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Soybean leaf nitrogen, chlorophyll content, and chlorophyll *a/b* ratio

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Abstract

The objective of this study was to assess genotypic variation in soybean chlorophyll (Chl) content and composition, and to test if these data could be used as a rapid screening method to predict genotypic variation in leaf tissue N content. Chl contents and composition were examined among 833 soybean (*Glycine max* L. Merr.) accessions and related to SPAD meter readings and leaf N content. In the initial year of the study (2002), the relationship between leaf Chl and leaf N contents ($r^2 = 0.043$) was not sufficiently close for Chl to be useful as a predictive tool for leaf N content. Therefore, leaf N content was not determined in 2004 but samples were again collected for determination of Chl content and composition. In 2002, the soybean accessions separated into two distinct groups according to leaf Chl *a/b* ratios, with the majority of a mean ratio of 3.79. However, approximately 7 % (60) of the genotypes could be readily assigned to a group with a mean Chl *a/b* ratio of 2.67. Chl *a/b* analyses in 2004 confirmed the results obtained in 2002 and of 202 genotypes, all but 6 fell into the same group as in 2002.

Additional key words: areal leaf mass; canopy; *Glycine max*; leaf insertion; light-harvesting complex; photosystem 2; SPAD.

Introduction

Synthesis of the photosynthetic apparatus requires large amounts of N, the proportion of leaf N allocated to the chloroplast amounts to approximately 75 % (Hák *et al.* 1993). When grown in high irradiance, leaves generally have a ratio of 1.0 : 1.4 mol N in ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) *vs.* thylakoid N, while under low irradiance more N is partitioned into the thylakoid components (Evans 1989). Significant correlations between photosynthesis and leaf N content have been documented for a large number of species, including soybean (Boote *et al.* 1978, Hesketh *et al.* 1981, Lugg and Sinclair 1981, Boon-Long *et al.* 1983, Buttery and Buzzell 1988, Evans 1989). A positive correlation between leaf N or N fertilization rate and chlorophyll (Chl) content is well documented for a large number of plant species and has been investigated for rapid N status determination using Chl meters in most major crops including corn (*Zea mays* L.), rice (*Oryza sativa* L.), cotton (*Gossypium hirsutum* L.), wheat (*Triticum aestivum* L.) as well as numerous other plant species (Evans 1989, Peng *et al.* 1993, Reeves *et al.* 1993, Bullock and Anderson 1998, Wu *et al.* 1998, Ntamatungiro *et al.* 1999, Nageswara Rao *et al.* 2001, Chang and Robison 2003, Mauromicale *et al.* 2006). Taking Chl meter readings is easy and quick, does not necessitate destructive sampling, and has been employed to predict Chl content in a large number of plant species, including soybean (Yadava 1986, Marquard and Tipton 1987). Thompson *et al.* (1996) observed strong correlations of Chl content with SPAD readings and areal leaf mass (ALM) in soybean, and suggested that a portable SPAD Chl meter may be used to select for genotypes differing in ALM. Facile and rapid assessments of soybean N status by Chl meter would make this a very attractive method to screen large plant populations for genotypic differences in leaf N content as well.

Generally, plants modulate leaf anatomy and physiology to irradiance, developing thicker leaves with a greater mesophyll to surface-area ratio (Boardman 1977, Lichtenthaler *et al.* 1981, Anderson 1986). Common adjustments to high irradiance also include a reduction in the total Chl per unit leaf area and an increased Chl *a/b* ratio (Björkman *et al.* 1972, Boardman 1977, Lichtenthaler *et al.* 1981, Anderson 1986). Due to its predictable response to irradiance, the Chl *a/b* ratio has been proposed as a bioassay to assess the light environment

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Abbreviations: ALM – areal leaf mass; Chl – chlorophyll; LHC2 – light-harvesting complex 2; PS2 – photosystem 2.
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of a plant (Dale and Causton 1992). Other modulations in response to irradiance include effects on chloroplast ultrastructure and Chl associations with photosystems (PS) 1 and 2 (Boardman 1977, Lichtenthaler *et al.* 1981, Leong and Anderson 1984, Anderson 1986, Evans 1989, Green and Durnford 1996). Under crop production conditions, the irradiance of most leaves is influenced by plant density and row distance and modulates various aspects of the photosynthetic apparatus. Thus, genotype specific Chl composition and content characteristics could have implications for crop management.

