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Disassembly of Chlorophyll-Protein Complexes in *Arabidopsis thaliana* during Dark-Induced Foliar Senescence

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The disassembly of Chl-protein complexes during dark-induced senescence (DIS) was investigated using detached third and fourth leaves of 21±1 day-old *Arabidopsis thaliana*. Although Chl content decreased linearly after 1 d, a significant decrease of photochemical efficiency (Fv/Fm) was observed after 2 d. In experiments using native green gel electrophoresis of Chl-protein complexes combined with additional two-dimensional SDS-PAGE analysis, we could observe the degradation of both photosystems after 2 d. Although light-harvesting complex(LHC) for PSI (LHCI) was degraded first in PSI complex, small PSII apoproteins including CP47/CP43 and D1/D2 apoproteins were degraded first in PSII complexes. LHC for PSII (LHCII) trimers were stable until 4 d. The level of LHCII monomers was increased until 3 d and decreased thereafter, resulting in the increase of free pigments. These results suggest that the disassembly process of PSI is different from that of PSII.

Keywords: dark-induced senescence, Chl-protein complex, Chl fluorescence, green gel, *Arabidopsis thaliana*

INTRODUCTION

Leaf development ends with senescence consisting of highly regulated process that lead to the death. A markedly visible sign of leaf senescence is the gradual loss of Chl and concomitant yellowing, which is due to preferential breakdown of Chl to carotenoids and is due to the synthesis of new compounds such as anthocyanins and phenolics (Gut *et al.*, 1987). Different tissues and cells in leaves have their own pattern and timing of senescence. Among many organelles, chloroplasts are the first organelles to show symptoms of disorganization when all other organelles are normal (Dodge, 1970).

Chl is noncovalently bound to at least 12 different intrinsic thylakoid membrane proteins (Markwell *et al.*, 1979). The reaction centers (RCs) of higher plant photosystems (P700 and P680) are surrounded by core complexes (CCI and CCII) and light-harvesting antenna complexes (LHCI and LHCII). Use of non-dissociating PAGE has allowed the fractionation of Chl-protein complexes with negligible displacement of pigment from the complexes and with their integrity retained. Recently, polypeptide composition in each separated green gel bands could be resolved by a two-dimensional SDS-PAGE (Dreyfuss and Thornber, 1994a; Dreyfuss and Thornber, 1994b; Allen and Staehelin, 1991), and many elaborate studies have been done on the assembly of Chl-protein complexes during the greening process (Dreyfuss and Thornber, 1994a; Dreyfuss and Thornber, 1994b; Anandan *et al*, 1993). On the other hand, only a few researches have dealt with the disassembly of Chl-protein complexes during leaf senescence (Hurkman, 1979; Thimann, 1980; Jenkins *et al.*, 1981; Bricker and Newman, 1982; Siffel *et al.*, 1991; Pancaldi *et al.*, 1996). Although dark incubation of detached leaves has been often used as an experimental system to study leaf senescence *in vitro* (Thimann,

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Abbreviations: CP47/CP43, 47 kDa and 43 kDa PSII Chl-binding antenna proteins; D1/D2, PSII Chl-binding reaction center proteins; DIS, dark-induced senescence; Fm, maximal level of Chl fluorescence; Fo, initial Chl fluorescence; FP, free pigment; Fv, maximum variable fluorescence (Fm minus Fo); LHC, light-harvesting complex.

1980), few research papers have paid attention to the disassembly of Chl-protein complexes in artificially senescing leaves.

In the present study, we investigated the disassembly process of Chl-protein complexes during artificially induced senescence in *Arabidopsis* leaves mainly using two-dimensional SDS-PAGE.

