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Photosynthesis, Light Use Efficiency, and Yield of Reduced-Chlorophyll Soybean Mutants in Field Conditions

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Reducing chlorophyll (chl) content may improve the conversion efficiency of absorbed photosynthetically active radiation into biomass and therefore yield in dense monoculture crops by improving light penetration and distribution within the canopy. The effects of reduced chl on leaf and canopy photosynthesis and photosynthetic efficiency were studied in two reportedly robust reduced-chl soybean mutants, Y11y11 and y9y9, in comparison to the wild-type (WT) "Clark" cultivar. Both mutants were characterized during the 2012 growing season whereas only the Y11y11 mutant was characterized during the 2013 growing season. Chl deficiency led to greater rates of leaf-level photosynthesis per absorbed photon early in the growing season when mutant chl content was ~35% of the WT, but there was no effect on photosynthesis later in the season when mutant leaf chl approached 50% of the WT. Transient benefits of reduced chl at the leaf level did not translate to improvements in canopy-level processes. Reduced pigmentation in these mutants was linked to lower water use efficiency, which may have dampened any photosynthetic benefits of reduced chl, especially since both growing seasons experienced significant drought conditions. These results, while not confirming our hypothesis or an earlier published study in which the Y11y11 mutant significantly outyielded the WT, do demonstrate that soybean significantly overinvests in chl. Despite a >50% chl reduction, there was little negative impact on biomass accumulation or yield, and the small negative effects present were likely due to pleiotropic effects of the mutation. This outcome points to an opportunity to reinvest nitrogen and energy resources that would otherwise be used in pigment-proteins into increasing biochemical photosynthetic capacity, thereby improving canopy photosynthesis and biomass production.

Keywords: photosynthesis, solar energy conversion efficiency, chlorophyll, canopy light distribution, soybean

INTRODUCTION

Increasing the yield potential (Y_p) of important agronomic crops is imperative for meeting predicted future production needs. Y_p is the maximum possible regional yield for a given crop in the absence of biotic and abiotic stresses (Evans and Fischer, 1999), but as radiation, water, nutrients, etc., become limiting or there is pest/pathogen pressure, realized yields decrease,

resulting in a yield gap (Lobell et al., 2009). Y_p for a given crop during a growing season is the product of several components: the incident solar radiation across the growing season (S_t), the proportion of S_t that is photosynthetically active radiation (PAR; estimated as 0.487), the radiation interception efficiency (ε_i), the conversion efficiency of intercepted radiation into biomass (ε_c), and the partition efficiency of biomass into harvestable product (ε_p; modified from Monteith, 1977). S_t and the proportion that is PAR vary but are largely predetermined by growing season length and location, although there can be substantial interannual variability in S_t at a given location (Monteith, 1965, 1972). Of the three efficiencies, plant breeders have already pushed ε_i and ε_p near their theoretical upper limits in highly productive crops (Evans, 1993; Hay, 1995; Sinclair, 1998). Breeding in soybean (*Glycine max* Merr.) has achieved ε_i season averages of approximately 0.60–0.75 (Koester et al., 2014) with peak midseason ε_i of >0.90 in modern cultivars (Dermody et al., 2008; Koester et al., 2014). ε_p has reached values of 0.60 for soybean (Dermody et al., 2008; Koester et al., 2014), which is at or near the estimated theoretical maximum of ~0.55–0.67 for major food crops (Austin et al., 1980; Bugbee and Monje, 1992; Khush, 1995; Smil, 1999; Hay and Porter, 2006; Prasad et al., 2006). These advancements in ε_i and ε_p leave only modest potential for further improvement of Y_p (Zhu et al., 2010). However, ε_c operates substantially below the theoretical maxima for C_3 (0.094) and C_4 (0.123; Zhu et al., 2010) crop canopies and therefore limits yield potential (Zhu et al., 2008, 2010), especially in soybean where the maximum realized ε_c (0.028) is estimated at less than a third of the C_3 theoretical potential (Slattery and Ort, 2015).

Reducing leaf chlorophyll (chl) content has been proposed as a strategy to improve ε_c in crop canopies. At low light levels, leaf photosynthesis (A_{leaf}) in crops such as soybean responds linearly to light intensity, but at approximately 25% of full sunlight, or 500 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), the linear relationship between absorbed quanta and A_{leaf} begins to plateau (Long et al., 2006). Light in excess of photosynthetic capacity is then wasted through heat dissipation, or photoprotection, which reduces light use efficiency (Niyogi, 1999; Ort, 2001). At normal chl levels, individual soybean leaves absorb approximately 85–90% of incident PPFD, which results in the uppermost ~25% of the canopy absorbing ~75% of incoming light (Campbell and Norman, 1998), much of which is wasted due to light saturation of photosynthesis in these leaves. Meanwhile, at full sunlight, leaves below the uppermost 25% receive half or less of the light needed to saturate A_{leaf} and are therefore light limited (Long et al., 2006). By distributing light more proportionately throughout leaf layers (Zhu et al., 2010; Ort et al., 2011, 2015), absorbed PPFD could be used more efficiently by mitigating both light oversaturation at the top of canopy and light limitation within the canopy. It is likely that some crops overinvest in chl content to the detriment of light distribution in the canopy similar to the manner in which soybean overinvest in leaf area (Srinivasan et al., 2016). Therefore, decreasing leaf absorbance (leaf$_{abs}$) through reduced chl content seems a potential strategy to achieve deeper light penetration into a crop canopy; thus, sun leaves would absorb

only enough photons at mid-day to sustain maximum A_{leaf} while allowing more light to reach the lower canopy and stimulate A_{leaf} in shade leaves, thereby potentially improving canopy photosynthesis (A_{can}), ε_c, and Y_p. Experimental evidence supporting the principle of this notion has been found in similar or greater rates of A_{leaf} in various crops with substantial reductions in chl compared to their dark-green counterparts (Highkin et al., 1969; Benedict et al., 1972; Edwards et al., 1993; Habash et al., 1994; Li et al., 2013; Kirst et al., 2017). In soybean, greater rates of A_{leaf} on an absorbed photon basis were evident in light-green soybean leaves, and the increase in A_{leaf} correlated with a more even light distribution among chloroplasts within leaves (Slattery et al., 2016). An analogous alteration of light distribution could therefore occur among leaves within a canopy. In addition, dense mass cultures of truncated light antennae (*tla*) green algae mutants demonstrated increased light penetration and improved solar energy conversion efficiency (Melis, 1999; Polle et al., 2002; Mitra and Melis, 2008), which ultimately led to increased hydrogen production (Kosourov et al., 2011).