The main objectives of this project were to assess genotypic variation in soybean Chl composition and content and to test the relationship between these characteristics and leaf tissue N content. To this end, a large number of soybean genotypes were sampled from the USDA-ARS Maturity Group V–VIII National Germplasm Collection at Stoneville, MS in 2002 and 2004.

Materials and methods

Germplasm survey: In 2002, a subset of soybean germplasm from the USDA-ARS National Germplasm Collection was grown on a sandy loam soil at the Delta Research and Extension Center at Stoneville, MS (W90"53, N33"25'). The 1 369 lines were sown on 14 and 15 May 2002 at a rate of 96–120 seed per row in plots 4 rows wide (0.9 m row spacing) and 3.6 m long (no replications). Fertilization, irrigation, herbicide, insecticide, and fungicide treatments were conducted according to standard management practices for the region (Heatherly and Hodges 1998). In 2004, the entire collection of the soybean germplasm maturity group (MG) V–VIII from the USDA-ARS National Germplasm Collection was grown within 0.5 miles of the 2002 field on the same soil type. The genotypes were sown on 5 and 6 May 2004 in the same manner as in 2002 and were managed according to the same production guidelines.

In 2002, leaf samples from 837 lines from MG IV (5), MG V (733), MG VI (70), MG VII (22), and MG VIII (6), and one of unknown maturity group were collected from the center two rows at the R5 (begin seed fill) developmental stage (Fehr *et al.* 1971). From each of the lines, 3 terminal leaflets of mature, fully irradiated leaves in the top quarter of the canopy were randomly selected within a row. The leaves were cut at the base of the petiole, placed in plastic zip-loc bags which were then inserted into manila envelopes, and placed on ice. Leaflets were kept in the dark and transported to the laboratory for processing. All samples were processed within approximately 2 h from collection in the field. The total area of three leaflets was determined using a *Li-COR 3100* leaf area meter (Lincoln, NE, USA). Prior to drying of the leaves at 65 °C, one leaf disk (1 cm^2) was excised from the lower half of each of the three terminal leaflets and immediately placed into opaque vials containing 12 cm^3 of 96 % ethanol for Chl extraction. The disks, suspended in 96 % ethanol, were placed on an orbital shaker (200 rpm) and incubated in the dark at room temperature (~26 °C) for 24 h. Chl *a* and *b* were determined by measuring absorbance at 649 and 665 nm wavelengths on a spectrophotometer (*µQuant*, *Bio-Tek Instruments*, Winooski, VT, USA) and computed according to Wintermans and de Mots (1965). On each leaflet, 3 SPAD meter (*Minolta 502*) readings taken near the spot of leaf disk excision were averaged and recorded. Once the SPAD meter readings were obtained, leaflets were combined, dried to constant mass at 65 °C, and ground to a fine powder using a sample mill (*Cyclotec*, *Foss*, Eden Prairie, MN, USA) with a 1-mm screen. Leaf N was determined using a *LECO FP428* Nitrogen Determinator (*LECO Corporation*, St Joseph, MI, USA) at the Agriculture Diagnostics Laboratory of the University of Arkansas, Fayettville, AR, USA.