MATERIALS AND METHODS

Plant material

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) (kindly provided by Dr. Hong Gil Nam, Pohang Univ. of Science and Technology, Kyungbuk, Korea) were grown on a compound soil mixture of vermicullite: peat moss: perlite (1 : 1 : 1) in a growth chamber under a 16 h/8 h (L/D) photoperiod (200 to 230 μmol · m^{-2} · s^{-1}) with a 23°C/18°C (L/D) temperature cycle. Bolting started about 3 weeks after germination when plants had 6-7 rosette leaves. Just prior to bolting, 21±1 day-old leaves were detached by cutting at the approximately middle part of the petioles of the third or fourth foliar leaves with a sharp scalpel to minimize wound effect (Oh *et al.*, 1996). For dark treatment, the detached leaves were floated on 2-(N-morpholine) ethanesulfonic acid (MES) buffer (pH 5.6) in 24-well Petri dishes with the abaxial side up.

Measurement of Chl content and Chl fluorescence

Chl content was measured according to Arnon (1949) after extraction of Chl in 80% (v/v) acetone with a glass homogenizer. Chl fluorescence of foliar leaves was measured using a potable plant efficiency analyzer (Hansatech Instruments Ltd, England). Leaves were dark-adapted for 10 min at room temperature prior to Chl fluorescence measurement. The initial fluorescence (Fo) and the maximum yield of fluorescence (Fm) were measured as described by Eu *et al.* (1996). The ratio of the maximum variable fluorescence (Fv: Fm minus Fo) to Fm was used to show the potential quantum yield of photochemical reactions of PSII or the photochemical efficiency of PSII.

Preparation of thylakoid membrane

Thylakoid membrane was prepared according to Aro *et al.* (1996) with some modifications. Detached leaves were ground with a glass homogenizer in ice-cold grinding buffer (50 mM Hepes, pH 7.6, 0.3 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$). The homogenates were filtered through four layers of cheese cloth and centrifuged at 4,500×g for 5 min. Pellets were then washed twice with washing buffer (identical to the grinding buffer except for 0.1 M sorbitol). Thylakoid pellets were resuspended in a small volume of washing buffer and were immediately used for next experiments after their Chl content was measured.

Electrophoretic analysis of Chl-protein complexes

Native Chl-protein complexes were separated by the low-ionic native green gel system of Allen and Staehelin (1991). The resolving gel contained 25 mM Tris · HCl (pH 8.3), 50 mM glycine, and 10% glycerol. The stacking gel contained 25 mM Tris HCl (pH 6.3), 50 mM glycine, and 10% glycerol. The electrode buffer contained 25 mM Tris, 192 mM glycine (pH 8.3), and 0.1% SDS. Eight percent polyacrylamide gel with acrylamide to bisacrylamide ratio of 100 : 1 was used. Polymerization was initiated at room temperature by the addition of 0.1% ammonium persulfate and 0.05% TEMED (*NNN'N'*-tetramethylethylenediamine). Thylakoid membrane pellets were washed twice in 2 mM Tris maleate (pH 7.0) and resuspended in solubilization buffer (0.45% octyl glucoside, 0.45% decyl maltoside, 0.1% lithium dodecyl sulfate, 10% glycerol, and 2 mM Tris maleate, pH 7.0). Solubilization buffer was added to yield a ratio of total nonionic detergent to chlorophyll of 20 : 1 (w/w). Samples were incubated on ice for 30 min and insoluble merterial was pelleted at 15,000 rpm (Micro17R, Hanil, Korea) for 7 min. A 30 μL aliquot of these solubilized thylakoid membrane containing 13.5 μg Chl was loaded per lane of 1.5 mm thick gel. Gels were run in precooled apparatus (Multi-ProteinR II Cell, Bio-Rad, USA) at 4°C with 6-mA constant current.

Two-dimensional SDS-PAGE

Two-dimensional SDS-PAGE was performed according to Allen and Staehelin (1991). Strips of 1.5 mm thick were excised from green gel lanes, incubated for 30 min at room temperature in denaturing buffer containing 1×stacking gel buffer, 2% sodium dodecyl sulfate, 2% β-mercaptoethanol, and 10% glycerol. The denaturated gel strips were loaded directly onto the stacking gel of 1.5 mm thickness using the buffer system of Laemmli (1970).

Electrophoresis was done at 30 mA constant current per gel using a vertical electrophoresis unit (LKB 2001, LKB, Sweden). Gels were stained with silver nitrate (Sambrook *et al.*, 1989).