Decreasing chl content could also have other benefits at the canopy level. Reducing light absorption and thereby increasing albedo at the top of the canopy could decrease leaf temperature (T_{leaf}) in the upper canopy, similar to the manner in which paraheliotropism reduces T_{leaf} in other species (Gamon and Pearcy, 1989). During times of above optimal temperatures, this should increase A_{leaf} by mitigating negative heat stress effects (Ainsworth and Ort, 2010) and in turn also improve water use efficiency (WUE). Cooler soybean canopies lower vapor pressure deficit, resulting in higher WUE (Baldocchi et al., 1985), which was reported for alfalfa with reduced chl content compared to the full green control (Estill et al., 1991). Greater light availability with depth in the canopy could also increase WUE by facilitating greater A_{leaf} in deeper layers where humidity is higher and therefore vapor pressure deficit is lower (Drewry et al., 2014; Ort and Long, 2014). If monoculture crops are overinvesting in chl biosynthesis, reallocation of nitrogen from an excess of pigment-protein complexes to other nitrogen-limited photosynthesis-related molecules might also be a benefit of chl reduction. In a modeling study, reallocating nitrogen resources among Calvin cycle enzymes predicted increased potential A_{leaf} without any additional nitrogen (Zhu et al., 2007). If nitrogen that would otherwise be used in pigment and pigment-proteins were reinvested in increased photosynthetic capacity, a similar increased nitrogen use efficiency would be expected.

Soybean is the world's third most economically important commodity crop (FAO, 2012). At agricultural planting densities, soybean develops a dense canopy with a leaf area index (LAI) often greater than six. This creates a situation in which the majority of leaves are experiencing light levels below the light compensation point during most daylight hours and makes it an ideal candidate crop for testing the effects of reduced chl content on ε_c. A large number of chl-deficient mutants have been identified in soybean, and two chl-deficient soybean mutants with robust canopy growth, *Y11y11* and *y9y9*, were previously reported to have greater A_{can} compared to

the nearly isogenic "Clark" wild-type (WT) throughout the growing season (Pettigrew et al., 1989). These mutants display a disproportionately large truncation in the antennae associated with photosystem II (PSII) compared to photosystem I (PSI; Ghirardi and Melis, 1988). This leads to higher chl a/b and PSII/PSI ratios, the latter of which serves to balance light absorption between the two photosystems (Eskins et al., 1983; Ghirardi and Melis, 1988). However, comprehensive studies of reduced chl effects on soybean at both the leaf and canopy scale have not yet been conducted in the same experiment. Therefore, the same light-green soybean mutants and the WT control were grown in the field during the 2012 growing season. The following year only the $Y11y11$ mutant was grown with the WT so that a row spacing treatment could be added. These field experiments were used to characterize the light-green mutants and to investigate the impact that reducing chl content has on leaf and canopy photosynthesis, photosynthetic efficiency, and yield.

MATERIALS AND METHODS

Site Description

Field experiments were conducted at the SoyFACE global change research facility at the University of Illinois at Urbana-Champaign (40°02′N, 88°14′W, 228 m above sea level) during the 2012 and 2013 growing seasons. The soil at this site is a deep and fertile Flanagan (fine, montmorillonitic, mesic aquic Argiudoll) with some low-lying blocks of Drummer [typic Haplaquoll; Rogers et al., 2004]. The site maintained a yearly maize-soybean rotation, and no nitrogen fertilizer was added prior to soybean planting in accordance with standard regional practices.

The experimental design consisted of a randomized complete block design with three replicates. WT soybean cultivar "Clark" and two nearly isogenic chl-deficient mutants, $Y11y11$ and $y9y9$ (Eskins et al., 1981), were grown in 2012. Only WT and $Y11y11$ were grown in 2013 in order to accommodate a row spacing treatment. Plots in 2012 consisted of 16–2.74 m rows running north-south with a row spacing of 0.38 m. Planting density was 30 plants m^{-2}. In 2013, a row spacing treatment was introduced by replicating the same design but adding a narrower row spacing treatment (0.19 m between rows) while maintaining a plant density of 30 plants m^{-2} (Supporting Information Figure S1). Since the $Y11y11$ genotype segregates (1 dark green: 2 light green: 1 yellow plant), it was planted at a higher density to account for the removal of $Y11Y11$ dark green and the seedling lethal $y11y11$ yellow plants before determining the final plant density. Planting in 2012 occurred on 16 May [day of year (DOY) 137], and harvest occurred on 17 October (DOY 291). In 2013, seeds were sown in the 0.38 m row spacing treatment on 7 June (DOY 158) with a cone planter. The narrow row spacing treatment was planted 1 day later on 8 June (DOY 159) with a push-planter. All plants were harvested on 11 October (DOY 284). Daily meteorological data spanning the growing season (planting to harvest) were obtained from the Illinois Climate Network monitoring station ~1.5 km from the field site (**Table 1** and **Figure 1**; Angel, 2009).

