The Structural, Biochemical, and Genetic Characterization of a New Radiation-induced, Variegated Leaf Mutant of Soybean [*Glycine max* (L.) Merr.]

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ABSTRACT

A variegated leaf mutant in soybean [Glycine max (L.) Merr.] has been identified and characterized. E25-10 was derived by exposure of seeds of the "Williams' 82" cultivar to γ -radiation. In this mutant, yellow leaf sectors contain defective chloroplasts, in which the thylakoid membranes are presented as long, parallel structures with little or no overlap. No starch grains have been detected in the mutant chloroplasts. Small vesicles and plastoglobuli can be found within the defective chloroplasts. Genetic studies revealed that a single nuclear-encoded gene is responsible for the mutation in E25-10. The total chlorophyll content is reduced in yellow leaf tissue by 70-80%. However, the chlorophyll a/b ratio is not altered. The absorbance spectrum of pigments in the mutant leaf tissue differed from that of the green extracts in the range of 400-500 nm. This reduction in total chlorophyll and the change in the absorbance spectrum pattern in the yellow tissue is related to a loss of certain photosynthetic complexes. Green gel analysis revealed that four major pigment-protein complexes (CP1, LHCP¹, LHCP², and CPa) of the thylakoid membranes were absent in the E25-10 mutant. Lithium dodecyl sulphate polyacrylamide gel analysis showed that at least 5-6 polypeptides (51, 44, 25, 15, 13, and 12 kDa) were missing in the thylakoid membranes of chloroplasts from the yellow tissue. Changes in chloroplast- and nuclear-encoded gene message levels were detected. The psaA transcripts which code for the P700 apoprotein in PSI were reduced in chloroplasts from the E25-10 mutant yellow tissue. The levels of the large subunit of ribulose bisphosphate carboxylase (rbcL) and light harvest complex protein (LHCP) of PSII mRNA appeared to be reduced slightly in the mutant plants. However, a much more significant reduction in the 16S rRNA and the small subunit of ribulose bisphosphate carboxylase (rbcS) expression was detected in the yellow leaf sectors. Our results suggest that the possible lesion in E25-10 is located in the photosystem I even though fewer grana were observed in the defective chloroplasts.

Key Words: photosystem I, pigment-protein complexes, soybean, variegated leaf mutant

I. Introduction

Genetically controlled patterns of somatic variegation have been found in the leaves, flowers, and seeds of soybean (*Glycine max*) and are known to be the result of unstable alleles in nuclear genes (Chandlee and Vodkin, 1989a; Groose *et al.*, 1988; Peterson and Weber, 1969). Two genes harboring unstable alleles produce products that contribute towards anthocyanin production and accumulation in soybean (Chandlee and Vodkin, 1989a; Groose *et al.*, 1988) whereas a third genetic locus contributes to chloroplast development (Chandlee and Vodkin, 1989b).

The soybean line *T225M* harbors an unstable allele (*Y18-m*) at the *Y18* locus which conditions variegated leaf sectors (green and yellow) from either homozygous (*Y18-m*)

m, *Y18-m*) or heterozygous (*Y18-m*, *y18*) plants (Peterson and Weber, 1969) due to a high rate of somatic mutation of the *Y18-m* allele. The *Y18-m* locus generates an unusual reverse variegation pattern because the unstable allele reverts from the dominant to a recessive form (*y18*) and not vice versa as in most systems in which transposable element activity has been implicated (Chandlee and Vodkin, 1989b). Germinal mutations of the unstable allele produce either dominant green or homozygous recessive yellow plants. The yellow plants (*y18*, *y18*) are generally lethal; however, they can survive under reduced light conditions. The instability of the *Y18-m* allele is affected by temperature (Sheridan and Palmer, 1977).

The Asgrow mutable line of soybean carries an unstable (recessive) allele of the w4 locus (w4-m) which affects soybean flower pigmentation (Groose *et al.*, 1988). Homozygous mutable plants produce flowers with purple sectors on near-white petals. The unstable allele can revert at a high frequency from the recessive form (white) to the dominant form (purple) either somatically or germinally. Germinal reversions produce stable purpleflowered progeny, and the frequencies have been estimated at a rate that varies from 5 to 10% per generation (Palmer *et al.*, 1989). Somatic reversions result in white flowers with purple sectors. Reversion of the *w4*-mutable allele in soybean is independent of temperature (Groose and Palmer, 1988).

The *r*-*m* allele of the *R* locus of soybean affects seed coat pigmentation patterns (Chandlee and Vodkin, 1989a). A variegated distribution of black spots and/or concentric rings of pigment on an otherwise brown seed coat is produced by somatic mutations of the mutable allele. The r-m allele exhibits instability both somatically and germinally (Chandlee and Vodkin, 1989a). Mixtures of seed with different seed coat colors (black + striped or brown + striped) can be produced in a single plant by non-heritable somatic changes of the unstable allele. Heritable changes of the *r*-*m* allele produce plants with all black or all brown seeds. These brown revertants (r^*, r^*) or homozygous black seed revertants (R^*, R^*) show continuous instability of the allele, giving rise to striped or striped + black seeded plants in subsequent generations. It has been suggested that an insertion sequence or transposable element residing in or near the *r*-*m* allele may be responsible for the changes in gene expression for this unstable allele and its derivatives (Chandlee and Vodkin, 1989a).

