Leaf Senescence in a Stay-Green Mutant of Arabidopsis thaliana: Disassembly Process of Photosystem I and II during Dark- incubation

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In this study the disassembly process of chlorophyll (Chl)-protein complexes of a stay-green mutant (ore10 of Arabidopsis thaliana) was investigated during the dark incubation of detached leaves. During this dark-induced senescence (DIS), the Chl loss was delayed in the mutant, while the photochemical efficiency of photosystem II (PSII) or Fv/Fm was accelerated when compared with the wild type (WT) leaves. This indicates that the decrease in Fv/Fm is a separate process and not causally-linked to the degradation of Chl during DIS of Arabidopsis leaves. In the native gel electrophoresis of the Chl-protein complexes, which was combined with an additional two-dimensional SDS-PAGE analysis, the delayed senescence of this mutant was characterized by the appearance of an aggregate at 1 d or 2 d, as well as very stable light harvesting complex II (LHClI) trimers until 5 d after the start of DIS. The polyepitope composition of the aggregates varied during the whole DIS at 5 d. D1 protein appeared to be missing in the aggregates. This result supports the idea of a faster depletion of functional PSII in the mutants compared with WT, as suggested by the earlier reduction of Fv/Fm and the stable Chl a/b ratio in the mutants. At 5 d, the WT leaves also often showed aggregates, but the polypeptide composition was different from those of ore10. The results presented suggest that the formation of aggregates, or stable LHCI trimers in the stay-green mutants, is a way to structurally protect Chl-protein complexes from serious proteolytic degradation. Detailed disassembly processes of Chl-protein complexes in WT and ore10 mutants are discussed.

Keywords: Aggregate, Arabidopsis thaliana, Chlorophyll-protein complex, Delayed dark-induced senescence, Stay-green mutant

Introduction

During leaf senescence, a gradual loss of chlorophyll (Chl) is normally accompanied by a decrease in photosynthetic activities, as well as the degradation of structural components of the photosynthetic apparatus. However, in some non-functional stay-green mutants, the Chl content is retained while other physiological functions are nearly the same as wild-type (WT).

One of the most comprehensively studied non-functional stay-green mutants is Bf993. It is a genotype of the pasture grass Festuca pratensis, in which the chloroplast disassembly during senescence is partially disabled (Thomas and Howarth, 2000). A mutation of a genetic locus sid in pasture grass renders the leaves permanently green. It is also expressed in the persistence of a number of Chl-binding proteins. The sid gene codes for or regulates phaeophorbide a oxygenase, an enzyme in the macrocycle-opening step of the Chl catabolism (Vicentini et al., 1995).

The assembly process of the Chl-protein complexes is a sequential process (Dreyfuss and Thornber, 1994; McCormac, 1996). Therefore, the disassembly process is expected to be the same. We investigated the disassembly process during the dark-incubation of detached leaves of the WT Arabidopsis thaliana (Oh and Lee, 1996). During this artificial dark-induced senescence (DIS), a light-harvesting complex (LHC) was degraded first in photosystem I (PSI), while the LHC was degraded last in the photosystem II (PSII). In the case of Bf993, the retained Chl was related to LHClI. The D1 protein was also unusually stable, while extrinsic proteins were labile as in the case of WT (Hilditch et al., 1989).

For the study of the disassembly process in the model plant, A. thaliana, several mutant lines are available. Among them, a non-functional stay-green mutant, ore11, is reported by Oh et al. (1997) to be an allele of another non-functional stay-green mutant, ore10, (personal communication).

In this study, we investigated the disassembly process of the Chl-protein complexes in the non-functional stay-green mutant, ore10, during DIS in comparison with the process in WT leaves. In ore10 mutants, LHClI was also a stable Chl-
protein complex form. In addition, this mutant could be characterized by the gradual appearance of aggregates in the process of DIS. The detailed disassembly process of the ore10 mutants is described comparing it with the process in WT.

Materials and Methods

Plant materials and growth Seeds of the Arabidopsis thaliana ecotype Columbia, as well as mutant lines, were grown on a compound soil mix (vermiculite/peat moss/perlite = 1:1:1) in a growth chamber with a cycle of 16h light /8h dark and a temperature cycle of 23°C day/18°C night. The non-yellowing mutant lines, ore10 and ore11, screened from seed pools mutagenized with ethylmethane-sulfonate, were kindly donated by Dr. Hong Gil Nam (Pohang Univ. of Science and Technology, Kyungbuk, Korea).