Gas Exchange Measurements

Diurnal Leaf Gas Exchange

All leaf gas exchange measurements were conducted with open path gas exchange systems equipped with leaf chamber fluorometers (LI-6400, LI-COR, Lincoln, NE, USA). Diurnal gas exchange measurements (5–6 time points at 2 h intervals throughout the day) were conducted six times during the 2012 growing season and five times during 2013 on sun leaves. A diurnal was conducted on shade leaves after canopy closure in 2012 as well. Sun leaves were designated as the youngest, fully expanded leaves exposed to full sunlight throughout the day. Shade leaves were designated as 3–4 nodes below the sun leaf on the same plant. Measurement chamber conditions were set to ambient PPFD and 60–70% relative humidity. For shade leaves, PPFD was measured within the canopy using a 1 m long quantum sensor (LI-190, LI-COR, Lincoln, NE, USA) before each set of measurements. The sensor was inserted into the canopy at the height of the shade leaves and at multiple locations within each block. The average light level across all blocks within each genotype was then used for that time point. Block temperature of the gas exchange system was set to ambient air temperature, and reference CO$_2$ concentration ([CO$_2$]) was set to 400 ppm. The daily integral of A_{leaf} (A') was determined as in Rogers et al. (2004) for each replicate, after which statistical analyses were conducted (see below). Daily means of A_{leaf}, g_s, intrinsic water use efficiency (iWUE; calculated as A_{leaf}/g_s for each point measurement before statistically analyzing as described below), and T_{leaf} (measured by the LI-6400 LI-COR, Lincoln, NE, USA) were also determined for sun leaves.
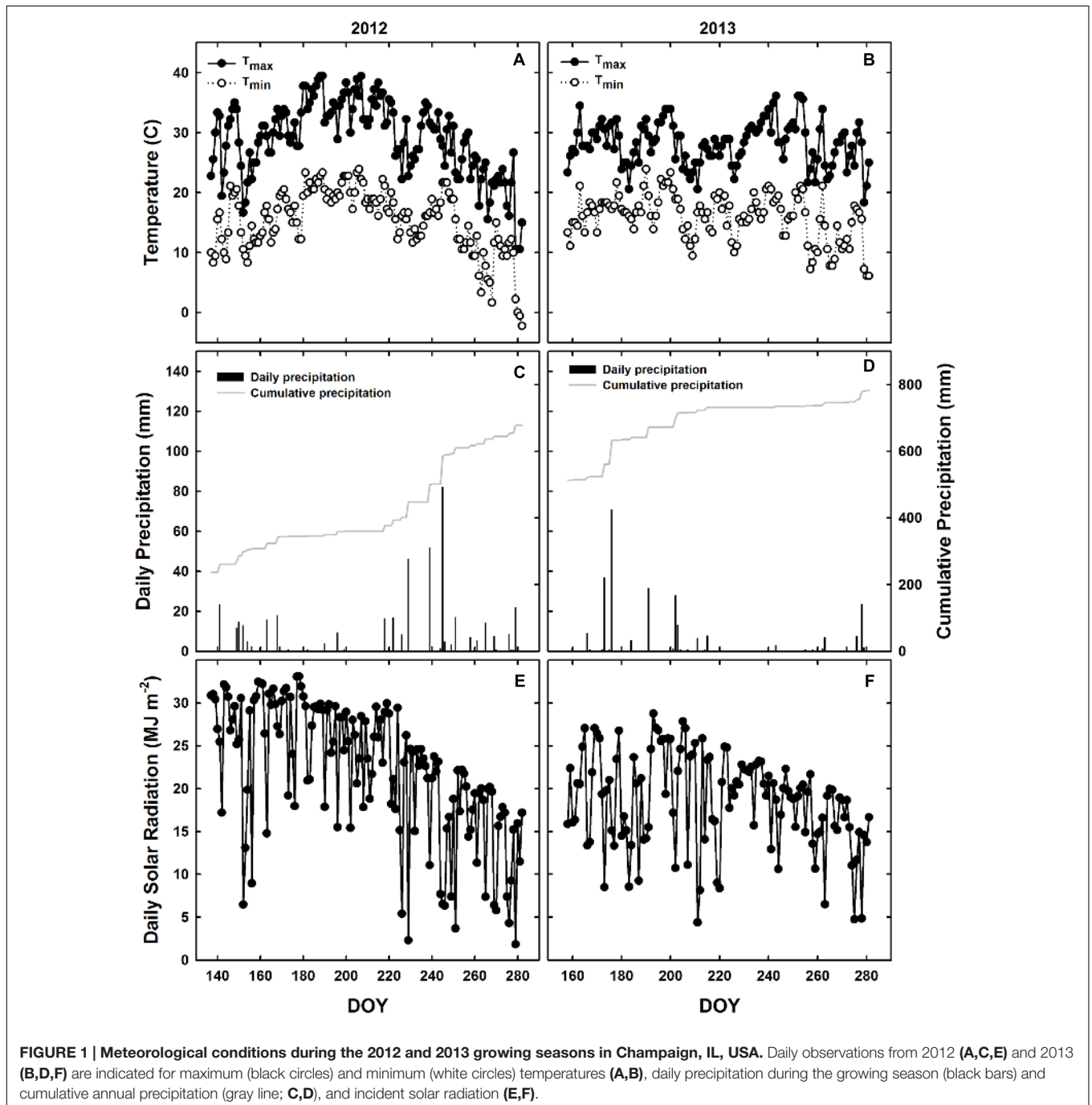
Light Response of Leaf Photosynthesis

Photosynthetic light response (A/Q) curves were conducted on sun leaves in the field at midday during the V5 (five true leaves) developmental stage in 2012 and during the V5, R1/R2 (flowering), and R5 (pod filling) developmental stages (Fehr et al., 1971) in 2013. Shade leaf A/Q measurements were only conducted in 2013 after canopy closure (R1/R2 and R5). Sun and shade leaves were designated as described above. Curves consisted of 12 points spanning from 0 to 2000 µmol m^{-2} s^{-1} PPFD. Dark-adapted minimal fluorescence (F_o) and maximal fluorescence (F_m) were measured pre-dawn the same day as A/Q measurements were conducted. Light-adapted steady state fluorescence (F_s'), minimal fluorescence (F_o'), and maximal fluorescence (F_m') were measured on each leaf during A/Q measurements according to Baker (2008). The operating efficiency of photosystem II ($\phi PSII$) was calculated as ($F_m'-F_s$)/F_m' and non-photochemical quenching (NPQ) was calculated as (F_m-F_m')/F_m'. The electron transport rate through PSII (ETR) was calculated as PPFD*Leaf$_{abs}$*f_{PSII}*$\phi PSII$ where f_{PSII} is the fraction of absorbed PPFD that goes to PSII (Baker, 2008). Maximum rate of photosynthesis (A_{sat}) was determined by fitting a non-rectangular curve to the data (SigmaPlot, Systat Software Inc., San Jose, CA, USA). Maximum quantum efficiency (ϕCO_2) was determined as the slope of the linear fit of A_{leaf} versus absorbed PPFD (Proc Reg; SAS 9.4, SAS Institute, Cary, NC, USA) at low light using data from light levels at or above the light compensation point to avoid any potential effects on the

TABLE 1 | Meteorological conditions during the 2012 and 2013 soybean growing seasons (planting to harvest) in Champaign, IL, USA.

Year	Row space (m)	Planting date	Emergence date	Harvest date	Precipitation[a] (mm)	T_{max}[a] (°C)	T_{min}[a] (°C)	T_{mean}[a] (°C)	Solar radiation[a] (MJ m^{-2})
2012	0.38	16 May	25 May	17 Oct	517[†]	28.4	14.9	21.4	3,337
2013	0.38	7 Jun	17 Jun	16 Oct	271	28.2	15.7	21.6	2,396
2013	0.19	8 Jun	21 Jun	16 Oct	271	28.3	15.7	21.6	2,380

Total precipitation, average maximum (T_{max}), minimum (T_{min}), and mean temperature (T_{mean}), and total available solar radiation over each growing season (planting to harvest) are indicated along with planting, emergence, and harvest dates for each experiment. [a]Data from the Illinois Climate Network (http://www.isws.illinois.edu/warm/datatype.asp) were used to find mean temperatures and sums of precipitation and solar radiation from each growing season. [†]Precipitation (481 mm) + two irrigations of 18 mm each during mid-July.

