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Characterization of Cytoplasmic and Nuclear Mutations Affecting Chlorophyll and Chlorophyll-Binding Proteins during Senescence in Soybean[1]

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ABSTRACT

Soybean plants (*Glycine max* [L.] Merr. cv Clark) carrying nuclear and cytoplasmic "stay-green" mutations, which affect senescence, were examined. Normally, the levels of chlorophyll (Chl) *a* and *b* decline during seedfill and the Chl *a*/*b* ratio decreases during late pod development in cv Clark. Plants homozygous for both the d_1 and d_2 recessive alleles, at two different nuclear loci, respectively, retained most (64%) of their Chl *a* and *b* and exhibited no change in their Chl *a*/*b* ratio. Combination of G (a dominant nuclear allele in a third locus causing only the seed coat to stay green during senescence) with d_1d_2 further inhibited the loss of Chl in the leaf. Whereas the thylakoid proteins seem to be degraded in normal Clark leaves during late pod development, they were not substantially diminished in d_1d_2 and Gd_1d_2 leaves. In plants carrying a cytoplasmic mutation, *cyt*G, Chl declined in parallel with normal cv Clark; however, the *cyt*G leaves had a much higher level of Chl *b*, and somewhat more Chl *a*, remaining at abscission, enough to color the leaves green. In *cyt*G, most thylakoid proteins were degraded, but the Chl *a*/*b*-binding polypeptides of the light-harvesting complex in photosystem II (LHCII), and their associated Chl *a* and *b* molecules, were not. Thus, the combination of d_1 and d_2 causes broad preservation of the thylakoid proteins, whereas *cyt*G appears to selectively preserve LHCII. The *cyt*G mutation may be useful in elucidating the sequence of events involved in the degradation of LHCII proteins and their associated pigments during senescence.

Chl degradation is the most conspicuous of the changes that occur during leaf senescence and is frequently used as an index of this process (14). Developing soybean embryos, seed coats, pod walls, and leaves normally are green but turn yellow during seed maturation (4, 18). Seed development is correlated with the yellowing of both the pod walls and the leaves (16, 18), which is part of a syndrome termed monocarpic senescence (14, 15).

In several species, there are genetic variants in which Chl loss in leaves during senescence is either blocked or attenuated, so that they stay green ("stay-green variants") while their "normal" counterparts are yellowing (22). The best studied of the stay-green variants is that of fescue grass (*Festuca pratensis* Huds.), in which a recessive nuclear gene (*sid*) causes the leaves to retain Chl (21). The retention of Chl in the nonyellowing *Festuca* is related to the maintenance of many thylakoid proteins, including the LHCII,[3] but not all thylakoid proteins are maintained (11, 22), and photosynthesis declines similarly to that in the wild type during senescence (10).

Several stay-green mutants of soybean have been isolated. A cytoplasmic gene, *cyt*G, or homozygosity for two recessive alleles, d_1 and d_2, at two different nuclear loci causes the leaves, pod walls, seed coats, and embryos to stay green at maturity (2, 8). Presumably, homozygosity at both nuclear loci is required because the two are homeologous (duplicate) loci in the tetraploid soybean genome (9). The dominant nuclear gene G inhibits yellowing of the seed coat but not the leaves, pod walls, or embryos (2). Chl retention is associated with a delayed decline of photosynthesis in the genotype $GGd_1d_1d_2d_2$, but not in $d_1d_1d_2d_2$ or *cyt*G (8). Here, we report the temporal changes in total Chl, Chl *a*, and Chl *b* and the composition of Chl-proteins in the thylakoid membranes of these soybean stay-green variants. This information is then related to the decline of photosynthesis (8) and also to thylakoid degradation during leaf senescence in these genotypes.

MATERIALS AND METHODS

Plant Material

Near-isogenic lines (1) of soybean (*Glycine max* [L.] Merr., cv Clark) were obtained from Dr. R. L. Bernard, Department of Agronomy, University of Illinois, Urbana. The genotypes used included d_1d_2, Gd_1d_2, *cyt*G, and normal Clark ($ggD_1D_1D_2D_2cytY$).

Culture

The plants were cultured as described earlier (12, 17). Seeds inoculated with *Bradyrhizobium japonicum* were grown in pots of soil on a glasshouse bench at the Matthaei Botanical

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[3] Abbreviations: LHC, light-harvesting complex; LHCII, LHC associated with PS II; CAB, chlorophyll *a*/*b*-binding (protein); FPE, full pod elongation; EMPF, early-mid pod fill; LPF, late pod fill; TPF, termination of pod fill; LY, leaf yellowing; d_1d_2, $ggd_1d_1d_2d_2cytY$; Gd_1d_2, $GGd_1d_1d_2d_2cytY$; *cyt*G, $ggD_1D_1D_2D_2cytG$.