Based on the results of the 2002 Chl analyses, a subset of 202 accessions (encompassing all the genotypes that had a low Chl *a/b* ratio and a subset of genotypes with a high Chl *a/b* ratio selected to cover the range in total Chl content that was observed in 2002) were selected for Chl determinations in 2004. Three leaf disks (1 cm^2) of the selected lines were excised in the field from mature, fully irradiated leaves from the top quarter of the canopy and immediately placed into amber vials containing 12 cm^3 of 96 % ethanol. To minimize irradiation, vials were placed immediately into covered boxes and transported to the laboratory within 2 h after collection. Chl extractions and determinations were conducted

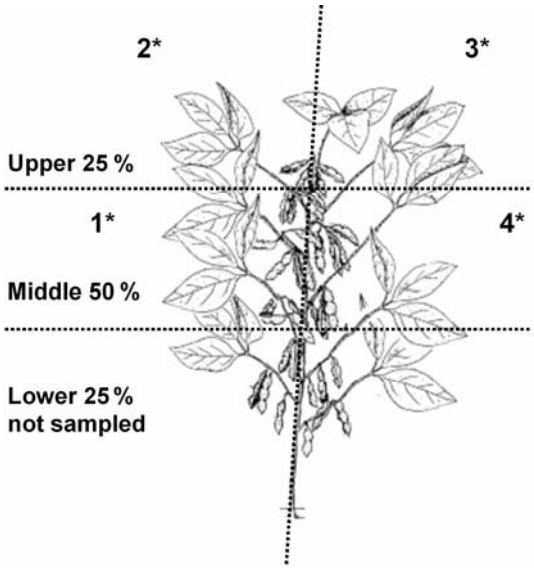

Fig. 1. Illustration of the soybean canopy sections from which leaf disks were collected for the analysis of chlorophyll composition and content. *Quadrants from which leaf samples were obtained.

as described above. On 135 of the selected lines, SPAD meter readings were taken immediately following and next to the site of leaf disk excision.

Additional tests investigated the effects of leaf position on the plant and leaf position in the row on Chl content and composition. To this end, 12 genotypes (6 with high and 6 with low Chl *a/b*) were randomly selected for sample collection. For three of these genotypes, samples were excised from leaflets of the uppermost 10–12 trifoliate leaves from 3 plants. For the other nine genotypes, the leaves to be sampled were selected based on their position on the canopy exterior (Fig. 1). The row spacing was wide enough that complete canopy closure was not obtained by the time of sampling. The canopy was first divided into the north face and the south face (rows were planted east-west). Each face was then divided into a top 25 % and a section immediately below encompassing the middle 50 % of the respective canopy face as illustrated in Fig. 1. Out of each of the four areas, three replications of fully expanded leaves from the canopy surface were selected and disks were excised from each leaflet.

Data analyses were conducted using the *SAS 9.1.2 for Windows* (*SAS Institute*, Cary, NC, USA) employing *PROC REG* for regression analyses and *PROC GLM* to test for differences between low and high Chl *a/b* groups and year effect by low and high Chl *a/b* groups. The *PROC MIXED* procedure was used to analyze effects of leaf position in a plant row or on a shoot with genotype and leaf position as fixed effects. *SAS MAP* files were used to generate latitude and longitude for the soybean lines for which origin data was available.

Results

Three genotypes were selected to test the effect of leaf position on the relationship between SPAD meter reading and Chl extraction. Leaf disks and SPAD meter readings were taken in parallel from a minimum of ten leaves starting at the tip of a shoot and moving basipetally along the same shoot. SPAD meter readings were conducted on the same leaves directly next to, and immediately after, leaf disks had been excised for Chl extractions. Good correlation between extracted Chl and SPAD meter readings was found for the two genotypes (PI603517B and PI603685B) with a large range in leaf Chl content between different aged leaves but a weaker correlation was observed for the genotype (PI424269C) with a narrower leaf Chl range (Fig. 2). The relationships between extracted Chl and SPAD meter readings differed among the three genotypes.

