome *b*6/*f* complex; cyt *f*, cytochrome *f*; *cytG*, a maternally inherited stay-green mutant of soybean; DAF, days after flowering; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4,-dichlorophenyl)-1,1,-dimethylurea; F_v/F_M, ratio of variable to maximum Chl *a* fluorescence in dark-adapted leaves; HEPES, (N[2-hydroxyethyl]piperazine-N′-[2-ethanesolfonic acid]); LHCI, light-harvesting complex associated with PSI; LHCII, light-harvesting complex associated with PSII; PSI and PSII, photosystems I and II, respectively; PPFD, photosynthetic photon flux density; q_N, non-photochemical quenching, q_P, photochemical quenching; TMBZ, tetramethyl benzidine.

escence, since senescent leaves may contain lower levels of zeaxanthin and other xanthophyll cycle carotenoids (Afitlhile et al. 1993). Senescence-related changes in leaf orientation of the flag leaf in rice may also increase light exposure at midday, thereby accelerating photoinhibition under field conditions (Murchie et al. 1999). These connections between senescence and photodamage prompted us to study the susceptibility of senescing leaves to photoinhibition of PSII.

Several mutations interfere with Chl degradation and cause the retention of Chl in senescent leaves of various species (Thomas and Smart 1993, Bachmann et al. 1994, Noodén and Guiamét 1996). Chl retention in these 'stay-green' mutants correlates with the preservation of Chl-binding proteins and certain other protein components of the thylakoid membranes. In soybean, the maternally inherited mutation *cytG* (Bernard and Weiss 1973) blocks the degradation of the light-harvesting complex of PSII and its associated pigments (Guiamét et al. 1991, Chao et al. 1995). *cytG* appears to act on Chl degradation rather than synthesis (Canfield et al. 1995) suggesting that at least one non-nuclear gene is involved in thylakoid membrane disassembly during senescence. The retention of excess LHCII in senescent leaves of *cytG* may increase the effective size of light-harvesting antenna in PSII with no concomitant increase in light energy utilization for CO_2 fixation in the mutant (Guiamét et al. 1990). This might favour photodamage of PSII reaction centres in senescent leaves of the mutant.

The main objective of this work was to determine if photodamage increases during senescence of soybean leaves, and if there are differences in rate of photoinhibition between wild-type and *cytG* leaves. Additionally, we report on the effects of *cytG* on the degradation of thylakoid proteins besides LHCII in senescing leaves. In this process, we have also characterized the normal breakdown of the photosynthetic apparatus.

Materials and methods

Plant material and growing conditions

Soybean (*Glycine max* [L.] Merr.) seeds of wild-type cv. Clark, and a near-isogenic line carrying the maternally inherited mutation *cytG* were obtained from Dr R. Bernard, Department of Agronomy, University of Illinois, USA. Soybeans were cultured as described previously (Guiamét et al. 1990) in pots with soil, watered daily and fertilized regularly with N-P-K (20:20:20, w/w/w). Plants were initially grown under long days (16 h, 350 μmol m^{-2} s^{-1} of photosynthetically active radiation) at 27/21°C day/night temperature. After 2–3 weeks (unifoliate leaves fully expanded), they were shifted to short days (10 h, 27/21°C day/night temperature) for the duration of the experiment.

In some experiments, leaf senescence was induced by darkness. These plants were grown under long days for 3 weeks until the unifoliate leaves were completely expanded and then transferred to continuous darkness at 25°C. Thereafter, all manipulations (e.g. watering, sampling) were performed under a dim (< 1 μmol m^{-2} s^{-1}) green safelight.

Sampling

For studies with plants undergoing monocarpic senescence, samples of the uppermost fully expanded leaf at flowering were taken at different stages of development, from leaf maturity to abscission. Measurements involving dark-induced senescence were performed on the unifoliate leaves. Since there were small differences in the timing of the degradation of thylakoid proteins between different batches of soybeans, we have indicated the changes in Chl content in wild-type for each particular experiment shown.

Thylakoid isolation

Thylakoids from mature and senescent leaves of both wild-type leaves (cv. Clark) and from *cytG* leaves were isolated using a method modified from Camm and Green (1980). Leaves were harvested and homogenized in chilled buffer (50 m*M* HEPES, pH 7.5, 0.4 *M* sucrose, 2 m*M* $MgCl_2$, 1 m*M* EDTA, 0.2% w/v bovine serum albumin) with a Waring blender. The homogenate was filtered through 4 layers of cheesecloth and a 10-μm pore nylon mesh, and centrifuged at 3000 *g* for 5 min at 4°C. The resultant pellet was resuspended in wash buffer (50 m*M* HEPES, pH 7.5, 10 m*M* NaCl) and centrifuged again as above. This second pellet was resuspended in a small volume of wash buffer containing glycerol (10% v/v) and stored at −70°C until further analysis. For measurements of senescence-induced changes in photosystem II activity, thylakoids were prepared essentially as described above, except that the homogenization buffer contained 0.4 *M* NaCl instead of sucrose, the wash buffer was 50 m*M* HEPES, pH 7.5, 0.25 *M* NaCl, 5 m*M* $MgCl_2$, and thylakoids were resuspended and frozen in 50 m*M* HEPES, pH 7.5, 0.4 *M* sucrose, 15 m*M* NaCl and 0.2% w/v bovine serum albumin. For photoinhibition experiments, thylakoids were isolated according to Pätsikkä et al. (1998).

Photoinhibition experiments

For in vivo photoinhibition measurements, mature and senescing leaves of wild-type cv. Clark and *cytG* were incubated overnight under very dim light with their petioles immersed in a solution of lincomycin (1 g l^{-1}). The leaves were then illuminated at a PPFD of 560 ± 40 μmol $m^{-2}s^{-1}$ at 30°C. After 0, 2 and 4 h of illumination, thylakoids were isolated and oxygen evolution activity was measured immediately. The rate constant of photoinhibition was calculated from first-order fits (Tyystjärvi and Aro 1996) of the loss of oxygen evolution activity. An aliquot of each thylakoid sample was used for determination of the D1 protein content by Western blotting.