RESULTS

Changes in Chl content during dark-induced senescence

The third and fourth foliar leaves were detached from 21±1 day-old *Arabidopsis* plants and kept in darkness for 5 d. In this condition, leaf yellowing was obvious after 3 d and leaves were almost completely bleached after 5 d. Although leaf yellowing started from the tip of aged leaf during natural senescence (also shown in Lohman *et al.*, 1994), simultaneous yellowing of a whole leaf was observed during this artificially induced senescence. Gradual decreases in the content of both Chl a and Chl b were observed after 1 d (Fig. 1A). However, Chl a/b ratio was not altered significantly in the beginning of dark-induced senescence (DIS), but increased after 3 d (Fig. 1B), which suggests that LHC started to degrade after 3 d.

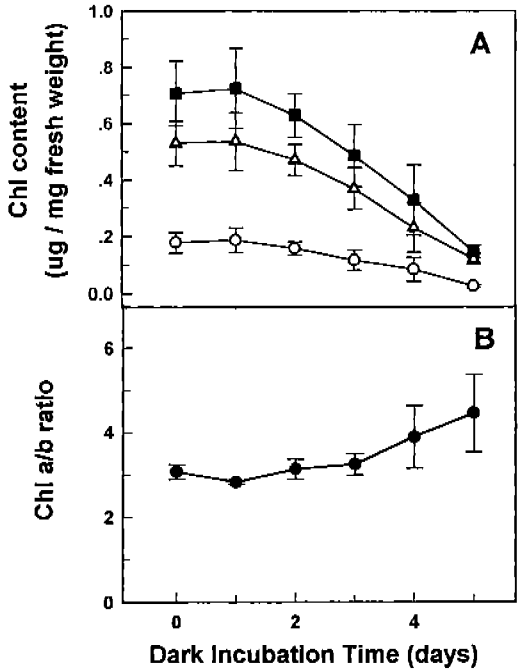

Fig. 1. Changes of Chl content and Chl a/b ratio of *Arabidopsis* leaves during dark-induced senescence. (A) Chl content, Chl a + Chl b (■), Chl a (△), Chl b (○), (B) Chl a/b ratio (●). Data presented are means and standard deviations of multiple replicates.

Changes in Chl fluorescence during DIS

Changes in the structure and function of photosynthetic apparatus during DIS were monitored by measuring Chl fluorescence directly emitted from the leaves. As shown in Fig. 2, Fm decreased gradually during DIS for 5 d. Fo increased after 1 d and decreased after 3 d. The reduction in Fm reflects a decrease in Chl content, a decrease of photochemical efficiency, and/or an increase in heat dissipation. Although the content of Chl decreased gradually after 1 d (Fig. 1), a significant decrease of photochemical efficiency (Fv/Fm) was observed after 2 d (Fig. 2C). After 4 d, the photochemical activity of the leaves was almost completely lost.