Sampling of leaves based on their position in the canopy, rather than nodal position, would strongly reduce time requirements for sampling. Nine genotypes were selected to investigate the variation in Chl composition and content with respect to the position of a leaf within four defined positions of the canopy exterior. Fig. 3 shows results from eight genotypes across the four leaf positions. Among the nine genotypes examined, three exhibited differences in total Chl and four in Chl *a/b* between any of the four leaf positions. However, importantly, samples collected from leaves at positions 2 and 3 (the upper quarter of the canopy) did not significantly differ in Chl contents and ratios in any of the nine genotypes tested. The total Chl content between the top quarter of the canopy (positions 2 and 3) and the side (positions 1 and 4) of the canopy was significantly different when analyzed across all genotypes. However, analyses by genotype only revealed significant differences for PI458260 [top = 353.3 mg(Chl) m^{-2}, side = 300.4 mg(Chl) m^{-2}] and PI458267 [top = 337.1 mg(Chl) m^{-2}, side = 392.7 mg(Chl) m^{-2}]. While the differences in Chl *a/b* ratio between top (positions 2 and 3) and side (positions 1 and 4) were only significantly different for 2 of the nine genotypes (PI458270: Chl *a/b* top = 2.55, side = 2.83; PI468967: Chl *a/b* top = 3.90, side = 4.08) when analyzed individually for each genotype, the difference was significant when the analysis was conducted combined across all nine genotypes. These results show that there was no need to determine the node position from which to collect samples as long as mature, fully expanded, irradiated leaves were collected from the top quarter of the canopy.

SPAD meter and Chl extraction data from 2002 (n = 833) and 2004 (n = 135) are shown in Fig. 4. While there were highly significant (p<0.0001) positive relationships between Chl extraction and SPAD measurements, neither the results from 2002 nor 2004 supported the use of the SPAD meter to compare soybean genotypes for variation in total Chl content (2002: Chl = –10.04719 + 1.34857 x, r^2 = 0.2451; 2004: Chl = –20.31590 + 1.14455 x, r^2 = 0.3568).

An important objective of this study was to test if ethanol-extracted Chl and/or SPAD meter could be used to compare leaf N content among a large number of soybean genotypes at beginning seed fill. While the correlations with leaf N content were significant for both ethanol-extracted Chl and SPAD meter readings (leaf % N = 4.222 + 0.011 Chl, r^2 = 0.043, n = 828, p<0.0001, Fig. 4; leaf % N = 5.456 – 0.015 SPAD, r^2 = 0.011, n = 828, p=0.0026, data not shown), the R2 values were very low and neither relationship provided the accuracy necessary to substitute N analysis for this purpose. Peng *et al.* (1993) illustrated that in rice, the estimate of leaf N content is improved if the Chl meter readings are adjusted for areal leaf mass (ALM; formerly specific leaf weight, SLW). Similar adjustment to our 2002 SPAD meter readings using the ALM validated their findings for soybean. However, the ALM correction for SPAD readings and total Chl content still did not result in relationships strong enough to be useful in

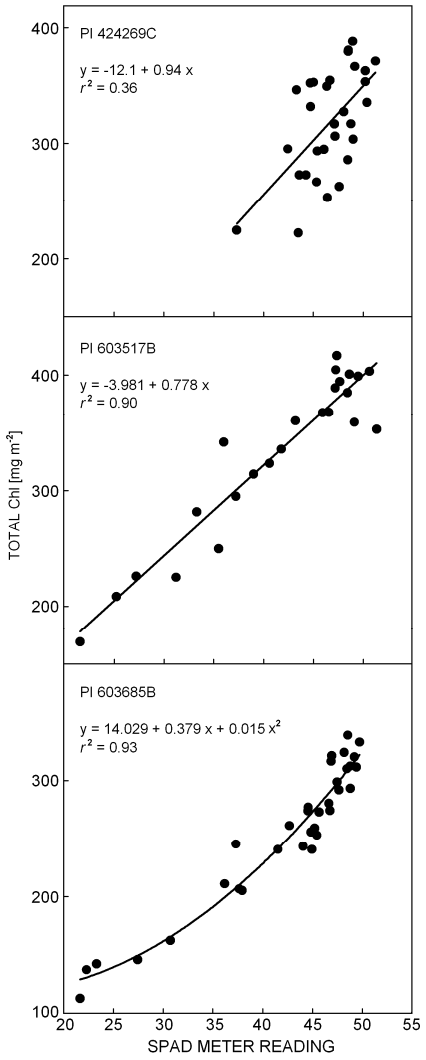

Fig. 2. Total chlorophyll (Chl) contents for leaf-disk samples from three different accessions plotted against corresponding SPAD meter readings. Leaf-disks were collected from unfolded leaves of three plants and up to 12 nodes per plant.

predicting leaf N content to screen a large number of diverse soybean genotypes (leaf % N = 3.337 + 0.194 ALM adjusted SPAD reading, r^2 = 0.248, n = 828, p<0.0001; leaf % N = 3.679 + 0.130 ALM adjusted total Chl, r^2 = 0.295, n = 828, p<0.0001, data not shown).