FIGURE 1 | Meteorological conditions during the 2012 and 2013 growing seasons in Champaign, IL, USA. Daily observations from 2012 **(A,C,E)** and 2013 **(B,D,F)** are indicated for maximum (black circles) and minimum (white circles) temperatures **(A,B)**, daily precipitation during the growing season (black bars) and cumulative annual precipitation (gray line; **C,D**), and incident solar radiation **(E,F)**.

slope due to the Kok effect (Kok, 1948). Proc Loess (SAS 9.4; SAS Institute, Cary, NC, USA) was used to determine 90% confidence intervals for all A/Q data where non-overlapping intervals indicated significant differences. The relationship between A_{sat} and chl content across all genotypes and growing seasons was also plotted with a second order logarithmic function (SigmaPlot, Systat Software Inc., San Jose, CA, USA).

CO$_2$ Response of Leaf Photosynthesis

Photosynthetic [CO$_2$] response (A/C_i) curves were conducted every 2 weeks throughout the 2012 growing season and during the V5, R1/R2, and R5 developmental stages in 2013. Measurements were conducted on sun leaves, and shade leaves were also measured in 2013 after canopy closure (R1/R2 and R5 stages). Maximum carboxylation rates of Rubisco ($V_{c,max}$), maximum electron transport rates (J_{max}), and the intercellular [CO$_2$] at the inflection point between Rubisco and RuBP limited A_{leaf} ($C_{i,inflection}$) were determined according to Long and Bernacchi (2003). $V_{c,max}$ versus chl content and J_{max} versus chl content were also plotted in the same manner as the A_{sat} versus chl content relationship described above.

Leaf Dark Respiration

Dark respiration (R_d) was measured 1–3 h after dusk using a LI-6400 equipped with a specially designed leaf chamber able to enclose an entire trifoliate leaf (Gillespie et al., 2012). Measurements were conducted at the three developmental stages in 2013 on sun and shade leaves as described above. After measurements, leaves were detached and leaf area was measured using a leaf area meter (LI-3100, LI-COR, Lincoln, NE, USA) in order to calculate R_d on a leaf area basis.

Midday Canopy Photosynthesis

A_{can} was measured at midday using a portable chamber on DOY 197 (V5), 213 (R1/R2), 221 [R3/R4 (pod initiation)], and 242 (R5) during 2013 on wide row widths planting in a manner similar to Prater et al. (2006). A chamber (0.914 m × 1.02 m base × 1.37 m height) with clear plastic siding was equipped with mixing fans and a rubber gasket on the bottom edge. An open path infrared gas analyzer (LI-7500, LI-COR, Lincoln, NE, USA) was mounted just above the canopy height within the chamber and was connected to a data logger (LI-7550, LI-COR, Lincoln, NE, USA) outside of the chamber. To reduce soil disturbance and prevent leakage during measurements, aluminum frames with vertical sides and a flat surface on top matching the dimensions of the chamber base (0.914 m × 1.02 m) were inserted into the soil in the area of measurement at least 1 day before measurements. The bottom surface of the chamber was lowered onto the flat top surface of the frame, rather than the uneven soil surface, with a seal created by the rubber gasket. CO$_2$ drawdown was measured on the two rows of plants encompassed within the chamber within 1 min of lowering the chamber over the canopy and onto the frames to minimize any microclimate effects. Soil respiration was measured using an infrared gas analyzer equipped with a soil CO$_2$ flux chamber (LI-6400-09, LI-COR, Lincoln, NE, USA). Soil respiration measurements were conducted in two locations within the measured A_{can} area within 1 h prior to or immediately

following the chamber measurements to account for any changes in [CO$_2$] within the chamber due to soil CO$_2$ flux. One row of plants from within the chamber area was used for biomass harvests (see below), and total leaf area within the chamber was estimated from those measurements. Canopy CO$_2$ assimilation rates were calculated after accounting for soil respiration rates and adjusted to a leaf area basis.

Leaf Tissue Sampling and Biomass Harvests

Leaf Tissue Sampling

Leaf disks 2 cm in diameter were collected at midday during each diurnal and dried to determine specific leaf weight (SLW; g m^{-2}). Leaf disks 1 cm in diameter were collected at midday during each diurnal to determine chl content, chl a/b ratios, and total carotenoid content using the methods of Lichtenthaler (1987) and Porra et al. (1989). Near the end of the 2013 season during developmental stage R5, 2 cm leaf disks were taken from leaves at the top (uppermost 0.25m), middle (0.25–0.50 m from the top of the canopy), and bottom (0.50–0.75 m from the top of the canopy) of the canopy to determine integrated WUE using isotope analyses (Farquhar and Richards, 1984). The samples were dried and ground to a powder, after which an elemental analyzer (Elemental Combustion System 4010, Costech Analytical Technologies, Inc., Valencia, CA, USA) in parallel with an isotope ratio mass spectrometry system (Finnigan Delta V Advantage Mass Spectrometer, Thermo Fisher Scientific, Waltham, MA, USA) were used to determine δ^{13}C on a per mass basis.

Leaf Absorbance Measurements

Leaf$_{abs}$ was measured during the three developmental stages of 2013 at various heights within the canopy using an integrating sphere (Spectroclip-JAZ-TR, Ocean Optics, Duiven, The Netherlands). Leaf$_{abs}$, or the fraction of light absorbed, was calculated as

$$\text{Leaf}_{abs} = I_o - I_t - I_r$$

where I_o is incident radiation, I_t is transmitted radiation, and I_r is reflected radiation. Absorbance of the blue (460 nm) and red (635 nm) wavelengths emitted from LEDs within the open gas exchange chambers (LI-6400, LI-COR, Lincoln, NE, USA) was used to calculate total absorbed PPFD during A/Q measurements. Since leaf$_{abs}$ was not measured in 2012, the relationships between chl content and blue and red light absorbance from 2013 (Supporting Information Figure S2) were used to estimate absorbed PPFD during 2012 A/Q measurements.