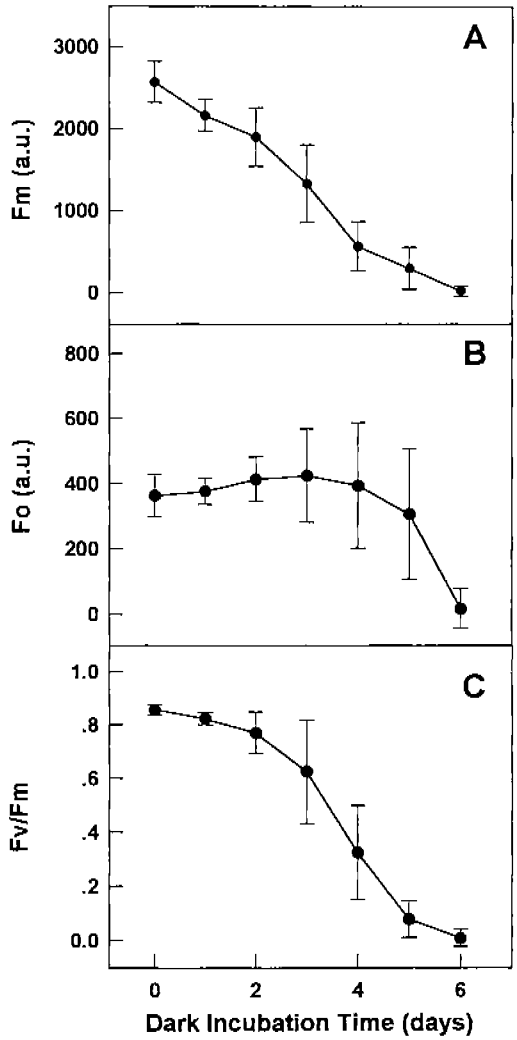

Fig. 2. Changes of Fm, Fo and Fv/Fm of *Arabidopsis* leaves during dark-induced senescence. Data presented are means and standard deviations of multiple replicates.

Changes in the Chl-protein complex composition during DIS

To investigate structural changes in photosynthetic apparatus, the sequential change in band patterns of separated Chl-protein complexes during DIS were examined(Fig. 3A). The separated band patterns were very similar to those reported by Allen and Staehelin (1991) using octyl glucoside/decyl maltoside/lithium dodecyl sulfate detergent system. The labels of regions shown in Fig. 3 were named according to Allen and Staehelin (1991). Senescence-related alteration of the green bands was also evident in the UV fluorescence images shown in Fig. 3B, which highlight the LHCs and free pigments that are not associated with intact reaction centers and are, therefore, highly fluoresent.

During DIS, we could noticc 5 major changes in band intensity (Fig. 3): (1) a decrease in RC-LHC and (2) an increase in RC-Core after 3 d, (3) a decrease in LHCII after 3 d, (4) a decrease in a slowly migrating band of SC after 3 d and a fast migrating SC band which increased until 3 d and decreased thereafter, and (5) an increase in FP after 3 d.

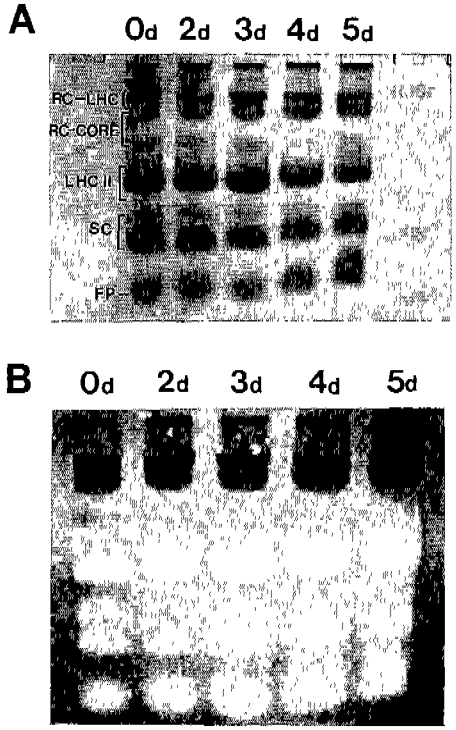

Fig. 3. Changes of Chl-protein complex patterns in *Arabidopsis* leaves during dark-induced senescence. (A) a picture of separated green gel and (B) its UV fluorescence image. Bands were grouped and labelled according to Allen and Staehelin (1991), and name of the groups are marked at left. 'RC-LHC' contains a number of large PSI and PSII complexes both with attached antenna; 'RC-Core', several partial PSI and PSII complexes that have been largely stripped of antenna; 'LHCII trimer', light-harvesting complex II trimer; 'SC', small complex; 'FP', free pigment.

Changes in polypeptide composition of each green band during DIS

From two-dimensional denaturing gel analysis using lanes excised from native green gel, the ploypeptide compositions of each green band were determined (Fig. 4). According to the composition of polypeptides of each band, the regions shown in Fig. 3 could be identified as follows: the region labelled as RC-LHC was found to contain a number of large PSI and PSII complexes attached to antenna in the later experiments. The RC-Core region contains mostly PSI and PSII complexes with few antenna. The LHCII region contains the trimeric form of LHCII and SC region contains small complexes which include monomeric forms of LHCII, smaller PSII complexes, oxygen evolving complexes, etc. FP denotes free pigments. The identification of each region agreed with that reported in Allen and Staehelin (1991).