Leaf variegated mutants of soybean affecting chloroplast development can arise through either nuclear or cytoplasmic mutation events (Honeycutt *et al.*, 1990), but nuclear controlled mutants are more interesting from a developmental and regulatory perspective since the precursor polypeptide is cytoplasmically synthesized and then has to be translocated to the interior of the chloroplast, where it is processed and assembled into its appropriate functional complex (Cline, 1988).

A fundamental process performed in plant chloroplasts is photosynthesis, which converts sun light into chemical energy (ATP and NADPH) and ultimately is responsible for the existence and survival of most living organisms on earth. Besides photochemical reactions, these intracellular organelles also participate in amino acid, nucleotide, lipid, and starch biosynthesis. The photosynthetic reactions and ATP-generating machinery are composed of several complexes, each one consisting of both nuclearand chloroplast-encoded components, which must be expressed in a synchronized manner in order to assemble the photosynthetic complexes at the right time and in the appropriate place (Barkan *et al.*, 1986) during chloroplast development. Nuclear mutations altering the process of photosynthesis should aid eventual understanding of the structure-function relationships of the protein subunits which contribute to the photosynthetic apparatus (Critchley and Bottomley, 1986).

Toward this goal, we are interested in characterizing variegated leaf mutants of soybean. E25-10 was generated by exposure of soybean seeds of the variety "Williams' 82" to γ radiation (Chandlee and Vodkin, 1989b). These plants produce a variegated leaf pattern similar to those produced by T225M (i.e., yellow or green leaf sectors). The variegated leaf patterns indicate altered chloroplast development in the mutant tissue sectors, leading to a reduction in thylakoid membrane proteins of one or more of the photosynthetic complexes. For this study of the foliar mutant E25-10, we examined (1) the patterns of somatic mutability produced by the mutation events, (2) the ultrastructure of the mutant chloroplasts, and (3) the inheritance patterns to determine if there is a nuclear origin for the E25-10 mutation. In addition, biochemical defects in the thylakoid membranes of the mutant were characterized.

II. Materials and Methods

1. Seed Stocks

Soybean cultivar used in this study (Williams' 82) was obtained originally from the USDA soybean germplasm collection at the University of Illinois, Urbana, and subsequently maintained in our laboratory at the University of Rhode Island, Kingston.

2. Light and Electron Microscopy

The ultrastructural features of chloroplasts from sectors of both normal green and mutant yellow tissues were examined either by light microscopy (thick sections) or transmission electron microscopy (thin sections). Leaf tissue (either green or yellow) was sectioned into about 1×1 mm² pieces and fixed overnight in 2.5% glutaraldehyde (in 0.05 M potassium phosphate buffer, pH 8.0). Each sample was washed in 0.05 M phosphate buffer (pH 8.0) and postfixed in 1% OsO₄ (in 0.05 M potassium phosphate buffer, pH 6.8), dehydrated in acetone, infiltrated with Spurr's low-viscosity resin for five days, and embedded flat in aluminum weighing dishes (Mueller and Beckman, 1988). For light microscope observation, thick sections $(1 \ \mu m)$ were cut with a glass knife and mounted on glass slides directly without staining. Samples were than observed under a phase contrast microscope and photographed. Ultrathin sections were mounted on 300-mesh uncoated grids and stained with uranyl acetate and lead citrate. Specimens were examined using a Hitachi HS-9 electron microscope at 75 kV.

3. Chloroplast Isolation and Protein Gel Electrophoresis

Fresh soybean leaves from E25-10 consisting of either all green or all yellow tissue (5-10 gram) were ground in 20 ml grinding buffer (50 mM Tris, pH 7.8, 1 mM MgCl₂, 1 mM EDTA, 330 mM sorbitol, 4 mM Na ascorbate, 0.5% bovine serum albumin (BSA)). Following established procedures (Chandlee and Vodkin, 1989b), intact chloroplasts were collected from the interface of a 40-90% discontinuous Percoll gradient and washed with resuspension buffer (grinding buffer without BSA). The pellet was resuspended in a final volume of 0.8-1.0 ml of resuspension buffer. The chlorophyll concentration was determined spectrophotometrically (Arnon, 1949). Total proteins were measured according to Bradford (1976), using BSA as a standard. Stromal and thylakoid fractions were prepared following the procedure of Fish and Jagendorf (1982). Polyacrylamide gel electrophoresis (PAGE) was performed (Laemmli, 1970) using lithium dodecyl sulfate (LDS) (Delepelaire and Chua, 1979) as a detergent to analyze both stromal and thylakoid membrane proteins.