In vitro photoinhibition was measured in thylakoids

isolated from both attached, normally senescing leaves at 20, 45 and 55 DAF, and from detached, dark-senescent leaves. To obtain the dark senescent material for in vitro photoinhibition, leaves of cv. Clark and *cytG* were allowed to senesce in darkness for 15 days until the Chl concentration of the wild-type leaves dropped to 26% and that of *cytG* to 49% of the initial value. Thylakoids isolated from control and senescent leaves were suspended in 50 m*M* HEPES (pH 7.6), 0.3 *M* sorbitol, 20 m*M* NaCl and 5 m*M* $MgCl_2$ at a Chl concentration of 50 μg ml^{-1} and illuminated at 20°C with a heat-filtered 250 W projector lamp delivering 1000 μmol m^{-2} s^{-1} of photosynthetically active radiation at the level of the thylakoid suspension. Aliquots of thylakoids were taken at regular intervals for PSII activity measurements as described below.

Protein electrophoresis

For SDS-PAGE, thylakoids were solubilized with Laemmli's solubilization buffer (Laemmli 1970). Each well was loaded either on the basis of Chl content or leaf area, as indicated in each figure. Proteins were separated in either 13% (w/v) or 11–16% (w/v) acrylamide concentration gradient gels.

Western blot analysis

Proteins separated by SDS-PAGE were electro-transferred to nitrocellulose membranes at 70 V for 3 h, or at 200 mA overnight, in a buffer containing 48 m*M* Tris, 39 m*M* glycine, 0.037% w/v SDS and 20% v/v methanol. Nitrocellulose membranes were blocked, incubated with antibodies and developed as described earlier (Guiamét and Giannibelli 1996) or with a chemiluminescent detection system based on horseradish peroxidase.

Heme peroxidase staining of cytochrome *f*

Mildly denaturing electrophoresis was performed as described by Camm and Green (1989). Cytochrome *f* was detected in mildly denaturing gels by staining for the peroxidase activity of its heme group as described in Thomas et al. (1976). After soaking the gels for 2 h in a solution of 1.9 m*M* tetramethyl benzidine (TMBZ) in 30% (v/v) methanol, 175 m*M* Na-acetate buffer (pH 5), the reaction was initiated by adding hydrogen peroxide to a final concentration of 30 m*M*. Band intensity was quantified by laser densitometry at 600 nm. Since staining intensity was linear for loads between 5 and 15 μg Chl and up to 15 min of incubation, gels were routinely loaded with 7.5–10 μg of Chl and developed for 15 min. All operations were performed in darkness or under subdued light.

Northern blot analysis

Leaves were harvested at midday and RNA was extracted as in John et al. (1995). RNA was electrophoresed in denaturing gels containing formaldehyde under standard conditions (Sambrook et al. 1989) and transferred overnight to nylon membranes (Gene Screen, Du Pont, USA) by capillary transfer. Blots were UV crosslinked at 1200 J. Blotted RNA was pre-hybridized at 42°C for 4 h in formamide (50% v/v), SDS (1% v/v), 1 *M* NaCl, dextran sulphate (10% w/v) and denatured salmon sperm DNA (0.1 mg ml^{-1}). Radiolabelled probes were added to this solution and hybridized overnight at 42°C. Membranes were washed twice for 5 min each with 2× SSC (1× SSC is 0.15 *M* NaCl, 0.15 *M* sodium citrate, pH 7.0) plus SDS (0.1% w/v) and twice for 15 min each with 0.1× SSC plus SDS (0.1% w/v).

Plasmids containing the soybean 18S rRNA gene and the barley *psbA* gene were digested with *Eco*RI/*Bam*HI (18S rRNA) and *Eco*RI/*Hin*cII (*psbA*), and the inserts were radiolabelled with a random primed labelling kit (Boehringer Mannheim) following the manufacturer's instructions. Blots were hybridized first with the *psbA* probe, then the probe was stripped and the same blot was hybridized with the soybean 18S rRNA probe. Levels of the *psbA* and 18S rRNA mRNAs were quantified with a Bio-Rad Molecular Imager System using a BI Imager Screen (Bio-Rad Laboratories, Hercules, CA, USA).

D1 synthesis in vivo

Eight leaf disks (12-mm diameter) were incubated for 1 h at 560 ± 40 μmol m^{-2} s^{-1} and 30°C in 4 ml of distilled water containing 0.37 MBq of ^{35}S-methionine (37.0 MBq μmol^{-1}). After this feeding period, thylakoids were isolated, polypeptides were solubilized with Laemmli's solubilization buffer (Laemmli 1970), separated by SDS-PAGE, and transferred to a nitrocellulose membrane as described above, and autoradiograms were developed using Kodak X-OMAT AR film (SIGMA Chemical Co., St. Louis, MO, USA).

Photosystem II activity

For photoinhibition experiments, light-saturated oxygen-evolution activity of PSII was measured polarographically from isolated thylakoids as described in Pätsikkä et al. (1998). 2,6-dichloro-*p*-benzoquinone was used as electron acceptor. Changes in PSII activity during senescence were measured according to Kim et al. (1994).

Chlorophyll a fluorescence measurements

The ratio of variable to maximum Chl *a* fluorescence (F_v/F_M), and the photochemical (q_P) and non-photochemical (q_N) components of fluorescence quenching were measured in attached leaves with an OS-500 Modulated Fluorometer (Opti-Sciences Inc., Tyngsboro, MA, USA). Leaves were dark adapted for 15 min before measuring F_0 (fluorescence with all PSII centres open) and F_M (fluorescence under a saturating flash of light).

For the analysis of the quenching parameters, leaves were illuminated continuously with an actinic light source (350 μmol m^{-2} s^{-1}), and saturating flashes of light (10 mmol m^2 s^{-1}, duration 0.8 s) were given every 20 s. At the end of each run, the quenched F_0 level (F_{od}) was measured with the actinic light off and illuminating the leaf with a far-red source. The coefficients q_P and q_N were calculated according to van Kooten and Snel 1990).

Chlorophyll content

Leaf disks were extracted overnight with dimethyl formamide and Chl content was calculated from the absorbances at 647 and 664.5 nm using the equations of Inskeep and Bloom (1985). The chlorophyll content of thylakoids was measured with the method of Porra et al. (1989).