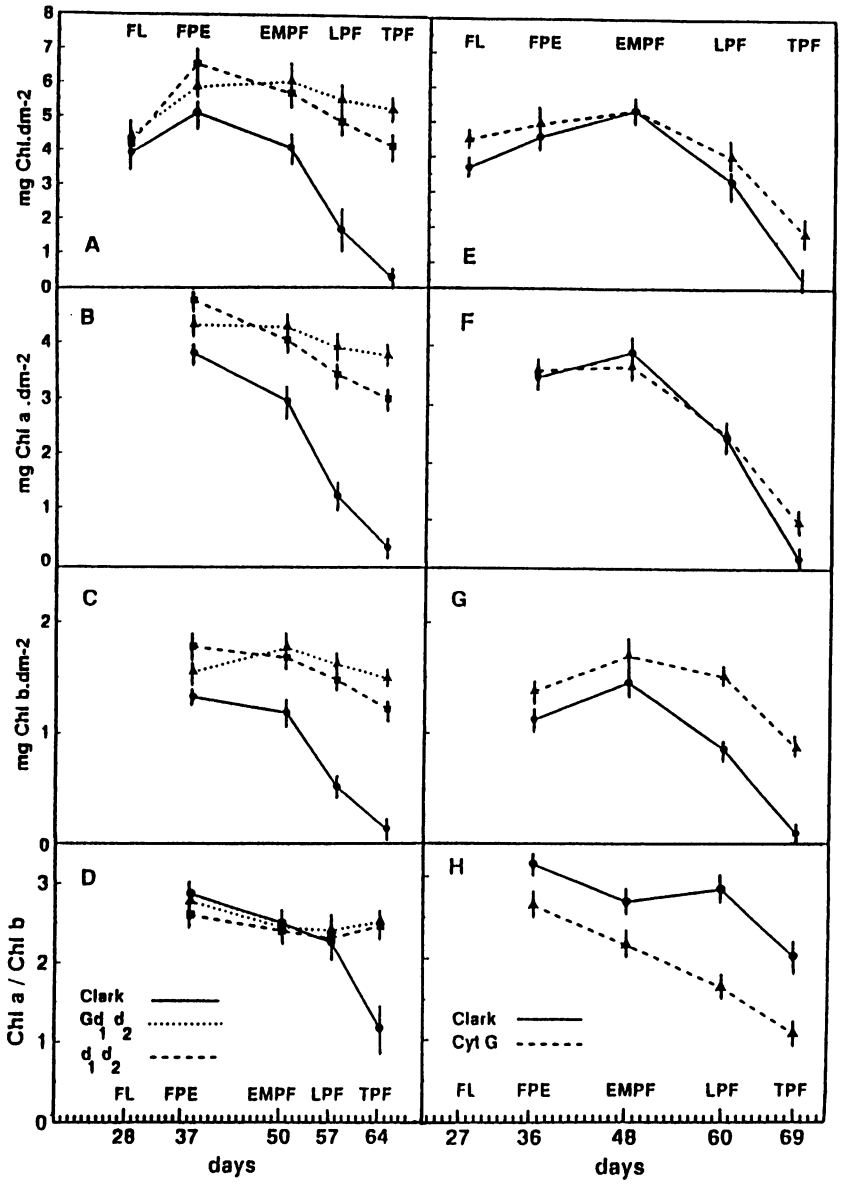

Figure 1. Chl *a* and *b* content per dm^2 and ratio of the same upper leaf (see "Materials and Methods") as related to pod development. The developmental stages are flowering (FL), FPE, EMPF, LPF, and TPF, which occurs approximately at the time of LY in Clark. A to D, Plants grown in chamber No. 1; E to H, plants grown in chamber No. 2 (see "Materials and Methods"). Vertical bars, SEM. A and E, Changes in total Chl; B and F, changes in Chl *a*; C and G, changes in Chl *b*; D and H, changes in Chl *a*/*b* ratio.

Gardens with extended days for 3 weeks. They were then transferred into environmental control chambers with short days (10 h). Owing to space limitations, plants for Chl extractions were sorted into two chambers as follows: No. 1: Clark, d_1d_2, and Gd_1d_2; No. 2: Clark and *cyt*G. Because irradiance and temperature differed slightly between the two chambers (No. 1: irradiance, 450 μmol m^{-2} s^{-1}; temperature, 26°C day/21°C night; and No. 2: irradiance, 500 μmol m^{-2} s^{-1}; temperature, 28°C day/21°C night), the plants in chamber No. 1 were slightly shorter, their pods developed earlier (2 to 3 d), and they senesced earlier (4 to 6 d, depending on the parameter used). Thus, the genotype effects must be compared against Clark plants grown in the same chamber.