4. Chlorophyll-protein Complex Analysis

Chl-protein complex analysis was performed using "green gel" analysis essentially as described by Allen and Staehelin (1991) with slight modifications. Intact chloroplasts (equal to about 200 μ l chlorophyll) were suspended in 100 μ l lysis buffer (6 mM Tris, pH 7.5, 50 mM glycine, 10% glycerol) and incubated on ice for 15 min. Thylakoid membranes were then dissolved by means of the addition of 1/10 (v/v) of 3% sodium dodecyl sulfate (SDS), 7% octyl glucoside. After an additional 15 min of incubation on ice, lysates were spun at 18,000 rpm for 10 min (SS-34 rotor, RC5-B centrifuge) to remove insoluble membranes. Samples (20 μ g chlorophyll per lane) were applied to a 7.5% LDS-PAGE minigel with a 4% stacking gel. The electrode buffer contained 25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 0.1% LDS. Gels were run for about 2 h at 5 mA of constant current per slab gel with the Mini-protean II dual slab cell (Bio-Rad, Richmond, CA, U.S.A.) at 4 °C in the dark. The gel was scanned densitometrically at 680 nm to determine the distribution of chl-protein complexes in the gel.

5. Immunoblot Analysis

Electrophoretic separation of thylakoid membrane proteins was performed on 15% LDS-polyacrylamide mini-gels at room temperature for 45 min. Proteins were then electroblotted onto nitrocellulose membrane using Towbin transfer buffer (20% methanol, 0.025 M Tris, pH 8.3 and 0.192 M glycine) (Towbin *et al.*, 1979) using the Trans-Blot[®] SD semi-dry cell (Bio-Rad, Richmond, CA, U.S.A.). The unreacted sites on the membrane were blocked with blocking solution (20 mM Tris-HCl, 500 mM NaCl, pH 7.5 with 3% skim milk powder). Subsequently, the membranes were reacted with specific antisera against OEC33, OEC23, and OEC16, obtained from Dr. B. Andersson (Stewart *et al.*, 1985). These antibodies were raised against purified spinach thylakoid membrane polypeptides of the oxygen-evolving complex of photosystem II.

6. RNA Isolation and RNA Gel Blot Analysis

Total soybean leaf RNA was extracted from either green or yellow tissue as described by McCarty (1986). RNA samples were separated on 1.5% agarose-formaldehyde gels, transferred to nylon membrane (Zeta-Probe blotting membrane; Bio-Rad Laboratories, Richmond, CA, U.S.A.) by passive capillary diffusion in 10X SSPE (1.5 M NaCl, 0.1 M NaH₂PO₄, 0.01 M EDTA, pH 7.4) (Farrell, 1990) overnight, and then cross-linked to the membrane using UV light (Stratalinker[™]; Stratagene Cloning System, La Jolla, CA, U.S.A.). A nonradioactive chemiluminescence method (ECL, Amersham Corporation, Arlington, IL, U.S.A.) was used for probe labeling, hybridization, and detection following the manufacturer's protocol with slight modifications. DNA probes (psaA, psbA, rbcL, rbcS, cab, and 16S rRNA; see below) in distilled water were denatured by boiling for 5 min and cooled immediately on ice for an additional 5 min. An equal amount of labeling reagent (horseradish peroxidasepolyethyleneimine complexes) was added, mixed, and incubated on ice for 5 min. Glutaraldehyde was then added, and the mixture was incubated at 37 °C for 15 min. The membranes were prehybridized for 1 hr at 42 °C in ECL hybridization buffer containing 6 M urea and 0.5 M NaCl for controlling stringency. After incubation, labeled probes were added to the hybridization buffer to a final concentration of 10 ng DNA/ml hybridization buffer. Hybridization was carried out at 42 °C in a shaking water bath for 16 h. Membranes were washed two times at 42 °C in the primary wash buffer (6 M urea, 0.4% SDS, and 0.5X SSC to control stringency) for 15 min each and rinsed two times in 2X SSC at room temperature. Regions of probe hybridization were detected using the ECL detection reagents. Membranes were incubated in the reagents for one min, wrapped in Saran wrap, and exposed to X-ray film. After hybridization, the labeled probe could be washed off from the membrane by boiling in 0.1X SSC and 0.5% SDS solution. Membranes were left in the solution, allowed to cool to room temperature with shaking, and then reprobed. To verify equivalent loading and transfer of RNAs, the membranes were probed with a soybean actin genomic clone (Shah et al., 1982).

7. DNA Probes

Clones of the chloroplast-encoded genes psaA, *psbA*, and *rbcL* from barley were kindly provided by Dr. J. Mullet (Texas A & M University). The *psaA* probe was a 1.8 kb BamHI fragment, the psbA probe was a 1.4 kb HindIII-EcoRI fragment, and the rbcL probe was a 1.3 kb PstI-HindIII fragment. The clone pSRS2.1 containing the coding region for the soybean small subunit of ribulose bisphosphate carboxylase (RubiscoSS) was provided by Dr. R.B. Meagher (University of Georgia). The clone AB2.3 containing the coding region for soybean lightharvesting chlorophyll protein (LHCPII) was received from Dr. D.E. Buetow (University of Illinois). Pea 16S rDNA (1.49 kb) containing the coding region for the chloroplast 16S rRNA was provided by Dr. A.T. Jagendorf (Cornell University). The clone pSAc3 containing a soybean actin gene on a 3 kb HindIII fragment of soybean genomic DNA was provided by Dr. R.B. Meagher (University of Georgia).