Results

Senescence-associated changes in thylakoid membrane composition

In wild-type leaves, the degradation of most thylakoid proteins on a leaf area basis occurred in parallel with the loss of Chl. Western blots showed that the amounts of light-harvesting complexes of PSII (LHCII) and PSI (LHCI) as well as PSII core proteins D1, CP47 and the PSII inner antenna protein CP26 declined at roughly similar rates after 26 days after flowering (DAF) (maximum Chl content, Fig. 1, lane 2) and reached non-detectable levels in abscising leaves of the wild-type, which contained less than 5% of the peak Chl content. Since the anti-CP26 antibody used in this work also recognizes CP29 in some species (Falbel and Staehelin 1992) the band labelled CP26 in Fig. 1 is actually a doublet of a CP26 band and slower migrating CP 29 band. The loss of the *β*-subunit of the ATP synthase was less rapid than other thylakoid proteins, and abscising leaves still retained significant amounts of this protein (Fig. 1). Immunoblots probed against cyt *f* and the Rieske Fe-S protein of the cyt *b*6/*f* complex, and quantification of cyt *f* in gels stained for the peroxidase activity of its heme group, show that in wild-type leaves the amounts of these proteins started to decline at 18 DAF (Fig. 2) before Chl content reached its maximum at 26 DAF (data not shown).

cytG retarded the degradation of Chl, particularly the degradation of Chl *b*, during monocarpic senescence causing a decline in the Chl *a*/*b* ratio (data not shown). At abscission, the cytG leaves retained about 30% of their Chl. Overall, this pattern is very similar to that observed earlier (Guiamét et al. 1991). Immunoblots confirmed earlier findings based on Coomassie-blue staining that senescing leaves of the *cytG* mutant retain the two main LHCII polypeptides. The *cytG* mutation did not cause retention of LHCI, the *β*-subunit of ATP synthase or the cyt *b*6/*f* complex (Figs 1 and 2). By contrast to LHCII proteins, the PSII core proteins D1 and CP47 as well as PSII inner antenna proteins CP26 and CP29 disappeared faster in *cytG* than in wild-type (Fig. 1).

Photosystem II activity and F_v/F_M

PSII activity measurements confirmed the accelerated degradation of PSII reaction centre proteins in senescing leaves of the *cytG* mutant (Fig. 3A). Likewise, the ratio of variable to maximum fluorescence (F_v/F_M) of attached leaves (Fig. 3B) remained fairly constant during senescence in the wild-type, except for a late decline of about 25%, but F_v/F_M decreased substantially to very low values during senescence in leaves of *cytG*, suggesting accelerated loss of photochemically active PSII centres.

Degradation of PSII and thylakoid membrane proteins in darkness

The accelerated degradation of PSII proteins and decline of F_v/F_M during senescence in *cytG* might be due to increased photodamage to PSII. Since this photodamage would not occur during senescence in darkness, the degradation of thylakoid proteins and loss of PSII activity was followed in leaves induced to senesce in continu-

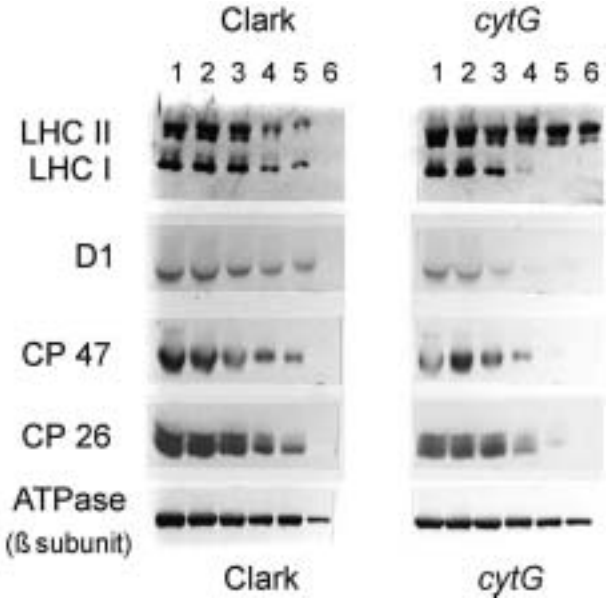

Fig. 1. Immunoblot analysis of the changes in steady-state levels of representative thylakoid proteins during senescence. Lane 1: 18 DAF; lane 2: 26 DAF; lane 3: 42 DAF; lane 4: 46 DAF; lane 5: 52 DAF; lane 6: 58 DAF (leaf abscission). For wild-type (cv. Clark), these stages represent: full leaf expansion (18 DAF), maximum Chl content (26 DAF), 25% (42 DAF), 50% (46 DAF) and 75% (52 DAF) of the Chl degraded, and leaf abscission (58 DAF). Each lane was loaded with an amount of solubilized thylakoids equivalent to 0.05 cm^2 of leaf. The anti-CP26 antibody used in this work was made against a 13-amino acid synthetic peptide, but it also recognizes CP29 in some species (Falbel and Staehelin 1992). In our blots, this can be seen as a slightly diffuse doublet, the slower-migrating band corresponding to CP29 and the faster one to CP26.

ous darkness. As with leaves undergoing monocarpic senescence in a normal photoperiod, *cytG* retarded the dark-induced loss of Chl, particularly of Chl *b*; therefore, the Chl *a*/*b* ratio decreased in leaves of *cytG* senescing in darkness (data not shown). Most thylakoid proteins were degraded to a similar extent in wild-type and *cytG* leaves incubated in darkness (Fig. 4A), with the exception of LHCII, which was lost more slowly in *cytG*. The immunoblot and activity measurements of PSII in Fig. 4B,C show that there was no difference in the rates of decline of PSII activity or in the loss of the D1 protein between wild-type and *cytG* leaves senescing in darkness, indicating that the accelerated degradation of PSII reaction centre proteins in *cytG* leaves senescing under a normal photoperiod is light-driven.

