Enzymes Related to Chlorophyll Biosynthesis in Mutant Soybean [*Glycine max* (L.) Merr.] Lines

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Variations in enzyme activities and pigment concentrations of Asgrow *w*4-*m* soybean [*Glycine max* (L.) Merr.] were used to evaluate plausible mechanisms for chlorosis in mutant soybean lines. Asgrow *w*4-*m* and three chlorophyll-deficient mutants, designated as CD-1, CD-2, and CD-3, were grown in an indoor growth room and harvested at the V3 to V4 stage of development. Crude extracts from leaf tissues were analyzed for malate dehydrogenase (MDH), cytochrome *c* oxidase (CCO), and aspartate aminotransferase (AST) activities, as well as concentrations of chlorophyll *a*, chlorophyll *b*, and carotenoids. Results revealed that MDH activity and pigment concentration were highest in Asgrow *w*4-*m*, followed by the mutant soybean lines ranked in descending order from CD-3 to CD-1. Relative differences in enzyme activities and pigment concentrations disclosed by these results suggest that a genetic alteration or decreased flow of metabolites may be responsible for chlorophyll abnormalities in the mutant lines of Asgrow *w*4-*m* soybean.

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] breeding programs sometimes produce cultivars with deleterious characteristics as well as cultivars with superior productivity. One such breeding program at the Asgrow Seed Company nursery, Stonington, Illinois, produced the highly mutable Asgrow *w*4-*m* line (Weigelt et al., 1990). Among the variants from this mutable line were three chlorophyll-deficient (CD) soybean lines possessing a null gene product for one malate dehydrogenase isozyme allele (*mdh*1-*n*) (Groose and Palmer, 1990; Hedges and Palmer, 1992). Several genetic hypotheses have been proposed to explain how a chromosomal aberration links the chlorophyll deficiency and the *mdh*1-*n* allele (Palmer, 1984; Groose and Palmer, 1987; Groose et al., 1988; Hedges and Palmer, 1992). Alternatively, physiological mechanisms have been suggested to explain how a chromosomal aberration produces soybean lines with the *mdh*1-*n* allele, resulting in chlorosis (Bidlack et al., 1991; Elmer et al., 1994).

Chlorophyll-deficient lines obtained from Asgrow *w*4-*m* exhibit different levels of chlorophyll concentration, chloroplast development, and growth rates (Groose et al., 1987). Isozyme analyses of Asgrow *w*4-*m* chlorophyll-deficient progeny also reveal the presence of a null gene product for one NAD-dependent malate dehydrogenase (MDH, EC 1.1.1.37) allele (Palmer et al., 1989). Subcellular localization suggests that two of the three mitochondrial MDH (mMDH) isozyme bands are absent. The dimeric nature of the mMDH isozymes (Gietl and Hock, 1982) is consistent with the hypothesis of a single deletion causing the disappearance of a homodimer and a heterodimer from mitochondria (Hedges and Palmer, 1992). Although the loss of two mitochondrial isozymes would sub-

stantially impact the flow of metabolites within mitochondria, similar work with corn expressing the mMDH null allele has shown that germination requires only a single active mMDH isozyme (Goodman et al., 1981). Additional studies have shown that the genetic aberration responsible for the chlorophyll deficiency and the missing mMDH isozymes occurs on a single chromosome (Hedges and Palmer, 1992).

Some mechanisms suggest that the relationship between the mMDH isozyme null allele and decreased chlorophyll biosynthesis may be attributed to a transposable element during reversion of an unstable allele or a deletion that affects the *mdh*1-*n* locus (Hedges and Palmer, 1992). Close linkage between genes encoding for a novel MDH and chlorophyll biosynthesis enzyme, geranylgeranyl hydrogenase, gives good reason for genetic alteration or physiological mechanisms. Recent cloning of this novel MDH gene from a genomic fragment indicates that there is a chlorophyll biosynthesis gene located immediately upstream (Imsande et al., 2001). If the mMDH null gene present in the chlorophyll deficient lines has a similar chlorophyll biosynthesis gene located upstream, the close linkage between the mMDH gene and a similar chlorophyll biosynthesis gene could explain the chlorophyll deficiency. Because of the difficulty in pinpointing an exact chromosomal aberration responsible for both the *mdh*1-*n* allele and the chlorophyll deficiency, physiological mechanisms, based on a single deletion in the *mdh*1-*n* locus, have been suggested (Bidlack et al., 1991; Elmer et al., 1994).