Major changes observed in Fig. 3 could be explained through the analysis of polypeptide maps as

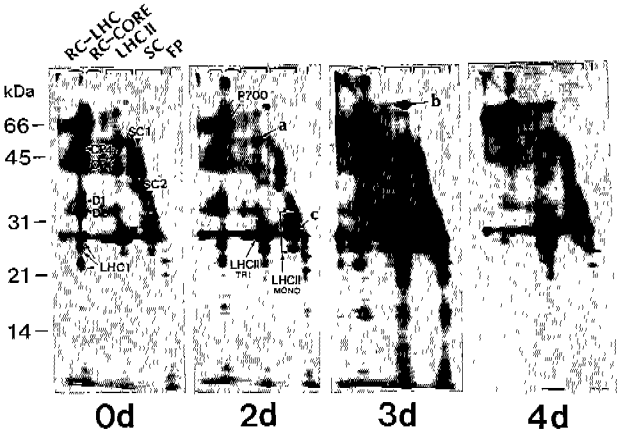

Fig. 4. Changes of polypeptide composition of Chl-protein complexes in *Arabidopsis* leaves during dark-induced senescence. Each lane of a green gel were excised and were applied to the top of a SDS-PAGE gel after 90° rotation from the direction of electrophoresis in the green gel. The positions of size markers are indicated as follows; LHCII tri, LHCII trimer apoproteins; LHCII mono, LHCII monomer apoproteins; P700, P700 apoproteins; CP47/CP 43, 47/43 kDa PSII core antenna proteins; D1/D2, PSII reaction center proteins; FP, free pigment.

follows: (1) After 2 d, we could observe a decrease in both P700 apoproteins (spots labeled as P700 in Fig. 4) and LHCI in the RC-LHC region, and an increase in P700 apoproteins in the RC-Core region, (2) the spot of LHCII trimer (according to the identification of Allen and Staehelin (1991)) in the LHCII region was relatively stable until 4 d and degraded thereafter, (3) a slight decrease in the slowly migrating form of SC (labelled as SC1) was observed at 2 d, which seemed to include smaller PSII complexes, oxygen evolving complexes according to previous reports (Dreyfuss and Thornber, 1994a; Dreyfuss and Thornber, 1994b; Preiss and Thornber, 1995), and (4) the fast migrating form of SC (labelled as SC2) seemed to contain LHCII monomer, which increased until 3 d.

Additional noticeable changes were the rapid degradation of CP47/CP43 and D1/D2 apoproteins in the RC-LHC region after 2 d. In addition, changes in unidentifiable spots were observed: (1) three spots in the LHCII region increased until 3 d, which migrated faster than the LHCII trimer spot, (2) several spots in the fast-migrating form of SC increased when LHCII monomers increased, which seemed to be other LHCII monomers according to Dreufuss and Thornber (1994a), (3) a spot labelled 'a' in Fig. 4 decreased after 2 d, and a spot labelled as 'b' increased during DIS, and (4) many small polypeptides in the FP region increased in the beginning and degraded after 4 d.

DISCUSSION

Using native green gel electrophoresis combined with two-dimensional SDS-PAGE, we examined the status of disassembly of the various pigmented-protein complexes throughout the senescence of the plastids. Although the disassembly process during DIS seemed very complicated, the major changes observed in the structure of photosystems were (1) the diappearance of P700 apoproteins in the RC-LHC region, (2) the corresponding increase of P700 apoproteins (LHCI depleted PSI complex) in the RC-Core region after 2 d, and (3) the rapid degradation of PSII RC core apoproteins after 2 d without any noticeable changes in LHCII (Fig. 3, 4).