Biomass Determination

Aboveground biomass harvests were conducted every 10–14 days each season by harvesting 1 m of a row in each plot at soil height while avoiding plot borders or previous harvest locations. Plant height was measured on three of the plants, and the number of plants per meter of row was recorded. Leaf area per plant was determined for five plants in each plot using a leaf area meter (LI-3100, LI-COR, Lincoln, NE, USA) and adjusted for total plant
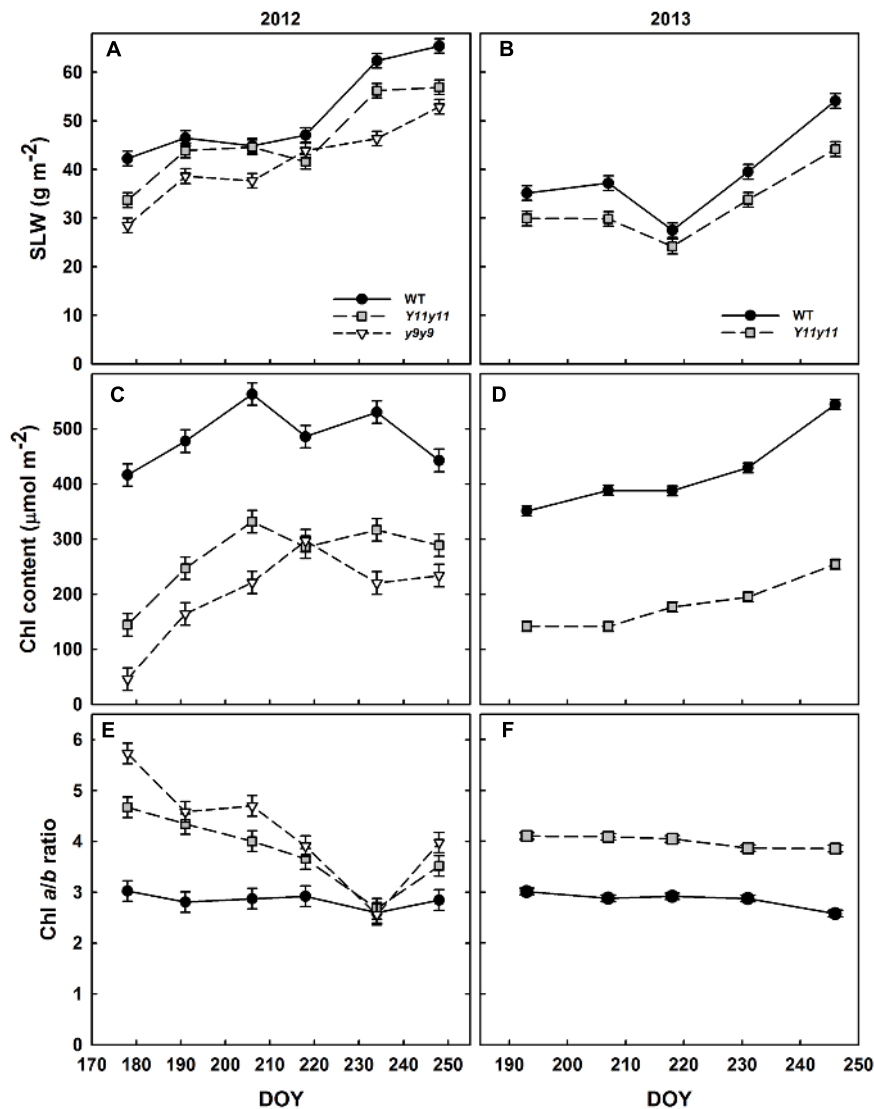
FIGURE 2 | Soybean specific leaf weight (SLW) and chlorophyll (chl) from two growing seasons. Mean SLW **(A,B)**, total leaf chl **(C,D)**, and chl *a/b* ratios **(E,F)** are indicated across the 2012 **(A,C,E)** and 2013 **(B,D,F)** growing seasons for WT dark-green soybean (black circles) and two chl-deficient soybean mutants (*Y11y11* = gray squares; *y9y9* = white triangles). Error bars represent standard errors ($n = 3$).

number to determine total leaf area within the canopy chamber (see above). Stems, leaves, and pods were then separated and dried at 65–70°C for 3 days to determine dry weights. Stem and leaf dry weights were converted to MJ of energy per land area using the tissue-specific energy contents from Amthor et al. (1994). Pod energy at various reproductive stages was determined in 2013 using a bomb calorimeter with a benzoic acid standard (Model 1261, Parr Instrument, Moline, IL, USA). This was used to convert pod mass to pod energy on a land area basis for each reproductive stage after pod initiation in both 2012 and 2013.

ε_i, ε_c, ε_p, and Yield

Daily canopy light interception fraction and season-long interception efficiency, ε_i, were determined as the fraction of

available PAR that was absorbed (APAR) by the canopy. APAR was calculated as

$$APAR = I_o - (I_t + I_r)$$

where I_o was incident PAR measured above the canopy with an upright quantum sensor, I_t was transmitted PAR measured at soil level using a line sensor, and I_r was reflected PAR measured with an inverted quantum sensor above the canopy. All data were collected using line (model SQ-311) and quantum (model SQ-110) sensors (Apogee Instruments, Logan, UT, USA) that had been calibrated with a high precision quantum sensor (LI-190, LI-COR, Lincoln, NE, USA) at the beginning of the season. All data were logged every 10 s using a datalogger (model CR3000 in 2012 and model CR10X in 2013, Campbell Scientific,

TABLE 2 | Light- and dark-green soybean leaf pigment and physiological parameters from two growing seasons and two leaf levels.

Leaf	Parameter		2012				2013		
			WT	$Y11y11$	$y9y9$	MSE	WT	$Y11y11$	MSE
Sun	SLW	(g m^{-2})	51.4	46.1*	41.3*	0.78	38.7	32.4*	0.61
	Chl content	(μmol m^{-2})	486	269*	197*	13	420	182*	4.9
	Chl a/b	–	2.84	3.81*	4.24*	0.065	2.85	3.99*	0.036
	Carotenoids	(g m^{-2})	59.3	40.1*	35.3*	2.5	55.3	35.6*	1.0
	Leaf$_{abs}$	–	–	–	–	–	0.86	0.72*	0.58
	A'	(mol m^{-2} d^{-1})	0.787	0.854*	0.758*	0.011	0.834	0.832	0.006
	A_{leaf}	(μmol m^{-2} s^{-1})	18.1	19.6*	17.5	0.26	20.3	20.3	0.15
	g_s	(mol H$_2$O m^{-2} s^{-1})	0.225	0.268*	0.300*	0.014	0.641	0.796*	0.011
	iWUE	(μmol mol^{-1})	95.9	87.5	73.0*	3.5	35.0	27.5*	0.34
	T_{leaf}	(°C)	29.8	29.4	29.0	0.081	23.9	23.4	0.057
	$V_{c,max}$	(μmol m^{-2} s^{-1})	121	127	116	3.3	107	106	3.5
	J_{max}	(μmol m^{-2} s^{-1})	162	167	158	2.8	166	178*	3.3
	$C_{i,inflection}$	(μmol mol^{-1})	187	176	182	6.5	182	233*	15
	A_{sat}	(μmol m^{-2} s^{-1})	33.2	47.4*	28.6	4.3	34.5	32.6	0.52
	ϕCO_2	–	0.068	0.061	0.036*	0.00004	0.054	0.061	0.005
	R_d	(μmol m^{-2} s^{-1})	–	–	–	–	−1.26	−1.10*	0.027
Shade	Chl content	(μmol m^{-2})	558	242*	105*	20	395	168*	21
	Chl a/b	–	2.27	3.26*	4.46*	0.15	2.67	3.37*	0.16
	A'	(mol m^{-2} d^{-1})	0.174	0.194	0.185	0.016	–	–	–
	$V_{c,max}$	(μmol m^{-2} s^{-1})	–	–	–	–	122	97.2	5.7
	J_{max}	(μmol m^{-2} s^{-1})	–	–	–	–	188	164	15
	$C_{i,inflection}$	(μmol mol^{-1})	–	–	–	–	206	247	27
	A_{sat}	(μmol m^{-2} s^{-1})	–	–	–	–	30.9	27.2	3.5
	ϕCO_2	–	–	–	–	–	0.060	0.060	0.004
	R_d	(μmol m^{-2} s^{-1})	–	–	–	–	−0.655	−0.542	0.060