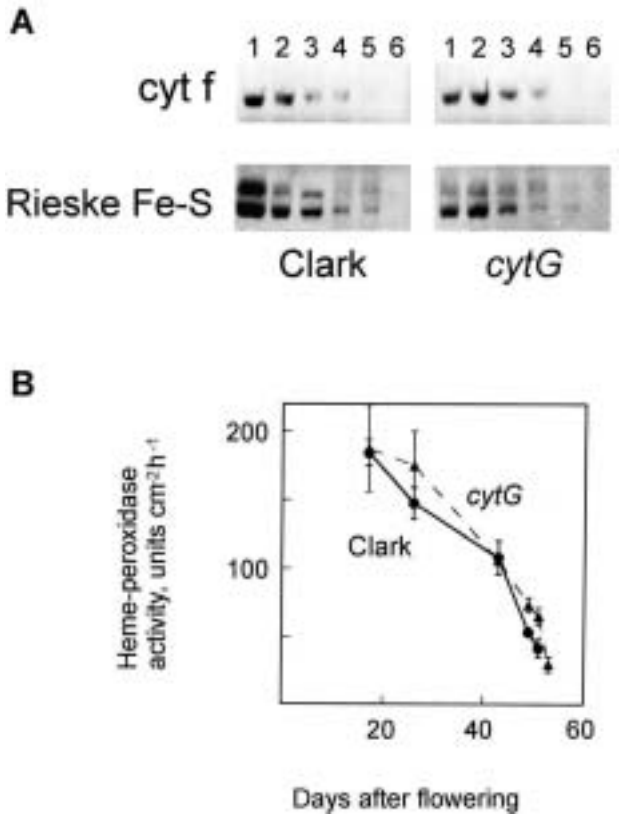

Fig. 2. Changes in the levels of key proteins of the cyt *b*6/*f* complex during senescence in wild-type (cv. Clark) and *cytG*. Panel A – immunoblot analysis of cytochrome f and the Rieske Fe-S protein of the cyt b6/f complex. Lane 1: 18 DAF; lane 2: 26 DAF; lane 3: 42 DAF; lane 4: 46 DAF; lane 5: 52 DAF; lane 6: 58 DAF (leaf abscission). For wild-type, these stages represent: full expansion (18 DAF), maximum Chl content (26 DAF), 25% (42 DAF), 50% (46 DAF) and 75% (52 DAF) of the Chl degraded, and leaf abscission (58 DAF). Each lane was loaded with an amount of solubilized thylakoids corresponding to 0.05 cm^2 of leaf. Panel B – quantification of the changes in cyt *f* by staining for its heme-peroxidase activity in mildly denaturing gels. Samples were taken when wild-type leaves reached full expansion (18 DAF), maximum Chl content (26 DAF), 26% (42 DAF), 60% (48 DAF) and 81% (52 DAF) of the Chl degraded. Each point is the average of three replicate measurements, and vertical bars represent standard errors (SE, standard deviation of the mean).

Senescent, lincomycin-treated leaves of *cytG* are more susceptible to photoinhibition in vivo

The light-accelerated loss of PSII reaction centre proteins in senescing leaves of *cytG* might be due to greater susceptibility to photoinhibition in the mutant. The rate of photoinhibition (i.e. light-dependent decrease of PSII activity) was measured in vivo during normal (monocarpic) senescence in wild-type cv. Clark and *cytG* soybeans. For the photoinhibition studies, we chose fully

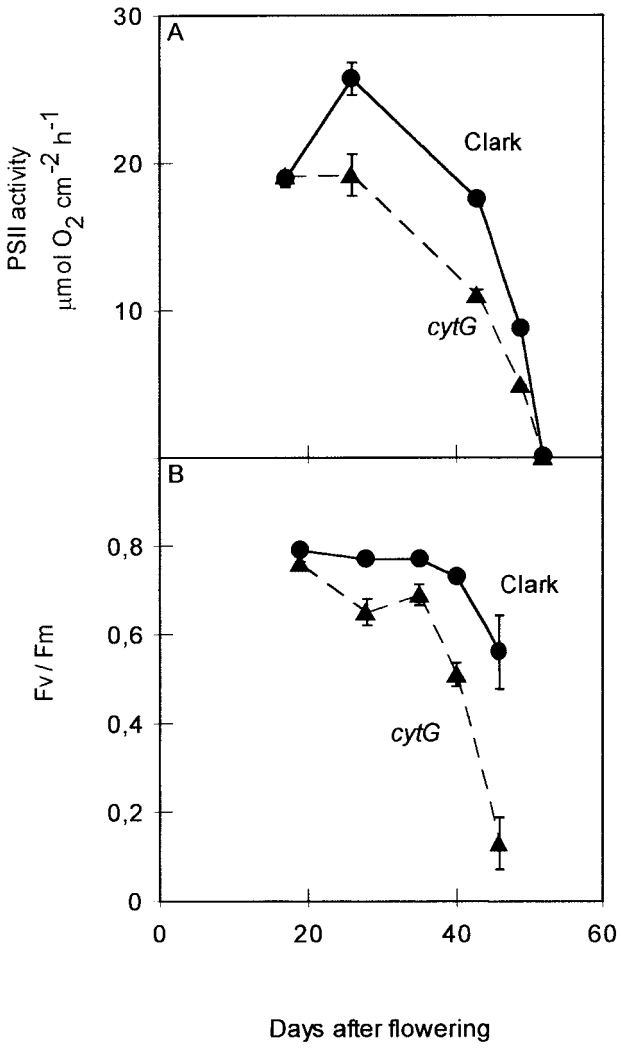

Fig. 3. A – Photosystem II activity of thylakoid membranes isolated from leaves of wild-type (cv. Clark) or *cytG* at different stages of senescence. The samples were taken at 18, 26, 42, 48 and 52 days after flowering. For wild-type, this represents: full expansion (18 DAF), maximum Chl content (26 DAF), 26% (42 DAF), 60% (48 DAF) and 81% (52 DAF) of the Chl degraded. Each point is the average of three replicate measurements, and vertical bars represent the standard error of the mean. B – The ratio of variable to maximum fluorescence in dark-adapted leaves of wild-type cv. Clark and *cytG* soybeans. For the wild-type, these stages represent maximum Chl content (19 DAF), 19% (28 DAF), 30% (35 DAF), 64% (40 DAF) and 92% (46 DAF) Chl loss. Each point is the average of five replicate measurements, and vertical bars represent the standard error of the mean.

expanded mature leaves (total Chl 65.3 ± 15.9 μg cm^{-2}; Chl *a/b* 3.2 ± 0.14) and senescing leaves (total Chl 40.3 ± 0.68 μg cm^{-2}; Chl *a/b* 3.3 ± 0.10) of the wild-type. The leaves were treated with lincomycin, which inhibits chloroplast protein synthesis and thus blocks the concurrent recovery of PSII from photoinhibition (Aro et al. 1993). Subsequent illumination caused a decline in PSII activity with nearly identical time-courses in mature and senescing leaves (Fig. 5A), indicating that in vivo the rate of photoinhibition does not change in wild-type leaves during the early phases of senescence. The PPFD used for the photoinhibitory illumination of the intact leaves was only slightly higher than used during growth, which ensures that the conclusions drawn from the photoinhibition experiments can be applied to the growth conditions.