III. Results

1. Morphological Analysis of the E25-10 Mutation

The mutant, designated *E25-10*, was generated by exposing soybean seeds of the cultivar "Williams' 82" to gamma radiation (25 kR; 60Co) (Chandlee and Vodkin, 1989b). Two M_2 plants (from *E25-10*, an M_1 plant) were weak and had variegated green/yellow leaves while the remaining plants in the progeny row were normal. The progeny of the weak plant (E25-10-1) have continued to produce the variegated leaf pattern through the M₈ plant generation (Chandlee, unpublished data). The variegated leaf patterns produced from somatic mutations in the leaves of E25-10 are similar to those observed for T225M (Y18m). Close examination of leaves from E25-10 variegated plants reveals that many different sectoring patterns (Fig. 1), suggesting random mutation in all stages of leaf development. For example, small or large green sectors have been found within large sectors of yellow tissue (Fig. 1(A) and (D)), suggesting the possibility that somatic reversions from a recessive form (yellow leaf sectors) to a dominant form (green leaf sectors) may occur. Yellow sectors, very light green sectors, and light green sectors can appear within sectors of green tissue (Fig. 1(B)). It has been previously suggested that the somatic instability observed in E25-10 is in some way regulated such that it occurs late in development (Chandlee and Vodkin, 1989b). However, the observation of large yellow sectors within green tissue (Fig. 1(B)) suggests otherwise. Leaf variegated patterns differ between plants, and patterns change from one type to another between branches in the same plant. Extensive distortion of leaf shape was observed in E25-10 variegated leaves (Fig.



Fig. 1. Somatic mutability patterns exhibited in leaves of the soybean mutant line *E25-10*. (A) A small green sector is present in a yellow leaf. (B) A variegated leaf exhibiting four different sector types, defined as green, light green, very light green, and yellow. (C) A trifoliate yellow leaf. (D) Large green sectors are present on a yellow leaf. The arrows in Fig. 1(A) and (D) indicate green sectors of leaf tissue that appear to have arisen from the surrounding yellow tissue.

1(D)). However, this was not observed in a completely yellow leaf (Fig. 1(C)). A completely uniform yellow plant was observed in a greenhouse grown population of the mutant line. This plant was stunted with distorted unifoliate yellow leaves, and no seeds were recovered from the plant because it died two months after germination (data not shown). This indicates that plant growth is affected by the mutation and germinal reversion, such that homozygous yellow plants may be produced in the *E25-10* plants. Field grown yellow plants observed in the summer of 1992 did not survive due to high light intensity in the field. Genetic analysis conducted with the *E25-10* mutant plants revealed that the mutation is controlled by a single nuclear-encoded gene (Table 1).

Observations were made to determine the cell layers involved in producing the different shadings of green in mutant sectors. The following four shadings (based on close examination of the dorsal surface of the leaves) were used to classify the sectors: normal green sectors (G), light green sectors (LG), very light green sectors (VLG), and yellow sectors (Y). Examinations under a phase contrast light microscope on thick sections (1 μ m) through the normal green leaves revealed that normal chloroplasts exist in the two upper palisade cell layers and in the lower spongy mesophyll (Fig. 2(A)); mutant chloroplasts are restricted to the uppermost palisade layer at the light green sectors (Fig. 2(B)). The very light green sectors lack normal chloroplasts in both of the upper palisade layers (Fig. 2(C)). Yellow sectors of the leaves lack normal chloroplasts in the upper palisade layers as well as lower spongy mesophyll layers (Fig. 2(D)). Similar observations on variegated leaves from the soybean line T225M have been reported by Chandlee and Vodkin (1989b).

A Soybean Leaf Variegated Mutant

Number of Plants								
Parentage	Generation	Green	Variegated	Total	X ² (3:1)	Р		
Clark E25-10								
Clark x E25-10	F1	20	0	20				
	F2	35	15	50	0.66	>.30		
	F2	37	11	48	0.11	>.70		
	F2	38	20	58	2.62	>.10		
	F2	45	18	63	0.43	>.50		
	F2	37	7	44	1.93	>.10		
<i>E25-10</i> x Clark	F1	18	0	18				
	F2	31	18	49	3.6	>.05		
	F2	27	15	42	2.57	>.10		
	F2	45	9	54	2	>.10		
	F2	46	14	60	0.09	>.70		

Table 1. Monogenic Inheritance of the E25-10 Variegated Leaf Mutation in Soybean



Fig. 2. Somatic mutations in leaves of *E25-10* are cell layer specific. (A) Micrograph of a cross section from a normal green leaf. (B) Micrograph of a cross-section from a mutant leaf sector. The upper palisade cell layer (UPa) has mutated to yellow while the rest of the cell layers are green. This is recognized as a light green leaf sector. (C) Similar to Fig. 2(B) but sectioned through a different leaf. The two uppermost palisade cell layers (UPa) have mutated to yellow. This is recognized as a very light green leaf sector. (D) Micrograph of a cross section from a yellow leaf. Leaf samples were embedded in Spurr's low-viscosity resin and sectioned (1 μ m). Specimens were observed under a phase contrast microscope without staining. Bar = 50 μ m.