Growing season means are reported for variables related to physical leaf properties, diurnal measurements, photosynthetic response curves, and dark respiration rates. Significant differences in relation to the WT are indicated with an asterisk at $p < 0.1$. Mean square error (MSE) is reported from each ANOVA. Dashes indicate effects not measured.

Logan, UT, USA). Measurements began on DOY 180 in 2012 and DOY 189 in 2013 and corresponded to the V5 developmental stage. The energy conversion efficiency (ε_c) was determined as the slope of accumulated aboveground biomass energy regressed on accumulated APAR from early vegetative stages to peak biomass energy. ε_p was determined as the ratio of seed energy: total aboveground plant energy at harvest maturity. Yield and seed mass were determined after harvesting and threshing seeds from pods of four complete rows per plot in each experiment.
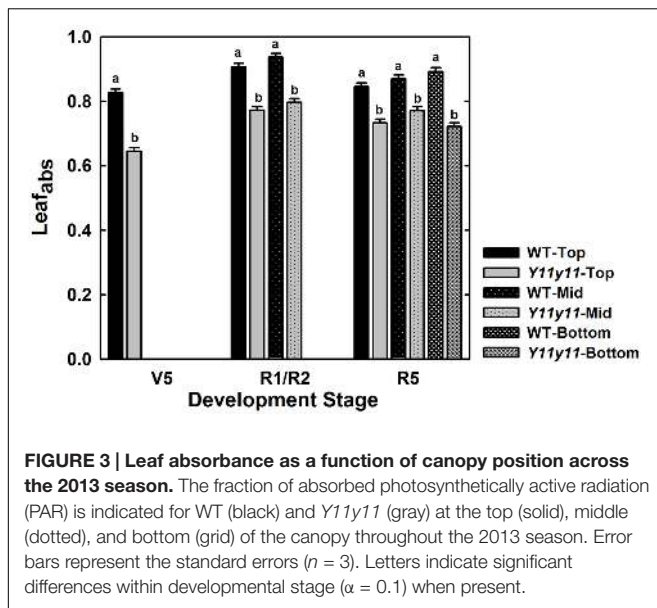
Statistical Analyses

Statistical analyses were conducted on the plot means using a mixed model ANOVA (Proc Mixed, SAS 9.4; SAS Institute, Cary, NC, USA) with genotype, time of day, and DOY considered fixed effects and block and block by genotype effects considered random. SLW, chl content, chl a/b, A', mean daily A_{leaf}, mean daily g_s, mean daily iWUE, mean daily T_{leaf}, leaf$_{abs}$, $V_{c,max}$, J_{max}, $C_{i,inflection}$, and LAI were analyzed as repeated measures with DOY as the repeated factor. Least squared means are reported and shown in figures with the associated standard errors. ε_c regressions and comparisons were performed on pooled plot data points (Proc Reg, SAS 9.4; SAS Institute, Cary, NC, USA). To reduce the probability

of type II errors, an α of 0.1 was used to determine significance.

RESULTS

Weather Conditions Differed Greatly between 2012 and 2013 but Still Resulted in Drought Conditions in Both Seasons

Planting occurred 3 weeks later in 2013 as compared to 2012 (**Table 1**), but mean daily temperatures were similar across both growing seasons (**Table 1** and **Figures 1A,B**). Overall precipitation during 2012 was almost double that of 2013 (**Table 1**), but annual cumulative precipitation was less than half as much at the start of the 2012 growing season as compared to the 2013 season (**Figures 1C,D**). Low precipitation amounts prior to planting in 2012 and the fact that most of the 2012 precipitation fell late in the season led to a significant drought from early to mid-season in 2012 (**Figure 1C**), which was prior to developmental stage R5 (pod filling; data not shown). A moderate drought also occurred late in the 2013 growing season (**Figure 1D**), the beginning of which corresponded to developmental stage R3 (pod initiation; data not shown). Total

FIGURE 3 | Leaf absorbance as a function of canopy position across the 2013 season. The fraction of absorbed photosynthetically active radiation (PAR) is indicated for WT (black) and *Y11y11* (gray) at the top (solid), middle (dotted), and bottom (grid) of the canopy throughout the 2013 season. Error bars represent the standard errors ($n = 3$). Letters indicate significant differences within developmental stage ($\alpha = 0.1$) when present.

S_t (from planting to harvest) was almost 30% lower during 2013 as compared to 2012 (**Figures 1E,F** and **Table 1**) and was slightly lower in the narrow row spacing due to planting 1 day after the wide row spacing (**Table 1**). Lower S_t in 2013 was only partially attributed to a later planting date in 2013 compared to 2012 (**Table 1**). Total monthly S_t was also greater during the main growing months of 2012. Total monthly S_t was 36% greater in June, 28% greater in July, and 8% greater in August of 2012 compared to the corresponding months in 2013.

SLW and Pigment Concentrations Were Significantly Altered in the Mutants Compared to WT, but Reductions in Leaf_{abs} Were Less Pronounced

Specific leaf weight was significantly reduced in the chl mutants in both growing seasons (Supporting Information Table S1). *y9y9* SLW was significantly lower than WT SLW on all days but one (DOY 218) during the 2012 season, whereas *Y11y11* SLW was significantly reduced on all days but DOY 206 and 218 (**Figure 2A**). In 2013, *Y11y11* SLW was significantly reduced compared to WT on all days except DOY 218 (**Figure 2B**).