While mature leaves of *cytG (*total Chl 68 ± 6.8 μg cm^{-2}; Chl *a/b* 2.7 ± 0.13) had photoinhibition rate constants similar to those of the wild-type, the rate constant of photoinhibition was 1.6 times higher in senescent (total Chl 39.0 ± 4.2 μg cm^{-2}; Chl *a/b* 1.8 ± 0.11) than in mature leaves of the mutant (Fig. 5A). Furthermore, in the presence of the chloroplast protein synthesis inhibitor lincomycin, the high-light-induced degradation of the D1 protein occurred more rapidly in senescent leaves of *cytG* than in the wild-type (Fig. 5C).

The higher rate of photoinhibition in intact leaves of *cytG* prompted us to compare in vitro photoinhibition in thylakoids isolated from untreated, senescent leaves of wild-type and the *cytG* mutant. Values for the in vitro rate constant of photoinhibition measured in thylakoids isolated from control and senescent leaves of both cv. Clark and *cytG* were similar (Table 1). Because differences in the composition of the starting material might affect this comparison, we repeated the experiment with thylakoids isolated from leaves induced to senesce in the dark, so as to have equal amounts of PSII reaction centre and D1 protein in both the wild-type and *cytG* thylakoids before the photoinhibition treatment. Likewise, in this experiment, thylakoids isolated from mature and senescent leaves of both the mutant and wild-type showed no difference in the rate constant of PSII inactivation under illumination in vitro (Fig. 5B).

Photochemical and non-photochemical quenching in wild-type and cytG leaves

The greater rate of photoinhibition in *cytG* leaves could be due to increased excitation pressure on PSII (i.e. decreased photochemical quenching, q_P) caused by its larger light-harvesting capacity (Park et al. 1997). Photochemical quenching (q_P) in wild-type leaves did not change much during the early stages of senescence (up to 30% Chl loss in wild-type cv. Clark) and declined only slightly thereafter (Fig. 6A). The changes in q_P of *cytG* leaves were similar to those in the wild-type at all stages studied, and therefore, differences in q_p, i.e. in excitation pressure, do not explain the faster photoinhibition of PSII in the mutant. Since non-photochemical quenching (q_N) has been claimed to protect PSII against photoinhibition (Park et al. 1997), another possible explanation for the enhanced photoinhibition-susceptibility of *cytG* may be that inefficient non-photochemical quenching of excitation energy renders senescing *cytG* leaves more sensitive to photodamage. However, in wild-type leaves, q_N declined markedly during senescence (Fig. 6B), but in *cytG*, q_N remained relatively constant, and therefore senescing leaves of the mutant maintained a higher q_N than the wild-type. Thus, non-photochemical quenching afforded *cytG* leaves a larger capacity than the wild-type to dissipate excitation energy absorbed by the light-harvesting pigments. We do not know whether the sustained capacity for

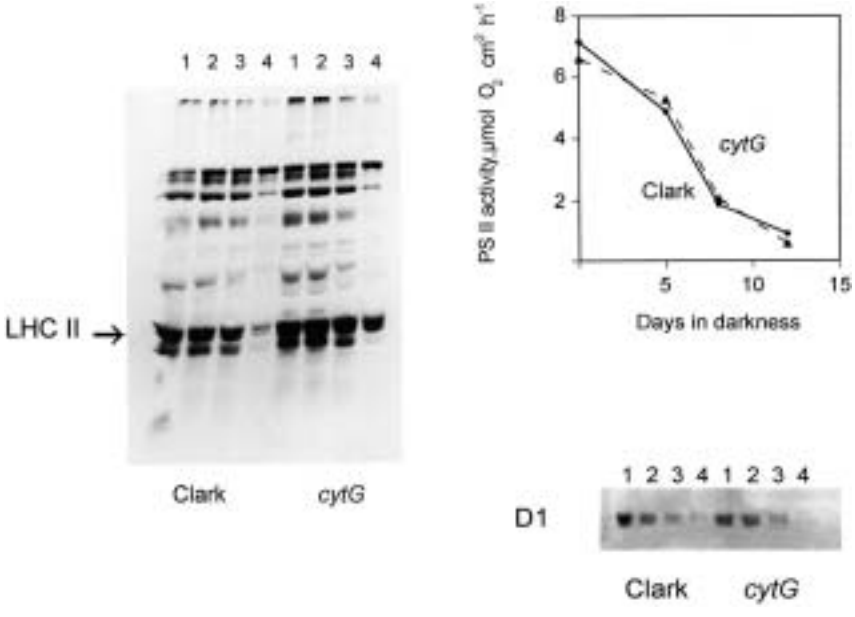

Fig. 4. A – Electrophoretic analysis (SDS-PAGE) of thylakoid proteins (equivalent to 0.25 cm^2 of leaf per lane) stained with Coomassie Blue, B – photosystem II activity of isolated thylakoid membranes and C – immunoblot of thylakoid proteins (each lane was loaded with an amount of solubilized thylakoids equivalent to 0.05 cm^2 of leaf) probed with an anti-D1 antibody. Wild-type cv. Clark and *cytG* leaves were incubated in continuous darkness for 12 days, lane 1: day 0; lane 2: day 5; lane 3: day 8; lane 4: day 12. In panel B, each point is the average of three replicate measurements; The SE bars were smaller than the symbols.