There are several metabolic avenues by which the lack of mMDH isozymes could reduce chlorophyll biosynthesis via decreased flux of metabolites through the Krebs cycle and malate/aspartate shuttle. The Krebs cycle intermediate, α-ketoglutarate, may be implicated in chlorophyll deficiency because it is an important precursor of glutamate, which is an essential metabolite for chlorophyll biosynthesis (Beale, 1990). The mechanism behind the chlorosis associated with mMDH involves the interconversion and transport of malate, via the malate/aspartate shuttle, into the cytoplasm and subsequently into the chloroplast. Interrelationships among the metabolites in the peroxisome, mitochondrion, cytosol, and chloroplast are shown in Fig. 1. Due to the selective transport of Krebs cycle intermediates across the inner mitochondrial membrane, only the mechanisms involving α-ketoglutarate (Bidlack et al., 1991) and the malate/aspartate shuttle (Elmer et al., 1994) were considered in this investigation.

In this study, enzyme activities and pigment concentrations of crude homogenates were evaluated to determine if significant differences exist among the four soybean lines. It was hypothesized that, if the level of enzyme activities followed the same ranking as the level of chlorosis in normal and mutant lines, then decreased pigmentation may be genetically or pleitropically related to enzyme activities involved in chlorophyll biosynthesis.

MATERIALS AND METHODS

Plant Germination and Maintenance

Soybean lines used in this experiment included the wild-type, T322 (Asgrow *w*4-*m*), and the chlorophyll-deficient mutants, T323 (CD-1), T324 (CD-2), and T325 (CD-3). All soybean lines were obtained from the USDA-ARS Soybean Germplasm Collection at the University of Illinois located at Urbana. Four replications of each soybean line were randomly arranged within blocks and planted in 35 cm pots containing 0.025 m^3 of Growing Mix No. 2 soil from Farfard Soil Co., Quebec, Canada. Planting was staggered to allow harvest of a single replication each week for four consecutive weeks. Lighting was provided by high output 110W wide spectrum Sylvania Grow-Lux (F96T12/ GRO/HO/WS) fluorescent bulbs. Plants were maintained with a 16/8 hour day/night cycle.