The mutations in *Y11y11* and *y9y9* caused clear reductions in chl content while increasing the chl *a/b* ratios and decreasing total carotenoids. Chl content was significantly affected by the genotype by DOY interaction in both years (Supporting Information Table S1). Mean chl content was significantly reduced by approximately 45–60% in *Y11y11* and *y9y9* across 2012 (**Table 2** and **Figure 2C**). Throughout 2013, *Y11y11* chl content was reduced on average by 57% (**Table 2** and **Figure 2D**). However, mutant chl content was lowest early in both seasons and increased with development (**Figures 2C,D**). Chl *a/b* ratios were significantly affected by genotype by DOY interactions in 2012, but only main effects were significant in 2013 (Supporting

Information Table S1). In both seasons the mutant chl *a/b* ratios were generally greater than WT ratios (**Table 2** and **Figures 2E,F**), but the differences decreased as the 2012 season progressed (**Figure 2E**), whereas the chl *a/b* ratios were consistent in both *Y11y11* and WT throughout the 2013 growing season (**Figure 2F**). Total carotenoid content was significantly reduced by > 30% in *Y11y11* and > 40% in *y9y9* compared to WT (**Table 2** and Supporting Information Table S1).

Although *Y11y11* chl content was reduced by approximately half in 2013, sun leaf_{abs} declined by only 16.6% compared to WT (**Figure 3** and **Table 2**). Leaf_{abs} was lowest for both WT and *Y11y11* during V5, at which time *Y11y11* leaf_{abs} was only 78% of the WT (**Figure 3**). Leaf_{abs} increased with development in both genotypes, but *Y11y11* leaf_{abs} was approximately 85% of the WT during reproductive stages (**Figure 3**). Leaf_{abs} also increased with depth in the canopy except for the lowest layer of the *Y11y11* canopy during R5 (**Figure 3**).

## Diurnal Measurements Indicated Transiently Greater *A′* in Mutant Sun Leaves

Sun leaf *A′* was significantly affected by genotype by DOY interactions in both years (Supporting Information Table S1). *Y11y11* *A′* was significantly greater than WT *A′* on 3 days during the 2012 growing season (DOY 191, 206, 248; **Figure 4A**). *y9y9* *A′* was significantly lower than the control on the 1st day of measurements in 2012 but became significantly greater on DOY 191, 206, 218, and 248 (**Figure 4A**). When averaged over all measurement days from the 2012 growing season, *Y11y11* *A′* was 8.6% greater than WT *A′*, but *y9y9* *A′* was reduced by 3.6% (**Table 2**), mainly due to extremely low *A′* on DOY 178 (**Figure 4A**). In 2013, *Y11y11* *A′* was significantly lower than the control on DOY 193 and significantly greater on DOY 246 (**Figure 4B**), and there was no significant difference between the mean season *A′* (**Table 2**).

Shade leaf *A′* was similar among all genotypes when measured in 2012 (**Table 2** and Supporting Information Table S1). Although *Y11y11* A_{leaf} was significantly greater than the other two genotypes at the 1200 time point and *y9y9* A_{leaf} was significantly greater than WT and *Y11y11* at the 1600 time point (Supporting Information Figure S3A), these increases were not large enough to significantly affect *A′* (**Table 2**). Incident PPFD within the leaf chamber was based on light measurements within each canopy before each set of gas exchange measurements and was higher for the mutants as compared to the WT (Supporting Information Figure S3B). The reduced chl content of the *Y11y11* mutant, however, resulted in a similar amount of absorbed PPFD as compared to WT (Supporting Information Figure S3C).

A_{leaf} and g_s were Greater in Mutant Sun Leaves whereas iWUE Was Reduced Despite Lower T_{leaf}

Diurnal measurements were used to calculate the daily means of A_{leaf}, g_s, iWUE, and T_{leaf} in sun leaves. Mean daily A_{leaf} was significantly affected by genotype by DOY interactions in 2012, but only DOY was significant in 2013 (Supporting Information
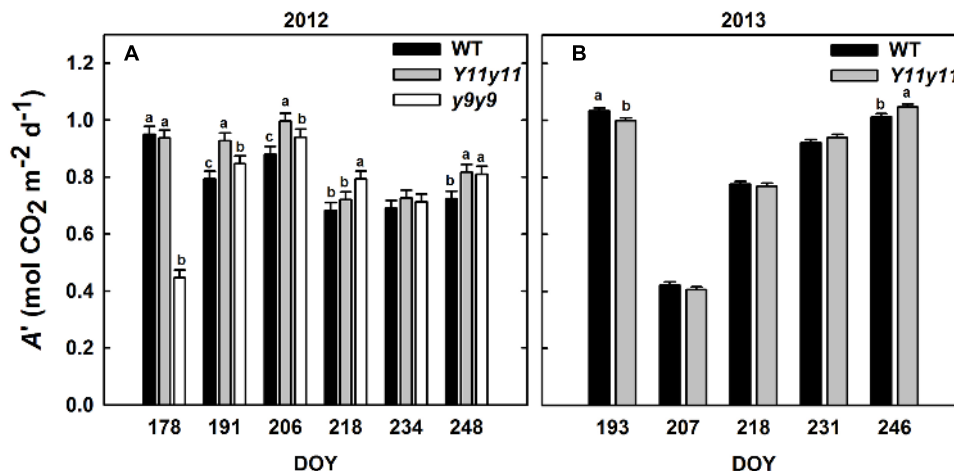
FIGURE 4 | Daily integrals of photosynthesis (A') from two growing seasons. Diurnal gas exchange measurements were used to calculate A' in 2012 **(A)** and 2013 **(B)** for WT (black), *Y11y11* (gray), and *y9y9* (white) soybean. Error bars represent the standard errors ($n = 3$). Letters indicate significant differences within DOY ($\alpha = 0.1$) when present.

Table S1). The within-day relationships between WT and mutant A_{leaf} were similar to those listed above for A'. In 2012, *y9y9* A_{leaf} was significantly lower than WT only on DOY 178 but was significantly greater than WT on DOY 191, 206, 218, and 248 (**Figure 5A**). A_{leaf} in *Y11y11* was significantly greater than WT on DOY 191, 206, and 248 in 2012 (**Figure 5A**), leading to a significant 8.5% increase in mean season A_{leaf} in *Y11y11* (**Table 2**). In 2013, *Y11y11* A_{leaf} was significantly lower than WT on DOY 193 but greater on DOY 246 (**Figure 5B**), resulting in no significant changes in season-long A_{leaf} (**Table 2**). g_s and iWUE were also significantly affected by the interaction effect (Supporting Information Table S1), but contrary to expectations, g_s was approximately 20–30% greater in the mutants across both seasons (**Table 2** and **Figures 5C,D**), resulting in lower mutant iWUE across both seasons (**Table 2** and **Figures 5E,F**). The diurnal iWUE data were supported by $\delta^{13}C$ signature, in which the mean *Y11y11* signature across leaf position (−28.8‰) was significantly lower than the mean WT signature (−27.9‰; $p < 0.0001$; Supporting Information Figure S4). This indicated greater ^{13}C discrimination in *Y11y11* and therefore higher g_s over the integral of leaf development across three layers of the canopy (Supporting Information Figure S4). T_{leaf} was significantly affected by the interaction between genotype and DOY in 2012 ($p < 0.01$) and the separate effects of genotype ($p < 0.0001$) and DOY ($p < 0.0001$) in 2013 (Supporting Information Table S1). As predicted, WT T_{leaf} was generally greater than mutant T_{leaf} in both seasons (**Table 2** and **Figures 5G,H**), which correlated with lower leaf$_{abs}$ (**Figure 3**) but also greater transpiration (data not shown) in the mutants, which correlated with the higher g_s.