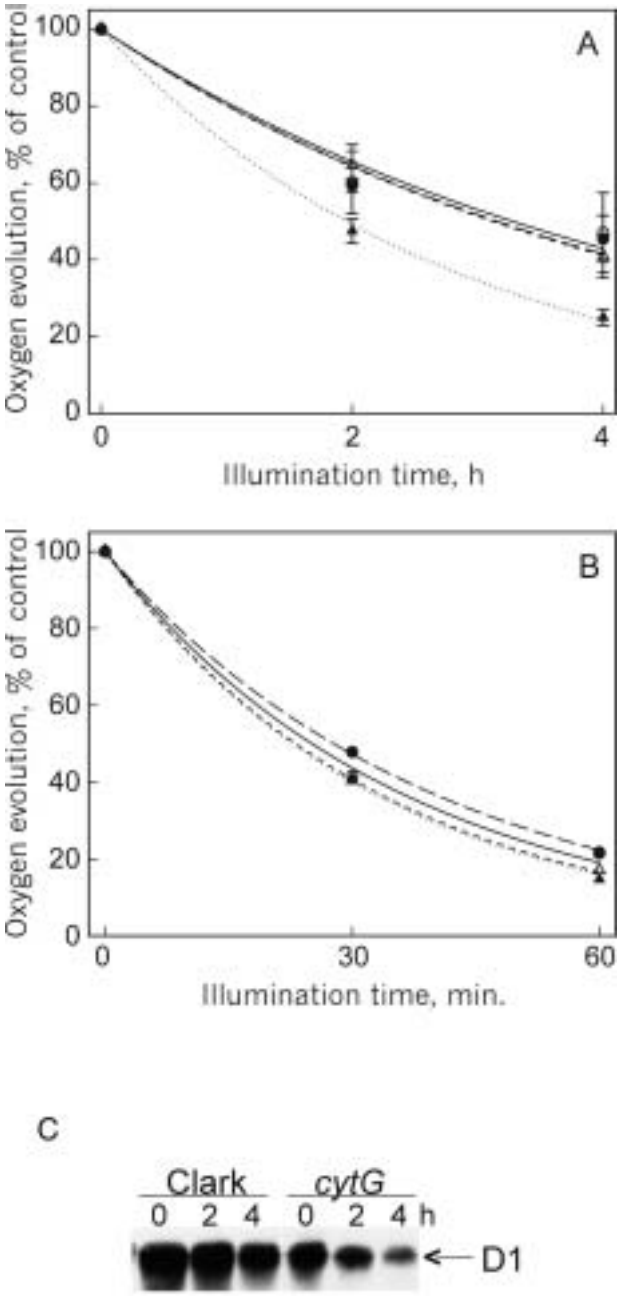

Fig. 5. A – Photoinhibition of lincomycin-treated leaves of wild-type cv. Clark (circles) and *cytG* (triangles) soybeans. Mature (open symbols) and senescent (solid symbols) leaves were irradiated at the PPFD of 560 μmol m^{-2} s^{-1}, and light-saturated oxygen evolution activity was measured in thylakoids isolated from treated leaves. Each data point represents an average of three independent experiments. The control oxygen evolution activities were 377, 327, 368 and 207 μmol O_2 mg^{-1} Chl h^{-1} in mature Clark, senescent Clark, mature *cytG* and senescent *cytG*, respectively. B – In vitro photoinhibition of thylakoids isolated from leaves of cv. Clark (circles) and *cytG* (triangles). Thylakoids isolated from mature (open symbols) and senescent (solid symbols) leaves were illuminated at the PPFD of 1000 μmol m^{-2} s^{-1} at 20°C, and light-saturated oxygen evolution activity was measured from aliquots taken at regular intervals. The control oxygen evolution activities were 283, 204, 100 and 74 μmol O_2 mg^{-1} Chl h^{-1} in mature Clark, senescent Clark, mature *cytG* and senescent *cytG*, respectively. The curves in A and B show the best fit to a first-order equation and the vertical bars represent SE. C – Western blot showing the decrease in the D1 protein content in senescent leaves of wild-type cv. Clark and *cytG* subjected to a similar photoinhibitory treatment as in A. The same amount of chlorophyll was loaded in each well.

q_N quenching in *cytG* is caused by retention of leaf xanthophylls, by a better capacity to maintain the transthylakoid pH gradient during senescence in the mutant, or by some other factor.

Synthesis of the D1 protein of PSII in senescing leaves of *cytG*

The D1 protein of the PSII reaction centre was degraded quite rapidly during photoinhibition of PSII activity (Fig. 5B), and indeed D1 protein levels appeared to decline faster than those of other PSII proteins, e.g. CP26 and CP47, in senescing leaves of *cytG* (Fig. 1). To exclude the possibility that the rapid loss of the D1 protein is caused by a reduced capacity for de novo protein synthesis, we measured the changes in translation activity of D1 protein and the steady-state levels of the psbA mRNA during senescence (Fig. 7). The results showed that the D1 protein was actively translated in senescent leaves of both wild-type and cytG (Fig. 7A). For reasons explained elsewhere (Noodén et al. 1997), we believe normalization on the basis of FW (Fig. 7D) better reflects the concentration of mRNAs in senescing tissues, and this decreases in senescing leaves. The amounts of psbA mRNA were similar in both genotypes during senescence (Fig. 7B,C,D); however, the concentrations of *psbA* expressed on the basis of FW were slightly higher in *cytG* (Fig. 7D).

Discussion

Breakdown of thylakoid proteins during senescence: alterations caused by the 'stay-green' mutation *cytG*

In wild-type genotypes of higher plants, the breakdown of the thylakoid membranes starts with the degradation of the cyt *b*6/*f* complex, and that is followed by the roughly simultaneous loss of Chl and decline of the pro-

Table 1. Photoinhibition rate constants (h^{-1}) of thylakoids isolated from non-treated leaves and subjected to photoinhibitory illumination (PPFD 1000 μmol m^{-2} s^{-1}) at 20°C. Oxygen evolution activity was measured periodically during the illumination and the rate constants were calculated from first-order fits of the decrease of the activity. Wild-type and *cytG* thylakoids were isolated from leaves harvested at different stages of senescence. For the wild-type cv. Clark, these stages represent: maximum Chl content (20 DAF), 38 (45 DAF) and 70% Chl loss (55 DAF). Figures in parenthesis represent standard errors.

DAF	Clark	*CytG*
20	1.8 (0.1)	2.1 (0.3)
45	2.0 (0.02)	1.9 (0.02)
55	1.7 (0.07)	1.8 (0.2)