Thompson *et al.* (1996) reported that SPAD meter readings could be used to distinguish high and low ALM genotypes in experimental lines selected to differ in this trait. Contrary to their findings, regression analyses of the 2002 data indicated that neither SPAD meter readings nor Chl content were good predictors for ALM among 833 lines examined in this study (ALM = 0.949 + 0.117 SPAD reading, r^2 = 0.13, p<0.0001; ALM = 5.813 + 0.009 total Chl, r^2 = 0.006, p=0.02, data not shown).

Chl *a/b* ratio differences in the USDA soybean germplasm collection: Analyses of Chl composition of the 2002 data revealed two distinct groups of soybean germplasm with respect to the Chl *a/b* relationship (Fig. 5). The two groups were clearly distinct with only a very small number of PI lines at the interface between the groups. Genotypes with a Chl *a/b* ratio greater or equal to 3.05 were assigned to the "high" group while those that had a ratio of less than 3.05 were assigned to the "low" group. The average Chl *a/b* ratio of the high group was 3.79 (Stdev = 0.227, Min = 3.10, Max = 5.04) and that of the low group was 2.67 (Stdev = 0.167, Min = 2.14, Max = 3.02) in 2002. The high group encompassed 773 genotypes whereas only 60 fell into the low group. While the difference in the Chl *a* content between the two groups was not significant (2002: low group = 415.4 mg m^{-2}; high group = 406.1 mg m^{-2}), Chl *b* content was significantly greater (p<0.0001) in the low group (155.8 mg m^{-2}) than in the high group (107.8 mg m^{-2}). The greater

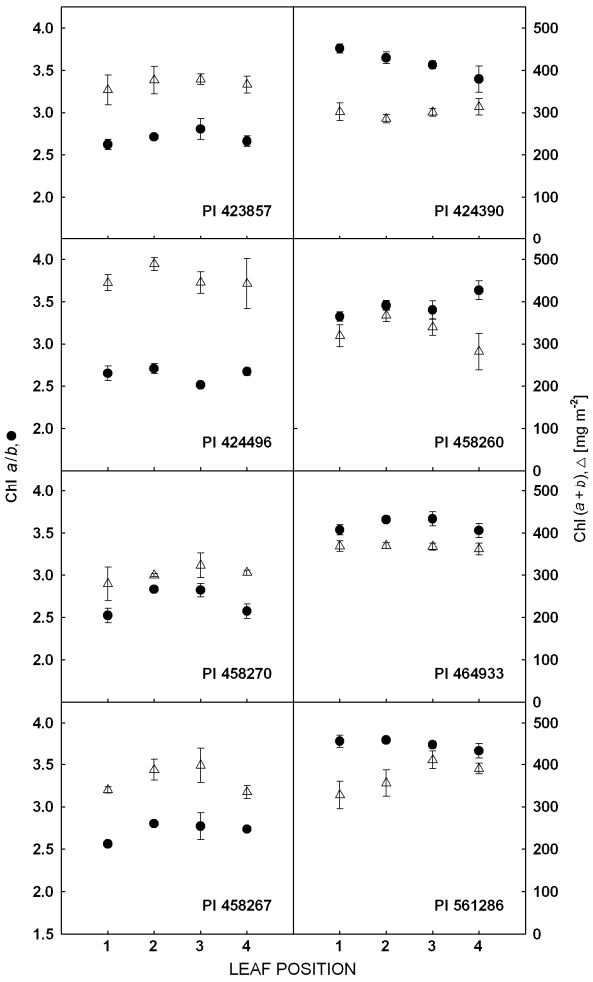

Fig. 3. Chlorophyll (Chl) *a/b* ratio and total Chl content for leaf-disk samples from eight different accessions plotted against the canopy position from which they were collected. Leaf-disks were collected from mature fully irradiated leaves on 1: the north facing central area, 2: the north top, 3: the south top, and 4: the south facing central area of the canopy. *Error bars* indicate SE of n = 3.