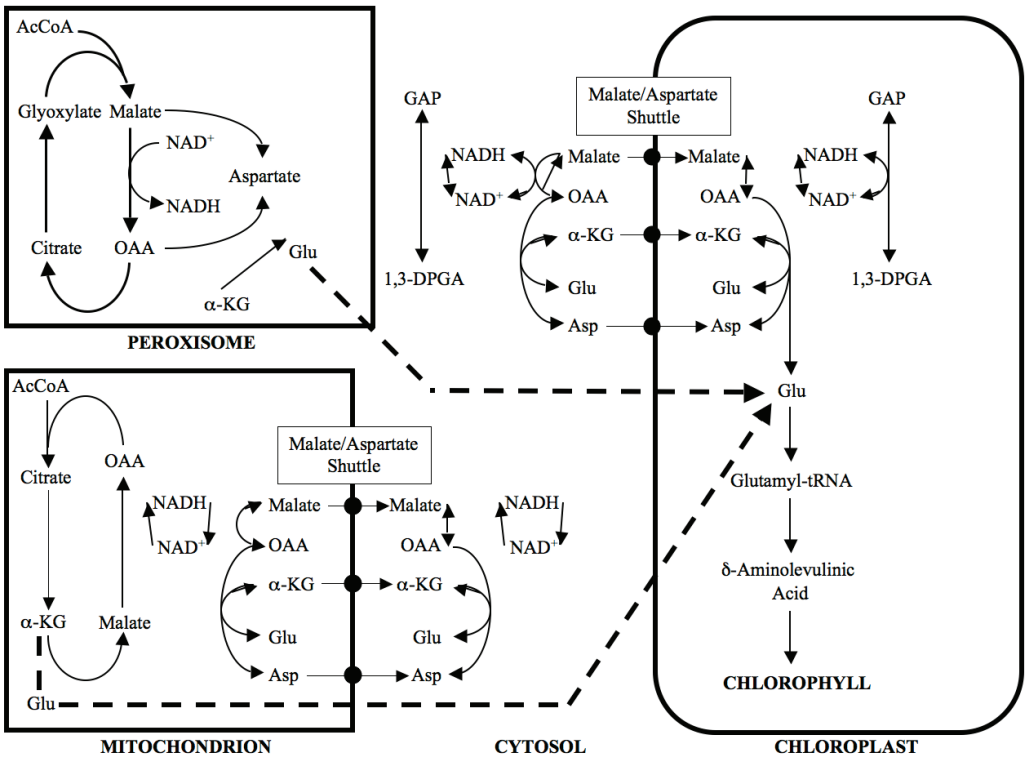

Figure 1. Hypothetical mechanism demonstrating interrelationships among subcelluar intermediates involved in synthesis of chlorophyll in mutant soybean lines. Solid lines represent known metabolic pathways and the two dashed lines suggest possible transport of glutamate from peroxisomes or mitochondria to chloroplasts for eventual conversion to chlorophyll.

Laboratory Supplies and Location

All procedures were conducted at the University of Central Oklahoma at Edmond and at the University of Oklahoma Health Sciences Center at Oklahoma City. Chemicals and reagents used in this experiment were obtained from Sigma Chemical Company, St. Louis, Missouri.

Harvest and Crude Enzyme Extract

On the fourth week, the V3-V4 stage of growth (Fehr and Caviness, 1977), a single replication was harvested. Leaf tissue was separated and sliced into 2 mm strips before being submerged in grinding buffer. The volume of the grinding buffer was kept at a ratio of 2.5 mL per gram of leaf tissue. Tissue samples were homogenized at 8,000 rpm using three 10-second bursts from an Ultraturrax-T25 homogenizer (IKA - Labortechnik, Staufeni. Br., West Germany) in an ice chilled grinding buffer containing 500 mM sucrose, 40 mM TES-NaOH, 1 mM DTT, 5 mM EDTA, 2.0% PVP-40, 0.1% BSA (Day and Hanson, 1977), and adjusted to a pH of 7.5. The crude homogenate was strained through four layers of cheesecloth and then filtered using Whatman® Number 54 filter paper to obtain a crude enzyme extract.

Protein Determination

Protein concentration (mg/mL) was determined using the Bio-Rad protein assay dye reagent concentrate (Bradford, 1976). Linear regression of the protein dilutions was used to determine the protein concentration of the samples.

Spectrophotometric Enzyme Assays

Malate dehydrogenase (MDH) was assayed using a Hewlett-Packard 8452A UV/VIS Diode Array Spectrophotometer (Hewlett Packard Company, Dallas, Texas) with a 1.0 mL quartz cuvette and a 1.0 cm light path. The substrate contained 20 mM TES-NaOH, 15 mM malate, 0.5 mM NAD, 70 mM KCl (Appels and Haaker, 1991) and 0.1% Triton X-100 (Hjelmeland and Chrambach, 1984). The substrate was modified to include 5 μM antimycin A (Day and Wiskich, 1978) and adjusted to a pH of 7.4. The linear increase in absorbance at 340 nm was measured for 45 seconds. Enzyme activity was calculated using an extinction coefficient of 6.233 mM^{-1} cm^{-1} (Netheler, 1974). Total enzyme activity was expressed as μmoles per minute.

Cytochrome *c* oxidase (CCO) was used to determine relative electron flow in mitochondria, mitoplasts (inner membrane and matrix), and inner membrane (Quail, 1979). The substrate contained 10 mM Tris-HCl, 0.5 mM EDTA, and 0.05 mM reduced cytochrome *c* (Day and Wiskich, 1974). The substrate was modified to include 75 mM KH_2PO_4 (Sen, 1975) and the pH adjusted to 7.2. The absorbance at 550 nm was measured for 1 minute. Enzyme activity was calculated using an extinction coefficient of 28.4 $mM^{-1}cm^{-1}$ (Van Gelder and Slater, 1962). Total enzyme activity was expressed as mmoles per minute.

Microplate Enzyme Assays

Aspartate aminotransferase (AST) was assayed using the colorimetric method, modified from the Sigma Diagnostics Kit #505, to accommodate the use of a microplate reader. Each microplate contained assay and blank samples for a single treatment. Standards included eight dilutions ranging from 0 to 0.050 mM oxaloacetic acid. The assay was initiated with 48 μL of substrate that contained 200 mM DL-aspartate, 200 mM α-ketoglutarate, 1.8 mM phosphate buffer, and adjusted to a pH of 7.5. The reaction proceeded at 37 ^{o}C for one hour. To terminate the reaction, 48 μL of color reagent (20 mg/dL 2,4-dinitrophenyl hydrazine in an acidic solution) was added to all of the wells. The color reagent reacted for 20 minutes at room temperature. The color reaction was then terminated by the addition of 94 μL of 1.60 N sodium hydroxide. Within five minutes, the absorbance was read at 550 nm. Absorbance units were converted to μmoles of oxaloacetate formed using linear regression of the calibration standards. Total enzyme activity was expressed as μmoles per hour (Tonhazy et al., 1950; Reitman and Frankel, 1957).