2. Ultrastructure of the Mutant Chloroplasts

All evidence suggests that chloroplast development in variegated sectors of soybean leaves of the mutable line E25-10 is affected by the mutation. Observations of the mutant chloroplasts using electron microscopy were made with thin sections of yellow and green sectors to determine the effect on chloroplast ultrastructure. A normal chloroplast of soybean is shown on Fig. 3(A). The double membrane of the chloroplast surrounds the stroma, in which the lamellar system is embedded. Well defined grana, consist-



Fig. 3. Electron micrograph of the variegated leaf mutant derived from the soybean line of E25-10. (A) A chloroplast from green tissue exhibits a thylakoid system that has well-developed grana (G) and thylakoid lamellae (L). Mitochondria (M), plastoglobuli (P), and starch grains (S) are present (Bar = 1 μ m). (B) A defective chloroplast from a yellow leaf of E25-10 plant exhibits an unstacked thylakoid membrane structure. No starch grains were found (Bar = 0.5 μ m). (C) Mutant cells of a trifoliate leaf from *E25-10* soybean containing abnormal chloroplasts (C). Unstacked grana were consistently found within chloroplasts of mutant yellow tissue. N = nucleus; V = vacuole; Bar = 1 μ m. (D) A defective chloroplast from a yellow leaf of an E25-10 plant exhibits irregular grana (G). W = cell wall; Bar = 0.5 μ m. Plastoglobuli (P) and small vesicles (Sv) were found in the degenerated chloroplasts (Fig. 4(C) and (D)). The typical defective chloroplast structure and mutated cells observed in mutant tissue are displayed in Fig. 4(B) and (C). The irregular grana observed in Fig. 4(D) was seen only rarely.

ing of thylakoids, are observed. Starch grains and plastoglobuli (osmiophilic bodies) are often found in the chloroplast (Fig. 3(A)). Ultrastructural studies of the defective chloroplasts from yellow (*E25-10*) tissue revealed that grana and stroma lamellar systems are disrupted. Thylakoid membranes are primarily represented as long and parallel structures with little or no overlaps (see Fig. 3(B),

	Mutant Chloroplast	
	T225M	E25-10
Lamellar Whorl	+	-
Small Vesicle	-	+
Plastoglobuli	+	+
Grana Stack	_	±
Separated Lamellae	+	+
Decreased Total Chl	+	+
Altered Chl a/b ratio	_	_

 Table 2. Comparison of the Properties of Defective Chloroplasts Between

 Variegated Leaf Mutants of Soybean, T225M and E25-10

Note: "-" not found; "+" found; "±" found but not common

a typical example), giving rise to poorly defined grana. No starch grains have been detected in the mutant chloroplasts. Irregular grana (rarely observed), small vesicles and plastoglobuli could be found within the defective chloroplasts (Fig. 3(C) and (D)). Lamellar disruption leading to the formation of unstacked grana thylakoids, following by the formation of loose and elongated lamellae, and the appearance of small vesicles revealed that degradation occur within the abnormal chloroplasts. However, lamellar whorls were not found in the defective chloroplast of *E25-10*, suggesting a distinct defect in *E25-10* as compared to *T225M*, a previously characterized soybean leaf variegated mutant (Table 2; Palmer *et al.*, 1979).

3. The Biochemical Defect in *E25-10*

To examine the biochemical defect in E25-10, thylakoid membrane protein extracts from chloroplasts isolated from green and yellow tissue were analyzed and compared on LDS-PAGE. A minimum of 5-6 polypeptides with estimated molecular weights of 51, 44, 25, 15, 13, and 12 kDa are consistently missing from chloroplasts of yellow tissue (Table 3). In addition, LDS-PAGE analysis of thylakoid membrane proteins revealed that a polypeptide with the estimated molecular weight of 60 kDa is absent from chloroplasts of yellow leaf tissue (data not shown). A ~36 kDa polypeptide was found in thylakoid membranes derived from mutant chloroplasts of yellow leaf tissue, but it was not detectable in thylakoid membranes proteins isolated from normal chloroplasts. This type of observation is not uncommon for this type of mutant and may result from an unprocessed polypeptide in the defective chloroplasts. The absorbance spectrum of acetone extractable pigments in the yellow tissue of E25-10 leaves differ from that of the green extracts in the range of 400-500 nm (data not shown). The absorbance by other pigments in the sample makes it difficult to conclude that changes in the absorbance pattern are entirely due to a loss of chlorophyll in the mutant. The total chlorophyll content in the mutant leaf tissue was measured spectrophotometrically and is presented as a percentage of the amount of total chlorophyll in green leaf tissue in Table 3. The amount of chlorophyll *a* and chlorophyll *b* is reduced in the mutant chloroplasts of *E25-10* by 82% and 55%, respectively. It is also clear that the total chlorophyll content is significantly reduced in yellow leaf tissue by 70-80%. However, the chlorophyll a/b ratio (2.8-3.0) is not altered by the mutation.

In order to determine whether the reduction in total chlorophyll and the change in the absorption spectrum pattern in the yellow tissue is related to a loss of specific photosynthetic complexes, non-denaturing lithium dodecyl sulfate polyacrylamide gel electrophoresis (or green gel analysis), which maintains the integrity of chlorophyllprotein complexes, was carried out. Six major pigmentprotein complexes (green bands) from green tissue were identified as CP1 (P-700-chl a complex), LHCP1 (oligomeric LHCIIb), LHCP² (dimeric LHCIIb), CPa (chl a-protein from PSII), LHCP³ (monomeric LHCIIb), and FP (Free Pigment; data not shown). Green gel analysis showed that four major pigment-protein complexes (CP1, LHCP¹, LHCP² and CPa) are absent in the *E25-10* mutant while the amounts of LHCP3 and FP are reduced (Fig. 4).