Chl *b* content in the low group resulted in significantly greater total Chl content as well (570.5 mg m^{-2} *vs.* 513.4 mg m^{-2}). Unlike total Chl content, the difference in leaflet N content was not significant between the two groups (low group = 4.69 % N; high group = 4.78 % N) in 2002.

The Chl *a/b* results from 2002 were highly repeatable in 2004 (Fig. 5). Out of the 202 genotypes tested in 2004 only six (PI458154, PI594811, PI597465, PI603538C, PI603703A, and PI605827B) did not fall into the same group as in 2002. All 6 of these genotypes were in the low group in 2002 but were in the high group in 2004. Four of these genotypes, (PI458154, PI594811, PI597465, PI603538C) were within 0.13 of the threshold Chl *a/b* ratio of 3.05 in 2002, only slightly missing the arbitrary cutoff. The other two genotypes clearly fell into the low group in 2002 and into the high group in 2004.

The average Chl *a/b* in 2004 was 2.67 for the low group (Stdev = 0.138, Min = 2.31, Max = 3.04) and 3.49 for the high group (Stdev = 0.186, Min = 3.08, Max = 3.90). A significant year by group interaction effect was observed for the Chl *a/b* ratio (p<0.0001) and the Chl *b* content (p<0.0001). For the low group, the Chl *a/b* ratio was not different between 2002 and 2004. However, the

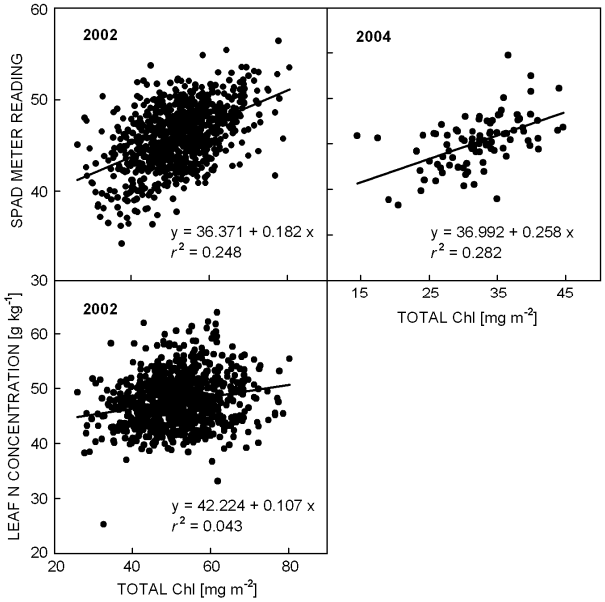

Fig. 4. Scatter plot of SPAD meter readings and leaf N content plotted against total chlorophyll (Chl) content for selected accessions of the USDA National Soybean Germplasm Collection grown in Stoneville, MS in 2002 and 2004.