Chlorophyll Quantification

Chlorophyll *a*, chlorophyll *b*, and carotenoid concentrations were determined by measuring the absorbance at 452, 644, and 663 nm. Chlorophyll *a*, chlorophyll *b*, and carotenoids were used as positive markers for chloroplasts (Quail, 1979). To extract chlorophyll from homogenates, 225 μL samples were placed into 1.5 mL microcentrifuge tubes. These mixtures were then brought to a final concentration of 85% acetone by the addition of 1.275 mL of 100% acetone, and centrifuged at 6000*g* to clarify the acetone-extracted pigment. Concentrations of the pigments were determined according to Shoemaker et al. (1985).

Statistical Analysis

Protein, enzyme, and pigment data were analyzed using PROC GLM (SAS Institute, 1985). One-way analysis of variance (ANOVA) provided a measure of significant differences among data collected from the four soybean lines. In cases where ANOVAs revealed significant differences, least significant differences and standard errors were used to graphically represent differences among specific sources of variation.

RESULTS AND DISCUSSION

Analysis of variance revealed significant differences among the four soybean lines for MDH and CCO activities as well as chlorophyll *a*, chlorophyll *b*, and carotenoid con-

centrations (Table 1). Malate dehydrogenase (Fig. 2A) and CCO (Fig. 2B) activities, as well as chlorophyll *a* (Fig. 3A), chlorophyll *b* (Fig. 3B), and carotenoid (Fig. 3C) concentrations, were higher in Asgrow *w*4-*m* compared with these same measurements in the CD-1 mutant soybean. Asgrow *w*4-*m* maintained the highest enzyme activity and pigment concentration, whereas measurements among the mutant soybean lines followed a descending order from CD-3 > CD-2 > CD-1. No significant differences were found among the soybean lines for protein concentration or AST activity (Table 1).

Consistent differences in crude MDH and CCO activities, following the order of CD-3 > CD-2 > CD-1 suggested these two enzymes are involved in the mechanism(s) resulting from mutations in Asgrow *w*4-*m* mutable lines. Low MDH activity would likely lead to a retarded flow of metabolites through the Krebs cycle, whereas low CCO activity would result from a loss of electron donors (e.g., NADH and $FADH_2$) needed for the electron transport system. It is possible that, in this study, low MDH activity could be responsible for lowering the pool of glutamate needed for chlorophyll biosynthesis in the chloroplast (see Fig. 1). Hence, in the mutant soybean lines, a lower amount of glutamate may contribute towards chlorosis.

In this study, the Asgrow *w*4-*m* soybean line had the highest MDH activity followed by that of the soybean mutants in the order, CD-3 > CD-2 > CD-1. Compared with Asgrow *w*4-*m*, crude MDH activity was 75%, 59%, and 52% in the CD-3, CD-2, and CD-1 mutant lines, respectively (Figure 2A). These data concur with previously published results for mMDH enzyme activity in the normal and mutant lines (Bidlack et al., 1991) in which mMDH activity was 86%, 68%, and 60% in the CD-3, CD-2, and CD-1 mutants compared with this activity in Asgrow *w*4-*m*.

Table 1. Significance among measurements of crude extracts.

From Asgrow *w*4-*m* soybean and associated mutants.

Measurement	Significance among soybean lines
Protein	NS
Malate dehydrogenase	*
Aspartate aminotransferase	NS
Cytochrome c oxidase	*
Chlorophyll a	*
Chlorophyll b	**
Carotenoids	**

*, ** Significant at the 0.05 and 0.01 probability levels, respectively; NS, not significant at the 0.05 probability level.