Siffel *et al.* (1991) also reported different stabilities of Chl-protein complexes in several plant species. They showed pronounced differences between two plant species, *Tilia* and Hibiscus. In yellowing *Tilia* leaves, Chl a/b ratio decreased due to the decrease of Chl content and LHC was very stable. On the other hand, PSII core complexes were more stable than LHC and PSI complexes, and Chl a/b ratio increased in yellowing *Hibiscus* leaves. In this study, *Arabidopsis* leaves showed similar degradation pattern as *Tilia* leaves; LHCII was very stable until 3 d during DIS (although Chl a/b ratio did not change significantly by 3 d), and PSII core complex and PSI complexes were degraded faster. During natural senescence of soybean cotyledons, Bricker and Newman (1982) reported that the degradation of PSI complexes and the decrease in PSI-dependent electron transport activity were faster than those related with PSII. However, they could not observe the appearance of a PSI form without LHCI as shown in Fig. 5. Pancaldi *et al.* (1996) reported a faster degradation of PSI compared with that of PSII during 1-2 d and high stability of LHCII in isolated chloroplasts of *Euglena* maintain for 10 d in darkness at low temperature. Similar observations were also reported by Jenkins *et al.* (1981) in the primary leaves of *Phaseolus vulgaris* during natural senescence.

There are few reports on the detailed fate of Chl-protein complexes during DIS. Although many workers has used dark incubation of detached leaves as an experimental system to study leaf senescence *in vitro* (Nooden 1988), the senescence observed during DIS is dissimilar to natural senescence in many ways. Hurkman (1979) also reported that the sequence of ultrastructural changes in chloroplasts of naturally senescing wheat leaves was different from that of detached leaves under DIS.

In the two-dimensional gel analysis of each lane of green gel, we could observe several additional information on the changes of polypeptide compositions during DIS. As already mentioned, we observed a rapid degradation of CP47/CP43 and D1/D2 apoproteins. Although we identified the bands according to their known molecular weights, evidences using radioactive isotopes and/or antibodies specific to them would be required to understand the structural changes during DIS in detail. In addition, several changes in mostly unidentifiable spots were shown in Fig. 4. Both the LHCII trimer spot and putative LHCII monomer spot (labelled as 'c' in Fig. 4) with the same mobility as LHCII trimer in fast-migrating form of SC were bound specifically with antiserum raised against Lhcb2 (data not shown). Because both Lhcb1 and Lhcb2 are components of LHCII trimer and their molecular weights are very similar (Jansson, 1994; Sigrist and Staehelin, 1992), we could observe their as a single spot. The LHCII

monomeric from labelled as 'c' in Fig. 4 also contains both Lhcb1 and Lhcb2 and, therefore, was shown as a single spot in Fig. 4. The degradation of LHCII trimer resulted in an increase of the LHCII monomer (Fig. 4). Other LHCII monomer spots in the fast-migrating form of SC are presumed to be CP24 and CP29 according to the mobility of their molecular weights. The spot labelled as 'a' in Fig. 4 seemed to be coupling factor 1 according to its molecular weight and its position in the two-dimensional polypeptide map reported by Allen and Staehelin (1991).

No significant changes in the structure of photosynthetic apparatus were observed during the first 2 d of DIS (Figs. 3 and 4), but the Chl content started to decrease after 1 d (Fig. 1), prior to the significant decrease in photochemical efficiency (Fig. 2). Jenkins *et al.* (1981) also reported a large decrease in Chl content without significant loss of electron transport activities of photosystems in the primary leaves of *Phaseolus vulgaris* during natural senescence. Therefore, the reaction centers whose pigments are degraded might stop functioning and the rests keep their high photochemical efficiencies during DIS for 2 days. As reported in Bricker and Newman (1982) and Pancaldi *et al.* (1996), our data seemed to indicate relatively faster decrease in PSI activities compared with that of PSII (data not shown). However, a detailed degradation mechanism of the decrease in their activities remains to be clarified.

In this report, we have shown differences in the stability of PSI and PSII complexes and several additional changes in polypeptide compositions during DIS. We are in search for conditions where we can separate the time for the onset of these two different degradation processes of two photosystems, for example, using chemicals which delay Chl degradation and/or using several delayed senescence mutants of *Arabidopsis*.

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