For the dark treatment, the third or fourth foliar leaves that were excised from about 21 day-old leaves just prior to bolting were floated on 3 mM 2-[N-morpholino]-ethanesulfonic acid (MES) buffer (pH 5.8) and then incubated in permanent darkness.

Measurement of Chl content and Chl Fluorescence Leaves were ground in 2 ml of 80% acetone with a glass homogenizer. The Chl content was calculated on a fresh tissue weight basis according to the method of Arnon (1949). The Chl content was presented as a relative value to that of a non-senesced control leaf.

The Chl fluorescence of foliar leaves was measured using a portable PAM-2000 fluorometer (Walz, Effelterich, Germany) after dark-adaptation for 10 min at room temperature. The fluorometer was connected to a leaf-clip holder (2030-B, Walz) with a trifurcated fiber optic (2010-F, Walz) and then to a computer with data acquisition software (DA-2000, Walz). This provided an on-line analysis of the fluorescence data. The photochemical efficiency of PSII, or photosynthetic efficiency in short, was deduced from the maximal efficiency of PSII photochemistry, Fv/Fm = (Fm-Fo)/Fm (Kitajima and Butler, 1975). The minimal fluorescence level (Fo) for all open PSII reaction centers was measured by the measuring modulated light, which was sufficiently low (<0.1 µmol m⁻²s⁻¹) in order not to induce any significant variable fluorescence. The maximal fluorescence level (Fm), with all of the PSII reaction centers closed, was determined by a 0.8 s saturating pulse at 8000 µmol m⁻²s⁻¹ in dark-adapted leaves.

Green gels and two-dimensional gels of thylakoid membrane proteins Thylakoid membranes were prepared as described in the preceding paper (Eu et al., 1996; Oh and Lee, 1996). The isolated thylakoid membrane pellets were washed twice in 2 mM Tris maleate (pH 7.0) and resuspended in a 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) to yield a ratio of total nonionic detergent to chlorophyll of 20 : 1 (w/w). In order to achieve a better solubilization of thylakoid membranes from WT leaves that were dark-incubated for 5 d, the amount of solubilization buffer added was doubled to yield a ratio of total nonionic detergent to chlorophyll of 40 : 1 (w/w). The native Chl-protein complexes were separated as described by Allen and Staehelin (1991). An aliquot of the solubilized thylakoid membranes, which contained 13.5 µg of Chl per lane, was loaded per lane of 1.5 mm thick gel. Samples were electrophoresed at 4°C and constant 6 mA.

For the second dimensional SDS-PAGE analysis, 1.5 mm thick strips were excised from green gel lanes and incubated for 30 min at room temperature in a denaturing buffer containing 1× stacking gel buffer, 2% sodium dodecyl sulfate, 2% β-mercaptoethanol, and 10% glycerol. The fully denatured gel strips were laid directly on 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

Morphological characterization of ore10 mutants The leaves of ore10 mutants are smaller and wrinkled compared with those of WT plants at a similar age (Fig. 1A). The flowerling time of this mutant, measured as the time of the visual appearance of the floral inflorescence, was similar to that of WT plants. Figure 1B also shows that the heights of inflorescence in both ore10 and WT are quite similar. In WT leaves, the progress of natural senescence is noticeable by the yellowing, which signals the loss of Chl. However, the senescing leaves of ore10 mutants started to lose rigidity without yellowing (Fig. 1C). The senescence shown in the 3rd and 4th foliar leaves after bolting generally started earlier in the mutant than in WT (pictures not shown). Mutant leaves stayed green even after most of the cells were dead. This is noticeable from the dry leaves with green color (Fig. 1C).

Changes in Chl contents and Fv/Fm during DIS in WT and mutants ore10 and ore11 leaves After the start of DIS in WT Arabidopsis leaves, the Chl content decreased gradually during the whole period of DIS for 5 d. The rate of

Fig. 1. Morphological characterization of a WT Arabidopsis plant and a non-functional stay-green mutant ore10. A. Phenotypes of a whole WT plant (left) and an ore10 mutant (right) prior to bolting. B. The floral inflorescence of WT (left) and an ore10 mutant (right) at the same age. C. Wilting-but-green leaves of a ore10 mutant during its natural senescence.
Chl degradation was slower both in ore10 and in ore11 (Fig. 2A). In both mutants, a significant amount of Chl remained at 5 d. However, the photosynthetic efficiency, or Fv/Fm, started to decrease after 2 d in WT leaves, while it began to drop rapidly after 1 d in both ore10 and ore11 (Fig. 3). Although Oh et al. (1997) described these mutants as non-functional stay-green mutants, the phenotype of the mutants was not only a delayed loss of Chl content, but also the DIS in the functional sense proceeded faster in these mutants than in WT (shown in Fig. 3). As noticed from the term non-functional stay-green mutants, the decrease in Fv/Fm is a separate process not causally linked to the degradation of Chl during DIS in Arabidopsis.