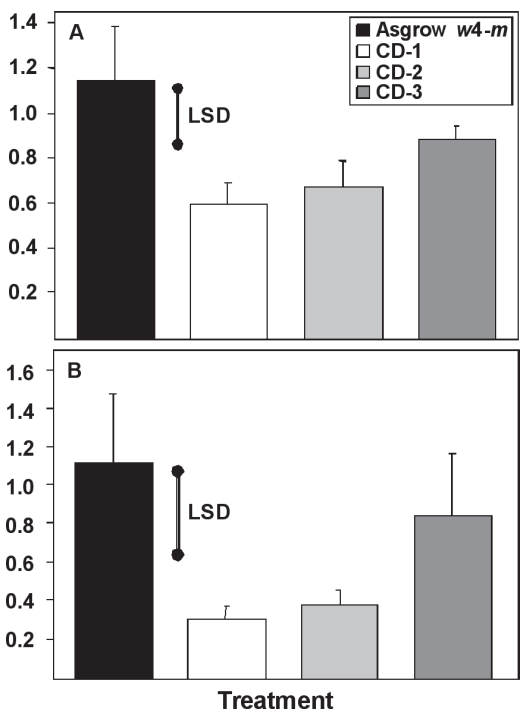

Figure 2. Crude malate dehydrogenase (MDH) and cytochrome *c* oxidase activities obtained from leaves of Asgrow *w*-4*m* and three chlorophyll-deficient (CD) lines. Vertical error bars represent standard error of the means (n-4). Least significant differences (LSD) are indicated in each graph.

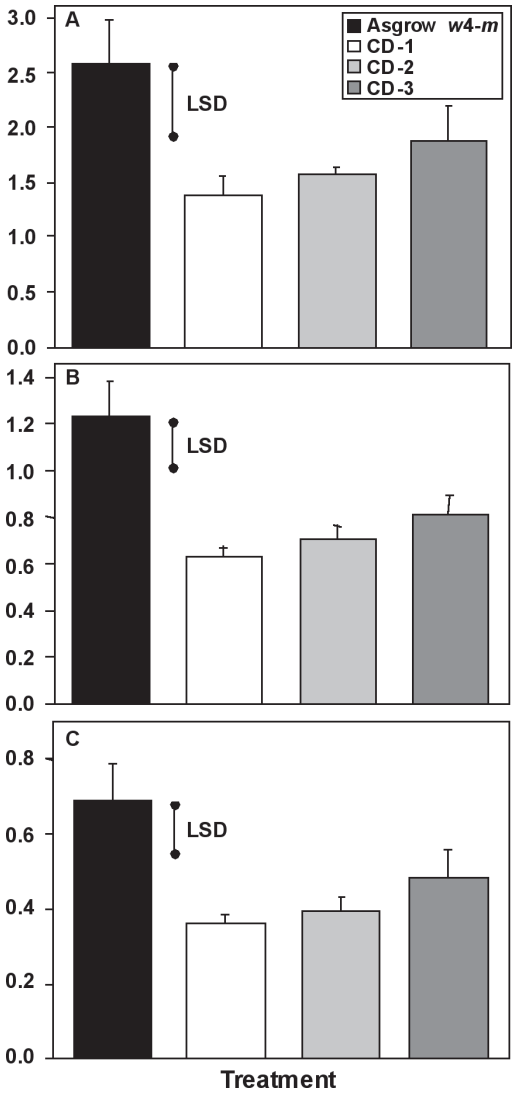

Fig. 3. Crude chlorophyll a, chlorophyll b, and carotenoid concentrations obtained from leaves of Asgrow *w*-4*m* and three chlorophyll-deficient (CD) lines. Vertical error bars represent standard error of the means (n-4). Least significant differences (LSD) are indicated in each graph.

Consistent differences in crude MDH activities and pigment concentrations among the four soybean lines substantiated a relationship between MDH activity and chlorophyll biosynthesis. Compared with Asgrow *w*4-*m*, CD-3 exhibited near-normal pigmentation (73% chlorophyll *a*; 66% chlorophyll *b*; 70% carotenoids; Fig. 3A), CD-2 demonstrated yellow-green to near-normal pigmentation (61% chlorophyll *a*; 57% chlorophyll *b*; 57% carotenoids; Fig. 3B), and CD-1 had less pigmentation than any of the other lines (53% chlorophyll *a*; 51% chlorophyll *b*; 53% carotenoids; Fig. 3C).

Results from this study show that the level of enzyme activities followed the same ranking as the level of chlorosis in normal and mutant lines. Hence, it is likely that decreased pigmentation in Asgrow *w*4-*m* may be genetically or pleitropically related to enzyme activities involved in chlorophyll biosynthesis.

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