Sampling

The uppermost, fully expanded leaves at flowering (sixth to seventh trifoliates) were used for Chl extraction and thylakoid isolation. Leaf samples were taken at key pod development stages (13) (Fig. 1). Leaves for thylakoid preparations were frozen in liquid nitrogen and stored at −80°C for later use.

Chl Measurement

Leaf discs (15 mm diameter) were excised with a sharp cork borer, ground with a mortar and pestle, and extracted twice with ethyl ether (18). The extract was cleared by centrifugation and the absorbance was measured at 642.5 and 660 nm with a Zeiss PMQ II spectrophotometer. Chl *a*, Chl *b*, and total Chl were calculated according to the method of Zscheile and Comar (25).

Soybean Thylakoid Preparation

Depetiolated soybean leaves (10 g) were homogenized in a Wareing blender containing 50 mL of ice-cold grinding buffer

(50 mM Hepes, 0.4 M NaCl, 2.0 mM MgCl, 1.0 mM EDTA, 2.0 mg/mL bovine serum albumin, pH 7.5). The resulting homogenate was filtered through four layers of cheese cloth and centrifuged in a Sorval SS34 rotor for 30 s at 175*g* to remove cell debris. The thylakoid membranes were then collected by centrifugation at 3300*g* in the same rotor for 10 min. The pellets were resuspended and homogenized into cold wash buffer (50 mM Hepes, 0.15 M NaCl, 4.0 mM MgCl, pH 7.5). The thylakoid membranes were pelleted again by centrifugation at 5000*g* for 10 min. The resulting pellet was resuspended in 0.5 mL of wash buffer and stored at −70°C until SDS-PAGE.

SDS-PAGE

The thylakoid membrane proteins were separated on a 13.5% acrylamide gel containing SDS as described by Piccioni *et al.* (19). Samples of 0.1 mL from a total of the 0.5 mL were loaded on each lane of the gel. Thylakoid membrane proteins were identified by comparisons with thylakoid preparations of tomato and spinach (5, 20).

RESULTS

Leaf Yellowing and Chl

In the control plants (cv Clark), pod walls, seed coats, and embryos turned yellow during the last stages of pod development. In plants carrying d_1d_2 or Gd_1d_2, the leaves, pod walls, seed coats, and embryos stayed green during the last stages of seed development, and the abscised leaves were green. In *cyt*G, there was some paling of these structures, but they were still green at the time the leaves were shed.

Generally, these visible changes were paralleled by the foliar Chl levels; however, differences in the rates of Chl loss and the degree of partial loss were revealed by quantitative measurements. In Clark, Chl levels peaked at slightly different stages in chambers No. 1 and No. 2 (Fig. 1, A and E); however, Chl declined rapidly in both after EMPF. At TPF, *i.e.*, a few days before leaf abscission, Chl was almost completely gone from these leaves. By contrast, the stay-green genotypes retained Chl to various degrees; at TPF, the leaves retained 34, 64, and 87% of their peak Chl levels in *cyt*G, d_1d_2, and Gd_1d_2, respectively, as compared to only 7 to 8% in Clark. Earlier, Caro and Hadley (3) reported that Chl decreased more rapidly in d_1d_2 than in *cyt*G, but these were field-grown plants and the Chl loss may have been driven by stress (16).

Chl *a*/*b* Ratio

Closer examination of the foliar Chl levels in terms of the Chl *a* relative to Chl *b* reveals further differences among these genotypes. The normal cv Clark lost both Chl *a* (Fig. 1, B and F) and *b* (Fig. 1, C and G) at about the same rate, and consequently the Chl *a*/*b* ratio (Fig. 1, D and H) decreased only slightly until the very end (after LPF), at which time it decreased sharply. The stay-green genotypes differed in the relative amounts of Chl *a* and Chl *b* retained. Both Chl *a* and *b* were similarly retained in d_1d_2 and Gd_1d_2 with the result that the Chl *a*/*b* ratio did not decrease significantly, even when the leaves abscised. Leaves of *cyt*G plants contained higher levels of Chl *b* than Clark from the onset of pod development, and Chl *a* was preferentially degraded. For example, at TPF, the *cyt*G leaves had only 26% of their peak Chl *a* levels but 51% of the Chl *b*. Thus, the Chl *a*/*b* ratio decreased steadily during pod development in *cyt*G (Fig. 1H).