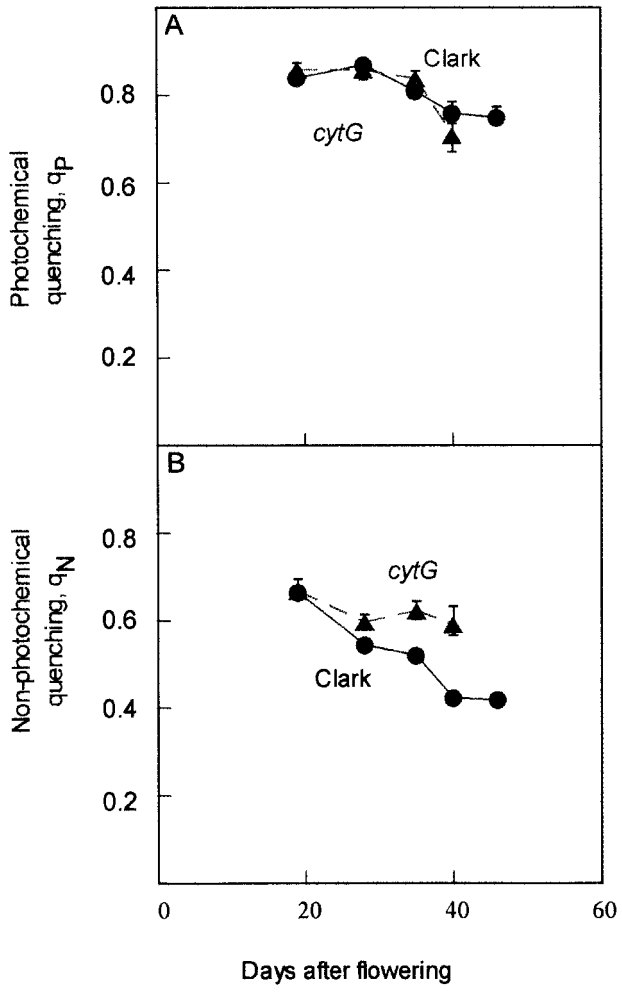

Fig. 6. Photochemical (A) and non-photochemical quenching (B) of Chl a fluorescence in leaves of wild-type cv. Clark and *cytG* soybeans at different stages during senescence. Measurements were made at 19, 28, 35, 40 and 46 DAF. For the wild-type, these stages represent maximum Chl content (19 DAF), 19% (28 DAF), 30% (35 DAF), 64% (40 DAF) and 92% (46 DAF) Chl loss. The fluorescence kinetics in *cytG* at 46 DAF precluded the accurate determination of q_P and q_N and therefore q_P and q_N for *cytG* at 46 DAF are not shown. Vertical bars represent the standard error of the mean.

tein components of both photosystems. Interestingly, the ATP synthase is broken down more slowly and persists at low levels even into very late senescence (Ben-David et al. 1983, Holloway et al. 1983, Roberts et al. 1987).

Since Chl degradation during senescence is partially inhibited in *cytG*, mutant leaves are shed pale green, and other green organs that normally yellow during senescence (e.g. stems, pod walls and seed cotyledons) also remain pale green when the plants die at seed maturity (Guiamét et al. 1990). The main effect of *cytG* on Chl levels is due to the inhibition of Chl *b* degradation (Guiamét et al. 1991), which correlates with substantial preservation of LHCII, the main Chl *a*/*b* light-harvesting complex associated with PSII (Fig. 1, Guiamét et al. 1991). By contrast, the breakdown of other Chl *a*/*b*-binding proteins like LHCI, CP29 and CP26, and of the other photosynthetic membrane protein complexes PSI, ATPase and cyt *b*6/*f* occurs normally in *cytG* (Figs 1 and 2). In some 'stay-green' mutants of higher plants (e.g. $sid^{G.}$ of *Festuca* and the NI 16 line of pea), Chl retention seems to be due to a deficiency in pheophorbide dioxygenase activity, the enzyme catalysing the third step in the Chl degradation pathway (Matile et al. 1996, Thomas et al. 1996). However, *cytG* does not seem to act through that mechanism. First, Chl *a*, some Chl *a*/*b* binding proteins (e.g. LHCI) and even Chl *b* albeit with a delay, are degraded normally in *cytG*. Second, *cytG* is a maternally inherited mutation, whereas sid^G and N16 are nuclear mutations. The specific inhibition of LHCII loss in *cytG* suggests that LHCII is degraded through a pathway different from that involved in the breakdown of other thylakoid proteins.

Susceptibility to photoinhibition during senescence

Susceptibility to (i.e. rate of) photoinhibition has been reported to increase during senescence in some species (Kar et al. 1993, Murchie et al. 1999). However, under constant environmental conditions, mature and senescent leaves as well as isolated thylakoids of wild-type soybeans are equally susceptible to photoinhibition of PSII, indicating that senescence per se does not affect the susceptibility to photodamage. Some earlier observations may actually reflect senescence-associated changes in leaf orientation that alter the amount of light absorbed by leaves of different ages (Murchie et al. 1999). It is also possible that the capacity to repair photodamage is lowered in senescent leaves (Kar et al. 1993). However, the results of the present study show that the capacity to synthesize the D1 protein is retained at least until mid senescence (Fig. 7). Likewise, translation of D1 remains active in senescing leaves of bean even in spite of decreasing levels of the corresponding *psbA* mRNA (Droillard et al. 1992).

Photoinhibition and loss of photosystem II core polypeptides in *cytG*

The *cytG* mutation exerts opposite effects on LHCII and the PSII reaction centre. While LHCII is retained in the mutant, the PSII reaction centre is degraded faster than in wild-type leaves. The accelerated degradation of the PSII core proteins (Fig. 1) and the enhanced loss of PSII oxygen evolution activity in *cytG* (Fig. 3A) are linked to photodamage of PSII, as these phenomena cannot be seen if senescence is induced in darkness (Fig. 4). The more rapid loss of the D1 protein compared to other PSII-core proteins in *cytG* suggests that the light-induced enhancement of PSII loss is related to photoinhibition of PSII. In fact, senescing leaves of *cytG* are more susceptible to photoinhibition than either senescing leaves of the wild-type or mature leaves of the mutant. Interestingly, thylakoids isolated from senescing leaves of *cytG* do not show any increase in the rate of photoinhibition. Fluorescence induction measurements in the

presence of DCMU indicate that the antenna size of PSII increases in senescing thylakoids of *cytG (*data not shown). Thus, the fact that *cytG* thylakoids are not more susceptible to photoinhibition confirms that the antenna size of PSII plays no significant role in susceptibility to photoinhibition (Tyystjärvi et al. 1992, 1994). Likewise, the photochemical capacity of PSII (F_v/FM) declines at similar rates during senescence of wild-type and the *sid*^{G} *Festuca* mutant, in spite of retention of LHCII in *sid*^{G} (Kingston-Smith et al. 1997), which lends support to our conclusion that retention of LHCII alone is not the reason for faster photoinhibition in *cytG*.

Fluorescence quenching in *cytG*

Several mechanisms dissipate energy absorbed in excess of that needed for CO_2 fixation (Horton et al. 1996), presumably helping to prevent the formation of active oxygen species that could impair photosystem function (Foyer et al. 1994). Photochemical and non-photochemical quenching phenomena have been shown to affect the balance between photoinhibitory damage and repair of PSII in several species (Park et al. 1997).