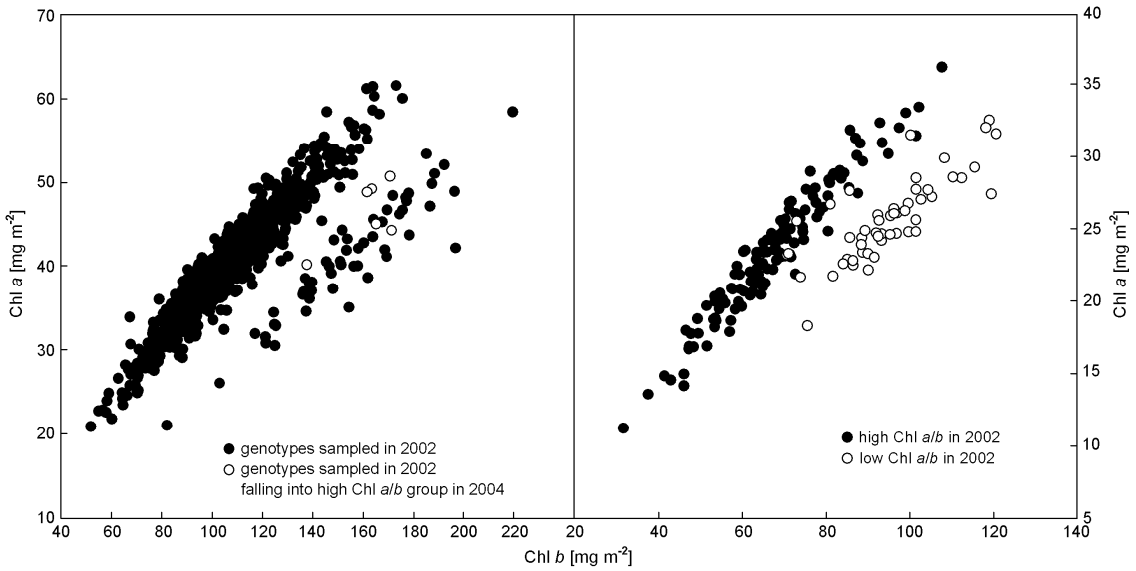

Fig. 5. Scatter plot of chlorophyll (Chl) *a* content plotted against Chl *b* content per unit area for selected accessions of the USDA National Soybean Germplasm Collection grown in Stoneville, MS in 202 and 2004.

Chl *a/b* ratio for the high group was significantly different between the two years (p<0.0001). Unlike 2002, both Chl *a* (p<0.01) and Chl *b* (p<0.0001) contents were greater in the low than in the high group, resulting in greater total Chl content of the low group (352.0 mg m^{-2} *vs*. 305.1 mg m^{-2}).

Of the 837 accessions evaluated in 2002, 326 were from Japan, 160 from China, 153 from North Korea and South Korea, 136 from Vietnam, 43 from the US, and the remaining 19 were either from various other countries or of unknown origin. An analysis of variance was conducted to investigate if there was a relationship between geographical location of origin and Chl *a/b* ratios of the two groups. While ANOVA indicated a marginally significant (p=0.03) difference in the latitude of origin for the two groups, geographical location did not explain the distinct grouping in the Chl *a/b* ratio observed in this study.

Discussion

Within individual genotypes, our analysis of the relationship between SPAD meter readings and Chl content was consistent with previous reports. SPAD meter readings were influenced by leaf developmental stage (Šesták 1985) and genotype. For two out of three genotypes for which SPAD meter readings were determined on leaves selected for a range in total Chl contents, the r^2 values from regression analyses were 0.9 or greater. In part, the weaker relationship in the third genotype may have been due to the narrower Chl content range observed. Unfortunately, neither the relationship of Chl content as determined by ethanol extraction nor the SPAD meter readings appeared to be useful to screen the USDA soybean germplasm collection for leaf N content or ALM. These findings differ from those reported by Thompson *et al.* (1996), and suggest that techniques other than SPAD meter readings should be used to effectively screen large soybean populations for leaf N content or ALM (at least at R5). The data reported here are based on a much larger and more diverse group of soybean germplasm than that examined by Thompson *et al*. (1996), which, when considering the close correlations within individual genotypes, may be the reason why different conclusions were reached. Consequently, when screening large soybean germplasm collections, SPAD meter readings should not be used to predict photosynthesis on the basis of reported positive correlations between ALM and apparent photosynthesis (Buttery *et al.* 1981, 1988, Hesketh *et al.* 1981). Additionally, nutrients other then N also affect Chl contents, further complicating the use of SPAD meter readings to assess N nutrition status. However, as results by others and those presented here for individual genotypes indicate, in studies comprising less diverse germplasm, SPAD meter readings may be useful to rapidly assess leaf N, ALM, and possibly photosynthesis (Buttery *et al.* 1981, 1988, Hesketh *et al.* 1981, Peng *et al.* 1993, Reeves *et al.* 1993, Thompson *et al.* 1996, Wu *et al*. 1998, Ntamatungiro *et al.* 1999).