Three extrinsic 33, 23, and 16 kDa proteins, which function as water splitting sites (oxygen-evolving complex, OEC) to produce protons, electrons, and oxygen molecules in the photochemical reaction II (Andersson, 1986) have been identified as nuclear gene products (Westhoff *et al.*, 1985). They are synthesized as cytosolic precursors of 39, 33, and 26 kDa, respectively and post-translationally translocated into chloroplasts (James et al., 1989; Westhoff et al., 1985). In T225M, the OEC16 polypeptide is absent from the y18 mutant tissue (Cheng and Chandle, manuscript in preparation). This appeared to not be the case in E25-10 defective chloroplasts since all three polypeptides in the complex (OEC33, 23, and 16) were detectable in yellow leaf extracts by Western blot analysis (Fig. 5). This reinforces the identification of distinct genetic defects in E25-10 and T225M.

Table 3. The Reduction of Chlorophyll and Molecular Weights of Thyla-
koid Membrane Proteins Missing in the Mutant Chloroplasts of
E25-10

Chlorophyll (%)		Total Chl	Missing Polypeptide kDa	
А	A B			
$18.4\pm4.0^{\rm a}$	$45.0\pm13.0^{\rm a}$	$25.0\pm4.0^{\rm a}$	51 ^b	
			44	
			25	
			15	
			13	
			12	

^a Average of five measurements.

^b Thylakoid membrane proteins were isolated from chloroplasts of both green and yellow leaf tissue, analyzed by LDS-PAGE, and detected by silver stain.



Fig. 4. Gel profile of chl-protein complexes. Thylakoid membranes from green or yellow leaf tissue of the soybean line *E25-10* were analyzed by mild LDS-PAGE (7.5%). Slab gels were scanned at 680 nm. The individual chl-containing bands were defined following the Anderson system (Anderson *et al.*, 1978): 1 = CP1 contains P700 and chlorophyll *a*; 2, 3, and 5 = LHCP¹, LHCP², and LHCP³, which represent the three complexes, oligomeric, dimeric, and monomeric LHCIIb, respectively, and contain the majority of chlorophyll *b* derived from the LHCII; 4 = CPa, which is derived from photosystem II and contains little chlorophyll *b*; FP = a zone of free pigment, which comprises 10-15% of the applied chlorophyll. CP1a, which contains LHCI (a chlorophyll-*b* containing antenna complex) and appears to migrate more slowly than CP1, is not shown. G = green, Y = yellow.

4. Reduced Levels of Photosynthetic Gene Expression are Associated with Mutant Yellow Sectors of *E25-10* Plants

To determine if the reduction in thylakoid membrane proteins was a consequence of reduced steady state mRNAs levels, we measured the accumulation of chloroplast mRNAs as well as nuclear mRNAs in both E25-10 green and yellow leaf tissue by Northern analysis. Equal amounts of RNA from wild-type and mutant leaves were separated on 1.5% denaturing gels and transferred to nylon membranes. The filters were then hybridized with clones of specific chloroplast- and nuclear-encoded photosynthetic genes. Changes in chloroplast-(psaA, psbA, rbcL, and 16S rRNA) as well as nuclear-encoded gene (rbcS and cab) transcript levels were examined (Fig. 6(A) and (B)). Equivalent levels of *psbA* mRNA, which encodes the 32 kDa protein in PSII, were detected in green and yellow tissue. However, psaA transcripts, which encode for the P700 apoprotein in PSI, were significantly reduced in *E25-10* mutant yellow tissue. The level of the large subunit of ribulose



Fig. 5. Western blot analysis of the three polypeptides which comprise the oxygen-evolving complex of photosystem II in wild-type (G) and mutant (Y) tissue of *E25-10* leaves. Thylakoid membrane proteins were isolated from chloroplasts of green or yellow soybean leaves of *E25-10*, separated on a 15% LDS-PAGE mini-gel, then transferred to a nitrocellulose membrane and detected with either anti-33, anti-23, or anti-16 sera, which exhibit specificity for OEC33, 23, and 16 proteins, respectively (Stewart *et al.*, 1985). The OEC33, OEC23, and OEC16 polypeptides can be detected in both green and yellow extracts, as indicated by a dash (–).

bisphosphate carboxylase (*rbcL*) mRNA appeared to be reduced slightly in the mutant plants whereas a much more significant reduction in the *16S rRNA* expression was detected in the yellow leaf sectors (Fig. 6(A)). Two nuclear gene transcripts encoding the small subunit of ribulose bisphosphate carboxylase (*rbcS*) and the light-harvesting chlorophyll a/b binding protein (LHCPII; *cab* gene) of PSII were also reduced in yellow tissue (Fig. 6(B)).