Photochemical quenching may decrease during senescence (Šesták and Šiffel 1997), but in leaves of wild-type soybeans q_P remained relatively constant until quite late in senescence. Photochemical quenching in *cytG* was similar to the wild-type at all stages of senescence. Non-photochemical quenching may increase (Šesták and Šiffel 1997, Murchie et al. 1999) or decline (Fig. 6B) in senescing leaves, probably depending on environmental conditions. Interestingly, while non-photochemical quenching declined during senescence of wild-type leaves, q_N remained constant in senescing leaves of the *cytG* mutant. The higher q_N of senescing *cytG* leaves may have helped to maintain a high q_P in spite of an increasing antenna size of PSII (data not shown). In any event, senescing *cytG* leaves appeared to dissipate absorbed energy more efficiently than the wild-type cv. Clark, reinforcing the conclusion that the light-accelerated loss of PSII in *cytG* is not caused by absorption of excess energy by the retained LHCII.

Repair of photosystem II in *cytG*

Photoinhibition occurs at low photon yield in illuminated leaves such as those used here (Tyystjärvi and Aro 1996). The resulting photodamage to the PSII reaction centre is repaired by degradation of the damaged D1 protein and re-synthesis of a new copy of D1 (Aro et al. 1993, Okada et al. 1996). Since we measured photoinhibition in the presence of the translation inhibitor lincomycin, the enhanced rate of photoinhibition in senescing leaves of *cytG* is not primarily due to inefficient repair of damaged PSII centres.

However, since PSII core proteins and many chloroplast ribosomal proteins are chloroplast-encoded, and *cytG* is a maternally inherited mutation, we tested whether the *cytG* mutation has an additional deleterious effect on chloroplast protein synthesis and therefore on the PSII repair cycle. The levels of the *psbA* mRNA in *cytG* soybeans were equal to or greater than those in the wild-type (Fig. 7B,C,D) suggesting that the accelerated loss of the D1 protein in *cytG* is not due to decreased

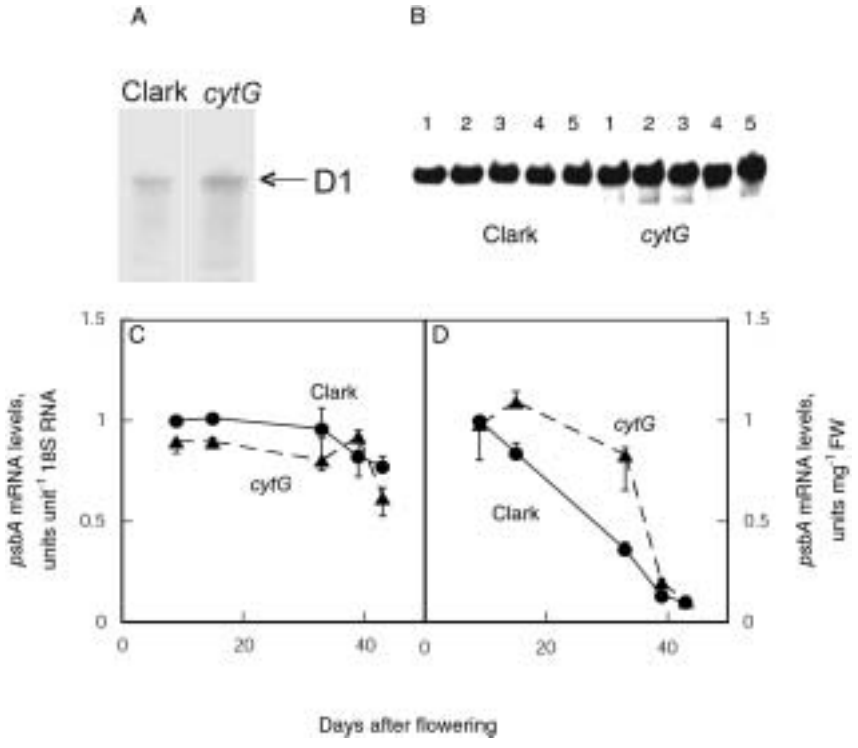

Fig. 7. A – In vivo synthesis of the D1 protein in senescing leaves of wild-type cv. Clark and *cytG*. The wild-type samples had lost 70% of Chl. Leaf disks were incubated for 1 h at 560 ± 40 µmol m^{-2} s^{-1} and 30°C on distilled water containing 0.37 MBq of ^{35}S-methionine. The gel was loaded on leaf area basis. B – A representative Northern blot hybridized with the *psbA* probe to show the changes in the steady-state levels of the *psbA* mRNA during senescence (lanes loaded with equal amounts of RNA). Blots were hybridized first with a *psbA* probe, then the probe was stripped and a soybean 18S rRNA probe was hybridized to the same blot. For wild-type, samples represent full leaf expansion (9 DAF), maximum Chl content (15 DAF), 25% (33 DAF), 56% (39 DAF) and 72% Chl loss (43 DAF). Panels C and D show a quantification of the changes in *psbA* mRNA abundance (average of three replicate extracts and blots) expressed on the basis of the content of 18S rRNA (panel C) or on the basis of leaf FW (panel D). Vertical bars represent the standard error of the mean.

transcription of the *psbA* gene. Moreover, D1 is synthesized at a similar rate by senescing leaves of wild-type and *cytG* soybeans (Fig. 7A). Thus, the accelerated loss of PSII in *cytG* is due to a higher rate of photodamage, rather than to decreased synthesis of the D1 protein.

In conclusion, susceptibility to photoinhibition does not change during senescence of wild-type soybean leaves. However, photoinhibition in senescing leaves is increased by the *cytG* mutation, even though fluorescence-quenching mechanisms are quite active in the mutant. This enhanced photodamage accelerates degradation of the D1 protein, which in turn may enhance the breakdown of other PSII polypeptides in the mutant. Since the rate of photoinhibition is not altered in isolated thylakoids of *cytG*, a stromal or cytosolic factor, rather than absorption of excess light by the retained LHCII, appears to be responsible for the faster photoinhibition in *cytG*. The nature of this factor remains to be elucidated, but it may be possible that the same factor conferring enhanced susceptibility to photoinhibition is responsible for the retention of LHCII in *cytG*.

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