Changes of Chl a/b ratio, Fo and Fm during DIS in WT and mutants ore10 and ore11 During DIS, the Chl a/b ratio increased significantly after 3 d in WT as we reported previously (Oh and Lee, 1996). However, the Chl a/b ratios in ore10 and ore11 mutants did not increase at all during DIS for 5 d (Fig. 2B). The increase in the Chl a/b ratio in WT was due to a rapid disappearance of Chl b (data not shown) that was bound mostly to the proteins in LHCs. Therefore, the degradation of the Chl b seemed to be partly blocked in mutant leaves during DIS. This suggests that the degradation of LHCs is delayed in the mutants.

The decrease in Fv/Fm in WT, shown in Fig. 3, was due to the decrease both in Fm and in Fo. However, such a noticeable drop in Fo was not observed in the two mutants. The size of error bars for both Frm and Fo are relatively large compared to that of Fv/Fm, because both Frm and Fo are roughly proportional to the Chl content. Therefore, the individual differences in the absolute Chl content are cancelled out in Fv/Fm. A decrease in energy coupling between LHCCI and the PSII traps leads to an increased fluorescence emission from LHCCI itself, which results in an increase of Fo. Because the Chl content drops significantly during DIS (shown in Fig. 1), the slow changes in Fo shown in the mutants during DIS support our idea of a preferential delay in the degradation of LHCCI during the disassembly of photosystems.

Changes of Chl-protein complexes during DIS in WT and ore10 mutants The non-functional stay green mutant of Arabidopsis retains its photosynthetic pigments without functional delay during DIS (Figs 1 and 3). Most of the Chls integrate as Chl-protein complexes by non-covalent binding with proteins (Markwell et al., 1979). Therefore, we examined the changes in band patterns of the Chl-protein complexes during DIS (Fig. 4).
Disassembly of Photosystems during Artificial Senescence

(3) In *ore10* mutants, a band that could not run through the stacking gel during electrophoresis was observed at 1 d or 2 d thereafter. Its band intensity was roughly the same from 2 d to 4 d and decreased at 5 d. A similar band could be observed only at 4 d and 5 d in the case of WT. However, it should be kept in mind that the amount of Chl in each aliquot loaded was equally adjusted for each lane, and the Chl content for each leaf was very low at 4 d and 5 d in the case of WT. Interestingly, the accumulation of aggregates in the *ore10* mutants is consistently matched with the loss of a band in the RC-LHC region and a band in the SC region.

**Disassembly of Chl-protein complexes in *ore10* mutants**

Detailed changes in the Chl-protein complexes can be examined by a two-dimensional (2D) electrophoresis with a native green gel in the first dimension and fully denaturing SDS-PAGE in the second. Therefore, the polypeptides composing each green band are shown as spots in a vertical lane corresponding to the band as depicted in Figure 5. The region labeled as RC-LHC in the first dimension contains a number of large PSI and PSII complexes that are attached with antennas. The PSII complexes can be identified in the 2D pattern by the presence of the apoproteins CP47, CP43, D1 and D2. Most of the P700 apoproteins are shown as pigmented CPI on the second-dimensional gel, and the positions of the other PSI subunits were in the range of 27-85 kDa as described by Allen and Staehelin (1994). However, we did notice the existence of two bands (marked with an arrow in Figs 4 and 5) composed of PSII core complexes without P700 apoproteins between the major RC-LHC band and the major RC-Cone band. Although these two bands are included in the RC-LHC region by Allen and Staehelin (1994), we think they are RC-Cone bands, since they are without LHCs. Therefore, we include them in a new RC-ConeII region, and the existing RC-Core region is named as a RC-CoreI region.