IV. Discussion

1. The Mutation in *E25-10* Produces Variegated Leaf Patterns and Affects Chloroplast Development

The existence of uniform yellow plants in either greenhouse or field grown populations of the soybean foliar mutant, *E25-10*, indicates that germinal mutations may occur in *E25-10* plants. Random somatic mutations occur in all stages of leaf development and lead to a broad range of variegated patterns observed from plant to plant. Small yellow sectors resulting from mutations that occur late during the ontogeny of the leaf and large yellow sectors within green tissue resulting from early mutation events suggest



Fig. 6. Northern blot analysis of steady state RNA levels for four chloroplast- (*psaA*, *psbA*, *rbcL*, and *16S rRNA*) and two nuclear-encoded (*rbcS* and *cab*) genes. 20 μg of total RNA from wild-type (G) and mutant (Y) leaf tissue was separated in 1.5% agarose-formaldehyde gels, transferred to positively charged nylon membranes, and hybridized with either *psaA*, *psbA*, *rbcL* and *16S rRNA* specific clones (Klein *et al.*, 1988; Cerutti and Jagendorf, 1991) or *rbcS* and *cab* (*LHCPII*) probes (Berry-Lowe *et al.*, 1982; Hsiao *et al.*, 1988). No apparent alteration in expression was detected for *psbA* whereas the *psaA* transcripts were barely measurable in the mutant. Accumulation of transcripts for *rbcL*, *rbcS*, *cab* and *16S rRNA* is also reduced in the yellow leaf tissue. The *psaA*, *psbA*, *rbcL*, *rbcS*, *cab* and *16S rRNA* transcripts are indicated by a dash (–).

that the timing of the mutation is not restricted to any particular developmental stage.

The ultrastructure of the defective chloroplasts of *E25-10* plants differs from that of normal chloroplasts. Characteristically, they consist of single or parallel thylakoid lamella and irregular grana in the presence of small vesicles and absence of starch grains. The chloroplasts from yellow tissue do not exhibit the "lamellar whorl" characteristic of the degenerated grana apparatus as observed in *T225M* (Palmer *et al.*, 1979). However, the distribution of the defective chloroplasts in *E25-10* plants is cell-layer specific, similar to the situation observed in other variegated leaf mutants.

2. Regulation of Pigment-protein Complex Accumulation in Mutant Yellow Tissue of *E25-10*

The abnormal chloroplasts also differ biochemically from normal ones in that there is a lack of certain thylakoid membrane polypeptides, an absence of certain pigmentprotein complexes, and a reduction of total chlorophyll content without an alteration in the chlorophyll *a/b* ratio. Because chlorophyll synthesis is required to stabilize thylakoid membrane proteins (Eichacker *et al.*, 1990; Mullet *et al.*, 1990), the newly synthesized P700 apoprotein is rapidly degraded if it is not associated with the pigment. Therefore, an assay of chlorophyll, its precursors, and/or other major pigments (e.g., carotenoids) for E25-10 yellow leaf extracts, using high performance liquid chromatography (HPLC), should be useful in determining what role these pigments play in protein accumulation during chloroplast development (Yamamoto et al., 1992). Further investigation into the presence or absence of protein components in the pigment-protein complexes is necessary to show if protein components play a key role in accumulation and degradation of photosynthetic complexes in the mutant. The existence of LHCP³ indicates that the light-harvesting chlorophyll *a/b* protein in the PSII may be present at a low level in the defective chloroplasts. These results of photosynthetic complex analysis, combined with the data from thylakoid membrane protein analysis and ultrastructural observations on defective chloroplasts, suggest that the primary lesion for the mutation is probably located at the PSI, even though a few grana stacks have been found within the mutant plastids.

3. The PsaA Polypeptide of PSI is Absent and its mRNA Level is Low in the Defective Chloroplasts of *E25-10* Mutant Plants

The molecular weight of the PSI P700 chlorophyll abinding proteins isolated from soybean is between 62 and 65 kDa (Henry et al., 1990). The absence of a ~60 kDa protein has been observed in the thylakoid preparations from E25-10 mutant tissues using 15% LDS-PAGE. Therefore, the presence and/or absence of the PsaA protein in E25-10 yellow leaves can be determined using Western blot analysis if the PsaA antibody is made available. This information will be particularly useful since the *psaA* transcript is barely measurable in the yellow sectors of E25-10 mutant plants (Fig. 6(A)). The accumulation of mRNAs during leaf development is regulated at the transcriptional and/or post-transcriptional level. Experiments performed with purified chloroplasts, root cell plastids, and light treated chloroplasts demonstrated minimal transcriptional regulation in these plastids as determined by transcription run-on assays (Deng and Gruissem, 1987; Mullet and Klein, 1987). There is general agreement that post-transcriptional effects on mRNA processing, transport, and stability are factors important in controlling gene expression (Brawerman, 1989). Nuclear mutants have been shown to affect the expression of photosystem II (PSII), PSI, and ATP synthase chloroplast and/or nuclear genes in barly (Kreps and Kay, 1997), Chlamydomonas (Drapier et al., 1992; Kuchka et al., 1989; Monod et al., 1992; Rochaix et al., 1989; Sieburth et al., 1991), soybean (Guiamet et al., 1991; Nakayama et al., 1998), and maize (Cook and Miles, 1992). Thus, an investigation into the mechanisms regulating expression of the psaA gene in the E25-10 mutant should provide insight into how a nuclear mutant may affect photosynthetic chloroplast gene expression and mRNA accumulation.