Thylakoid Proteins

Clark, d_1d_2, Gd_1d_2, and *cyt*G did not differ appreciably in their thylakoid protein composition as revealed by SDS-PAGE at FPE and EMPF (not shown). However, their thylakoid protein bands at TPF were strikingly different (Fig. 2). In Clark, the major thylakoid proteins declined to about the same extent as Chl decreased. Compared with Clark, d_1d_2 and Gd_1d_2 retained high levels of all the major polypeptide components of the thylakoids, *i.e.* the 47, 43, 33, 28, 26, and 17 kD polypeptides (Fig. 2). These proteins are known components of PSI and PSII (including the LHCs) (5, 7, 23). In contrast, *cyt*G retained only the 28- and 26-kD polypeptides, the CAB polypeptides of LHCII (7, 20), whereas all the other bands were as faint as those in Clark.

DISCUSSION

Effect of d_1d_2 and Gd_1d_2

The stay-green isolines d_1d_2 and Gd_1d_2 retain much more Chl than Clark at comparable times during senescence, but some Chl degradation does occur in these stay-green mutants. The Chl content of Gd_1d_2 decreases only by 10% from its peak, whereas the Chl concentration in d_1d_2 decreases about 36%. In d_1d_2 and Gd_1d_2 plants, the Chl *a*/*b* ratio does not change, even at TPF. These data are concordant with the observed lack of thylakoid protein degradation. SDS-PAGE analysis reveals that, in d_1d_2 and Gd_1d_2, the major PSI and PSII polypeptides of the thylakoids show no appreciable loss at TPF, whereas at this stage only traces of these polypeptides remain in Clark. The retention of the thylakoid proteins in d_1d_2 and Gd_1d_2 correlates well with the maintenance of leaf N and specific leaf weight (JJ Guiamét, JA Teeri, LD Noodén, unpublished data). Interestingly, photosynthesis in the d_1d_2

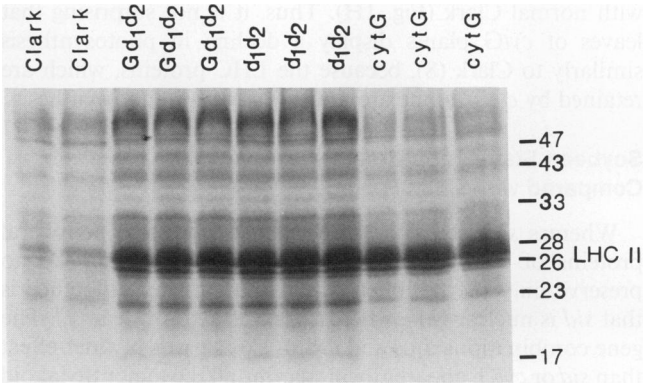

Figure 2. Electrophoretic profiles (SDS-PAGE) of thylakoid proteins from leaves sampled at termination of pod fill. Loads represent equal fresh weights (see "Materials and Methods"). The gel was stained with Coomassie blue.

isoline declines the same as in Clark (8). However, Gd_1d_2 retains photosynthetic activity in the leaf until very late in pod development (8). The only visible effect of the nuclear dominant G is to cause the seed coats, but not the leaves, to stay green (2), and it does not seem to alter the major thylakoid proteins when combined with d_1d_2 (Fig. 2 and additional data not shown). Thus, the massive decline of photosynthesis in d_1d_2 cannot be accounted for by the loss of the entire set of thylakoid proteins. The observations that Gd_1d_2 plants retain photosynthetic activity in the leaves and that the G allele by itself appears to affect only the seed coat suggest that the photosynthetic apparatus of d_1d_2 leaves at abscission is intact and that the retention of photosynthetic activity in Gd_1d_2 leaves, but not in d_1d_2 leaves, may be due to maintenance of the main sinks, the seeds, by G (8).