The major differences in the 2D gel profiles between WT and *ore10* mutants (Fig. 6) are as follows:

(1) In WT, we have shown in Fig. 4 that a decrease in the RC-LHC was matched by an increase in the RC-Cone. During this process, there is a noticeable increase in the P700 apoproteins without the small PSI subunits, including the LHCI polypeptides in the RC-CoreI region. This confirms our previous report (Oh and Lee, 1996). However, in the *ore10* mutants we observed no sign of an increase in the band intensities in the RC-CoreI regions. Instead, the P700 apoproteins and LHCI proteins in the RC-LHC region and P700 apoproteins in the RC-Core region seemed to be degraded faster in the *ore10* mutants than in WT. In addition, in the *ore10* mutants a new band containing PSII core complexes with LHCII was observed at 2 d. These migrate slower than the RC-LHC band. After 2 d, the PSII core complexes in this new band, as well as those in the RC-CoreII region, disappeared more rapidly than PSI subunits.

(2) In *ore10* mutants, the disappearing PSII core complexes may be located in the aggregates, which increased while RC-
LHC and RC-Core bands decreased. These aggregates are different than the ones that are often located on top of the stacking gel in non-senescing leaves of WT and mutants, which contain only a small amount of the P700 apoproteins. The aggregates that were observed in the ore10 mutants at 2 d contained P700 apoproteins, PSII core complexes and LHCI. The increase in LHCI in the aggregates was roughly matched by a decrease in the LHCI monomers in the SC region. After 2 d, aggregates showed PSI subunits, including LHCI, in addition to the P700 apoproteins, which had a significant decrease in the bands in the RC-LHC region. The polypeptide composition of the aggregates at 3 d in the mutants was similar to the composition of the band in the RC-LHC region. After this stage, PSI/II core proteins were significantly degraded in the aggregates, which had a concurrent accumulation of small polypeptides.

The polypeptide composition of aggregates (shown in WT at 4 d and 5 d) was different than those in the mutants, which consisted of P700 apoproteins, LHCI monomers and a small amount of PSI small subunits.

(3) As shown in Figures 4 and 6, the LHCI trimer bands in the green gel, and the corresponding spots in the 2D gel, were quite stable during DIS in the ore10 mutants (Fig. 6). At 5 d in senescing ore10 mutant leaves, most of the Chls were bound with LHCI trimers, LHCI monomers and aggregates, and all of the other Chl-protein complexes seemed to be degraded. After 5 d, only the LHCI trimer proteins were left even after all of the other proteins disappeared in the 2D gel (pictures not shown).

Discussion

During DIS, we compared the disassembly process of the photosynthetic apparatus among WT, ore10 and ore11 mutants. These mutants were characterized by their 'stay-green' leaves, but the senescence was rather accelerated in a functional sense, especially in the photosynthetic machinery.

Relationship of the changes in the photosynthetic efficiency or Fv/Fm with the changes in Chl contents

In WT leaves, high (>0.8) photosynthetic activity was retained for 2 d, although the gradual degradation of Chls during DIS resulted in the reduction of the amount down to 70% of the control (Figs 2A and 3). When Fv/Fm decreased less than 10% at 3 d, the Chl content decreased to 60% of the control value. This result suggests that the function of each existing photosynthetic apparatus was not hindered during DIS for 2 d, but the number of functional apparatus was decreased. At 3 d and thereafter, some functional units might be damaged, resulting in a reduction in the average value of Fv/Fm. A
similar phenomena was demonstrated in the leaves of young spinach grown in a Mg- and S-deficient medium (Dannehl et al., 1995).

However, Fv/Fm dropped rapidly even after 1 d in the mutants, while the degradation of Chl was delayed. This result indicates that the photosynthetic apparatus of the mutants are more vulnerable to damages in the functional sense, but Chls in some Chl-protein complexes seemed to be protected against degradation. The stable Chl-protein complexes were found to be LHCIIIs in this report.

Relationship of LHCII, Chl a/b ratio with F0 Because the Chl a/b ratio of LHCII is normally near 1 or below 2, and this value is much lower than the values of other Chl-protein complexes (Thornber, 1995), the stability of LHCII shown in the green gel patterns, as well as their 2D gel pattern, can explain the stable Chl a/b ratio in the mutants during DIS for 5 d (Fig. 2B).

If all of the Chl-protein complexes were degraded, except LHCII, the excitation energy coupling between LHCII and PSII core complexes would be blocked. This would result in an increase in Fo. The time points for the changes during DIS in these three parameters are roughly similar (shown in Figs 2, 3 and 4.)