4. Certain Nuclear- and Chloroplast-encoded Photosynthetic Gene Expression is Affected by the *E25-10* Mutation

The *rbcS*, *cab*, *rbcL*, and *16S rRNA* transcript levels are low in the yellow leaf sectors of E25-10 mutant plants (Fig. 6(A) and (B)). It is known that expression of *rbcL* and rbcS genes must be coordinated during chloroplast development in certain plants (e.g. Simpson and Herrera-Estrella, 1990). The reduction of *rbcL* and *rbcS* mRNAs in the E25-10 mutant reflects interactions between the nuclear and chloroplast genomes, in general. Further investigations are needed to determine if the interactions between nuclear and chloroplast genomes are interrupted by the mutation. However, it is not surprising that the coordination between *rbcL* and *rbcS* events is mediated by protein degradation since failure to synthesize *rbcL* polypeptides results in rapid degradation of the *rbcS* polypeptides in the cytoplasm. A simillar regulatory mechanism prevents the accumulation of LHCPII in the absence of chlorophyll (Bennett, 1981). In the *E25-10* mutant, the total chlorophyll is reduced by 70-80% (the chlorophyll *a/b* ratio remains the same), and the LHCPII proteins are expressed at a reduced level. As shown on the Fig. 4, the chlorophyll binding proteins, LHCP¹ and LHCP², are not detectable in the chloroplasts of mutant tissue. Similar observations were obtained from LDS-PAGE gel analysis, where the levels of LHCP² appeared to be reduced in the mutant. The light-harvesting chlorophyll a/b binding proteins of PSII are the most abundant thylakoid membrane proteins (Gounaris et al., 1986). These polypeptides are encoded by a small family of nuclear genes (cab). The major LHCP is the 27 kDa protein in soybean, and there are three additional LHCP polypeptides of 23-29 kDa (Walling et al., 1988). A soybean LHCP gene clone was used to quantify mRNA steady state levels in total RNA preparations extracted from the green and yellow leaf sectors of the E25-10 plants. The level of LHCP transcripts in yellow leaves is slightly reduced (Fig. 6(B)). Therefore, a transcriptional regulatory mechanism is likely involved in the *cab* gene expression in *E25-10* yellow leaf. Finally, the translational capability of specific plastid genes in the defective chloroplasts may be affected by the mutation. This is suggested because of the reduced levels of 16S rRNA transcript in chloroplasts from E25-10 yellow tissue (Fig. 6(A)).

In summary, the effects of the mutation in E25-10 on chloroplast development have been analyzed. The results of this study suggest a nuclear origin of the E25-10 mutation. A degenerated chloroplast membrane structure and absence of certain thylakoid membrane proteins and chlorophyll-protein complexes were revealed. This mutation differs from T225M in that no lamellar whorls are found in the defective chloroplasts of E25-10 as has been observed in T225M. Also, all three oxygen-evolving complex com-

ponents could be detected in the yellow leaf tissue using Western blot analysis whereas the OEC16 protein was not found in the homozygous yellow plants of *T225M*. The expression of *psaA* is apparently reduced in the mutant. Therefore, a transcriptional and/or post-transcriptional regulation in the expression of the *psaA* gene in *E25-10* mutant chloroplasts is suggested. Our results indicate that the primary lesion for *E25-10* mutation is possibly located at the PSI in spite of the fact that fewer grana were observed in the defective chloroplasts.

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大豆葉斑紋突變E25-10葉片構造遺傳與生化之研究

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摘要

本研究採用生理、生化與遺傳方法對經放射線站六十誘發之大豆葉斑紋突變種E25-10進行分析。E25-10葉片的黄 色斑紋葉肉細胞內含缺失的葉綠體,由葉表顏色深淺不一且挾雜相間,及其缺失葉綠體所位在葉肉細胞層的排列方式 判斷,E25-10可能具有自發回復突變的能力。在電子顯微鏡下觀察缺失的葉綠體,其葉綠餅膜以相互平行、無或甚少 重疊方式排列,不含澱粉粒,而小囊袋或油狀體則可見。遺傳研究則顯示E25-10為核基因突變體。E25-10黃色斑紋葉 肉組織所含葉綠素較正常葉肉組織減少70-80%,雖然其葉綠素a/b比值仍維持不變,但是光譜分析顯示在波長400-500 nm間,其葉綠素吸收光譜與正常葉肉組織所含葉綠素並不相同。這種葉綠素含量減少與吸收光譜改變,應與缺失 的葉綠體內缺損某些光合作用複合體有關。綠色膠體電泳分析顯示,在缺失的葉綠體內四個主要的色素一蛋白複合體 (CP1, LHCP¹, LHCP²和CPa)皆不存在。LDS-PAGE電泳分析發現,至少有5-6個葉綠餅膜蛋白在缺失的葉綠體內不可 見,其分子量分別是51,44,25,15,13及12 kDa。在黃色斑紋葉肉細胞內,葉綠體或細胞核基因表現研究結果顯示,葉 綠體基因psaA (PSI),rbcL,16S rRNA與細胞核基因rbcS, cab都有不同程度的衰減。綜合以上結果,雖然E25-10缺失的葉 綠體內甚少重疊之葉綠餅膜,但是我們認為突變發生的部位仍以光反應系統1的可能性較大。