Collection of mature, fully irradiated leaves from the top quarter of the soybean canopy proved to be a robust, quick, and simple method for sampling of leaf Chl composition and content. This technique allows the efficient screening of large soybean populations at beginning seed fill. While not tested in this study, it is conceivable that it may also be valid at other stages in soybean development or to rapidly examine specific treatment effects on Chl composition and content.

The over 800 soybean genotypes screened in this study separated into two distinct groups based on their Chl *a/b* ratio. While the majority of the genotypes had a Chl *a/b* ratio greater than 3.05, about 7 % of the lines screened exhibited a Chl *a/b* ratio smaller than 3.05 (Fig. 5). The irradiance of plants modulates leaf anatomy and physiology (Boardman 1977, Lichtenthaler *et al.* 1981, Anderson 1986). In high-irradiance environments leaves are often thicker and possess increased mesophyll to surface area. Typically, total Chl content per unit leaf area is lower and the Chl *a/b* ratio is greater in sun compared to shade leaves. The effect of photosynthetic photon flux density on the leaf Chl *a/b* ratio is one of the most characteristic differences between sun and shade leaves (Björkman *et al.* 1972, Boardman 1977, Lichtenthaler *et al.* 1981, Anderson 1986). Due to its predictable response to irradiance, the Chl *a/b* has been proposed as a bioassay to assess the irradiance of a plant (Dale and Causton 1992). In fact, Chl content and Chl *a/b* ratio are sensitive to changes in the irradiance within the mesophyll of individual leaves (Terashima and Inoue 1983, Terashima *et al.* 1986, Cui *et al.* 1991). Chloroplasts in shade-leaves develop a high thylakoid to stroma volume with more and broader grana stacks, and more thylakoids per granum (Boardman 1977, Lichtenthaler *et al.* 1981, Anderson 1986). While the relative proportion of Chl associated with the photosystem (PS) 1 complex and the PS2 core reaction center complex decreases with a reduction in the Chl *a/b* ratio, the relative proportion of Chl associated with the light-harvesting Chl *a/b* protein complex increases (Leong and Anderson 1984). The light-harvesting complex 2 (LHC2) has a lower Chl *a/b* ratio than other Chl-binding proteins associated with PS2 because LHC2 contains the majority of Chl *b* (Evans 1989, Green and Durnford 1996). Thus, the Chl *a/b* ratio can serve as an indicator of the protein makeup within a chloroplast as well as the ultrastructure of a chloroplast. In fact, Terashima and Inoue (1983), Terashima *et al.* (1986), and Kitajima and Hogan (2003) used the Chl *a/b* ratio as an indicator of N partitioning within a leaf based on the positive relationship of Chl *a/b* with the ratio of PS2 cores to light-harvesting Chl-protein complex. In brief, these well documented effects of differential irradiances may indicate that the 60 lines with lower Chl *a/b* possibly originated or evolved in environments with reduced irradiance. If the Chl *a/b* characteristics of the two groups are also associated with some of the other irradiance linked chloroplast attributes discussed above, is unclear at this point. Unfortunately, we could not test for the light environment from which these genotypes originated. An examination of genotype and geographic location of origin did not result in significant findings.

That a distinct subgroup of soybean genotypes exists based on differences in Chl *a/b* ratios has not been previously reported. Further study of the contrasting groups is needed to better understand the causes and implications of these findings. As discussed above, typical results from studies into the effects of irradiance on plants indicate that there may be differences in thylakoid associated protein composition and in the ultrastructure of chloroplasts between the two groups. The Chl *a/b* ratio of a soybean genotype may have management implications under production conditions. Questions as to how

genotypes from the two groups differ in adjustment to changing irradiance have not been addressed to date, but could potentially impact management decisions such as sowing density and row spacing.

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