An example for the relationship of LHCII with Fo can be found in Zucchielli et al. (1988). They showed an increase in Fo when LHCII was partially uncoupled from the PSII reaction centers at low cation concentrations. Ottander et al. (1995) also suggested that the increase in Fo in a winter stressed leaf indicated damaged photochemistry. This resulted from the detachment of LHCII from PSII reaction centers upon the decrease in D1 content of the reaction centers. In this report, the PSII core complexes, indicated by an arrow in between the RC-LHC and the RC-Core region of the 2D gel (Fig. 6), decreased significantly at 3 d. A significant increase in Fo is a well known phenomenon in plant leaves under high temperature stress (Renger and Schreiber, 1986).

Disassembly process and stability of Chl-protein complexes in stay-green mutant During DIS, the disassembly of photosystems is probably a sequential process, as is their assembly process (Dreyfuss and Thornber, 1994; McCormac, 1996). In detached leaves of A. thaliana, the disassembly process for PSII was different than that of PSI. Oh and Lee (1996) described the detailed processes for both PSI and PSII. As described in this report, more detailed information about the disassembly process could be obtained by a comparison of the WT with mutants that are defective in the disassembly process.

In mutants ore10 and ore11, the rapid decrease in Fv/Fm, shown at 2 d and thereafter (Fig. 3), is probably due to the blockage of energy transfer from LHCII to the reaction centers in CCII. Although the increase in Fo, or the stable Chl a/b ratio as supporting evidence for this assumption, could be observed clearly at 3 d (Figs 2B and 3) we expect a mature PSII is separated into CCII and LHCII as an early process after the start of DIS. According to Ghanotakis et al. (1987), Barbato et al. (1989) and Camm and Green (1989), the treatment with octyl-β-D-glucoside in high-salt conditions specially removes LHCII and CP24, while CP29 and CP26 are enriched with CCs, and a more complete treatment with detergent results in CCII being attached to CP29 only. Therefore, monomeric LHCIIIs, including CP26 and CP29, are expected to be bound with CCII, leaving LHCII mainly in its trimeric form, or together with a monomeric CP24.

The stability of the Chls in mutants is probably related with the early appearance of aggregates and the very stable LHCII trimers (shown in Figure 6.) The formation of the aggregates can explain most of the characteristic differences of the mutants in the disassembly process compared with the process in WT. This includes the disappearance of PSI in the RC-LHC region, without the increase of P700 apoprotein in the RC- Corel region, the disappearance of LHCII monomers in the SC region at 2 d, etc.

The appearance of aggregates that could not run through the stacking gel during electrophoresis was also reported in Ottander et al. (1995) in winter leaves of Pinus sylvestris. After the completion of the Chl loss, the aggregates were formed during winter by the reorganization of the remaining Chl and Chl-protein complexes from PSII and PSI. The aggregates in pine leaves were devoid of D1 protein and efficiently quenched excitation energy through non-radiative dissipation. Although the aggregates formed in the mutants during DIS at 1 d or 2 d seemed to be also devoid of D1 proteins, their polypeptide compositions differed from those reported by Ottander et al. (1995) and also from the composition of the aggregates shown in WT (Fig. 6). The polypeptide composition of the aggregates was not static, but kept changing during DIS as described earlier. Funk et al. (1994) also observed similar aggregates on top of the stacking gel when the Chl-protein complexes were separated from spinach using octyl-thioglycol.pyronoside. Although we did not use the same detergent as Ottander et al. (1995), the formation of aggregates is nevertheless probably due to the hydrophobic attractions of the Chl-protein complexes, which are modified during DIS by proteases.

As mentioned earlier, the aggregates formed in the mutants are probably devoid of the D1 protein. However, the D1 protein could be observed in WT even at 4 d in the RC-LHC region, but no D1 proteins were observed in the ore10 mutants even in the RC-LHC region (Fig. 6). This result supports the idea of a faster depletion of functional PSII in the mutants compared with WT, as suggested by the earlier reduction of Fv/Fm and the stable Chl a/b ratio in the mutants (Figs 2B and 3).

The results presented suggest that the formation of aggregates, or stable LHCII trimers in the stay-green mutants, is a way of protecting Chl-protein complexes from serious proteolytic degradation. However, the protection cannot cover the functionally important proteins, such as D1 proteins,
although the depletion of D1 protein in the aggregates needs to be confirmed. The factors causing the formation of the aggregates are under investigation. Finally, the results presented provide experimental evidence showing that the protection of protein degradation, as in the ore10 or ore11 mutants, is an important way of delayed senescence in addition to the mere blockage of Chl catabolism.

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References