Effect of *cyt*G

The paling of *cyt*G leaves during pod development is due to substantial Chl loss. However, the *cyt*G leaves are visibly green when shed, retaining about 34% of their peak Chl levels. Chl levels, especially Chl *b*, are higher in *cyt*G than in Clark, and Chl *b* is degraded less rapidly during senescence in *cyt*G, whereas Chl *a* is lost at about the same rate in both. Nonetheless, at TPF, *cyt*G leaves also contain more Chl *a* than Clark plants (Fig. 1F). The preferential retention of Chl *b*, and to a lesser extent Chl *a*, in *cyt*G suggests the involvement of the LHCs, which are rich in Chl *b*. It is known that the Chl pigments are associated with PSI and PSII in the thylakoid membranes of chloroplasts. Several proteins in the "core" PSI and PSII complexes bind Chl *a*, but Chl *b* is found only in the peripheral LHCs (7). These LHCs contain both Chl *a* and Chl *b*, with about 1.1- to threefold more Chl *a* than Chl *b* (24). Both kinds of pigment molecules are bound in the LHCs by CABs (7). In plants grown in a well-lit chamber, the LHCII (corresponding to PSII) predominates, and more than 80% of the Chl *b* is found there (23, 24). The *cyt*G plants, unlike d_1d_2 and Gd_1d_2, retain only the CAB polypeptides of LHCII. The observation that only LHCII CAB proteins (and the population of the Chl *a* and Chl *b* pigments bound to them) are maintained at TPF, whereas the core PSI and PSII (and their associated Chl *a* molecules) are lost, explains the greatly reduced Chl *a*/*b* ratio of the *cyt*G plants at TPF as compared with normal Clark (Fig. 1H). Thus, it is not surprising that leaves of *cyt*G plants display a decline in photosynthesis similarly to Clark (8), because the LHC proteins, which are retained by *cyt*G, alone are not sufficient for photosynthesis.

Soybean Stay-Green Genes Compared with *sid* in *Festuca*

Whereas *sid* seems to protect all the core and peripheral proteins of PSII during senescence (11), *cyt*G appears to preserve only the peripheral LHCII. A further difference is that *sid* is nuclear (21), whereas *cyt*G is extranuclear (2). The gene combinations d_1d_2 and Gd_1d_2 exert a much wider effect than *sid* or *cyt*G, apparently preserving all the major thylakoid proteins and perhaps the entire photosynthetic apparatus (8). Thus, the stay-green phenotype can be caused by more than one molecular mechanism and is controlled by more than one gene.

How Does *cyt*G Act?

The *cyt*G mutant, unlike the d_1d_2 and the *Festuca sid* mutants, appears to affect only a very limited part of the senescence program. The preferential retention of the CAB proteins of LHCII, which are nuclear encoded (20), during senescence in *cyt*G is also intriguing because of the cytoplasmic, and most likely plastidic, mode of inheritance of *cyt*G. Although it is known from analysis of Chl *b*-deficient mutants that CAB polypeptides are degraded if the Chl *b* molecules are not synthesized (23), the sequence of degradative events during senescence is not known. It is possible that either Chl or CAB proteins must be degraded first, and if the first obligatory degradation event does not occur, the second component is protected from degradation. Because both Chl *a* and Chl *b* are clearly degraded in the *cyt*G mutant but the amount of CAB polypeptide per fresh weight does not appreciably decrease (Fig. 2), it is our hypothesis that this mutation is likely to affect CAB protein rather than Chl degradation. We further note that to date no gene related to Chl degradation has been identified in the chloroplast genome, whereas recently a plastidic ATP-dependent protease that had one subunit encoded in the plastid genome and a second subunit encoded in the nucleus was identified (6). Alternatively, *cyt*G could modify the activity of a nuclear-encoded, specific degradative enzyme (*e.g.* a protease or a chlorophyllase) or change the accessibility of LHCII to degradative enzymes. The characterization of the mechanisms of action of mutants, such as *cyt*G, which specifically affect the degradation of different components of the LHCs should enable us to elucidate the sequence of events leading to the complete dismantling of these protein-Chl complexes during senescence.

Dissecting the Senescence Syndrome

Our results with *cyt*G show that the degradation of some thylakoid constituents may be separated from other senescence syndrome processes and from degradation of other thylakoid proteins. The *cyt*G mutation, like *sid* in *Festuca*, alters a relatively limited part of the senescence program. Similarly, photosynthesis declines (8) without a corresponding loss of Chl, thylakoid proteins, and leaf N (JJ Guiamét, JA Teeri, LD Noodén, unpublished data) in d_1d_2, whereas all these components of the senescence syndrome are prevented, or at least attenuated, in Gd_1d_2. Another component of the senescence syndrome, leaf abscission, is not prevented by *cyt*G, d_1d_2, or even Gd_1d_2 (8). Thus, single-gene modifications appear capable of producing well-defined alterations in the senescence syndrome, therefore suggesting that its different components are under separate genetic control. Several time-course experiments have shown that the order in which Chl, protein, photosynthesis, etc. decline during senescence can vary, depending on environmental conditions, the type of senescence-altering treatments, etc. (13). These earlier observations (13) now converge with the results reported here for the stay-green lines which show selective blockage of components of the senescence process and strengthen the hypothesis that senescence is a set of parallel, coordinated events, which are not obligatorily interconnected, rather than a single chain of changes (13).

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