## *Y11y11* Sun Leaves Demonstrated Greater Light Use Efficiency Compared to WT Early in the Growing Season

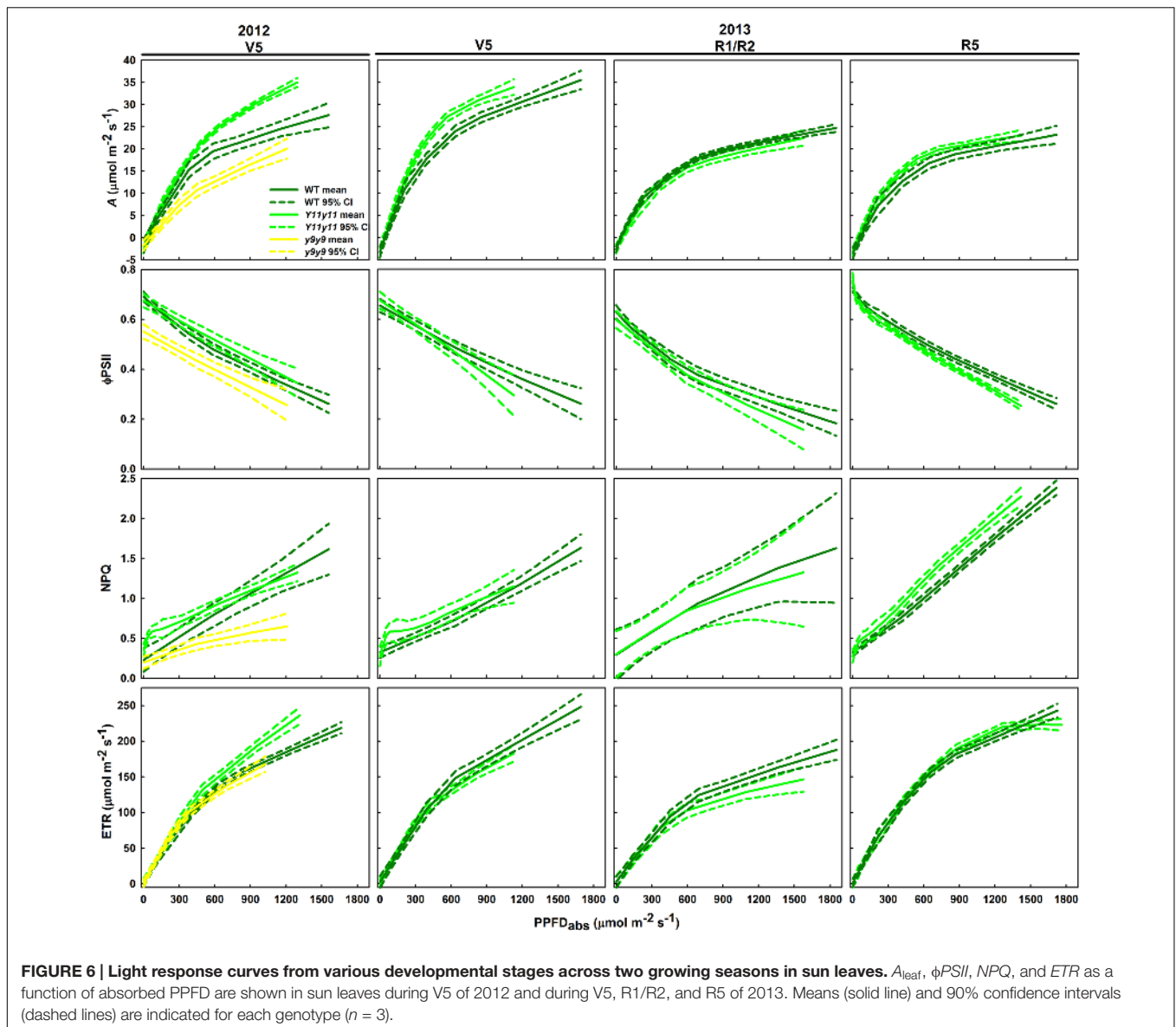Since leaf$_{abs}$ significantly differed across genotype and DOY, A/Q measurements were based on absorbed PPFD instead of incident PPFD. In both seasons, *Y11y11* sun leaves reached greater rates of A_{leaf} with fewer absorbed photons at high light levels during the V5 stage, demonstrating greater light use efficiency (**Figure 6**). Genotype main effects on sun leaf A_{sat} were significant in both years (Supporting Information Table S1). *Y11y11* sun leaves had a 43% higher A_{sat} early in 2012, whereas *y9y9* A_{sat} was lower by 14% compared to WT (**Tables 2**, **3**). In 2013, significant differences in A_{sat} occurred during R1/R2 (**Table 3**) but did not result in season-long differences (**Table 2**). The relationship between A_{sat} and chl shows a steep increase at chl contents less than 100 µmol m^{-2} and a more graduate decline with chl contents greater than 200 µmol m^{-2} (Supporting Information Figure S5A). ϕCO_2 was greater in *Y11y11* sun leaves compared to WT during the V5 stage of 2013, but season averages did not differ between the two genotypes in either year (**Tables 2**, **3** and Supporting Information Table S1). On the other hand, *y9y9* sun leaf ϕCO_2 was significantly impaired early in 2012 when chl content was severely reduced (**Tables 2**, **3**). Genotype effects were not significant within shade leaf A_{sat} or ϕCO_2 analyses in 2013 (**Tables 2**, **3** and Supporting Information Table S1).

Chl Fluorescence Parameters Varied with Chl Content and Developmental Stage

Sun leaf $\phi PSII$ was significantly lower in *y9y9* in 2012 and *Y11y11* during R5 of 2013 (**Figure 6**). In 2012, the decrease in *y9y9* $\phi PSII$ was accompanied by substantially lower *NPQ* compared to the other two genotypes in mid to high light conditions (**Figure 6**). However, *NPQ* was similar between WT and *Y11y11* early in both seasons and greater in *Y11y11* during R5 in 2013 at mid to high light levels (**Figure 6**). *Y11y11* *ETR* was greater than WT *ETR* at high light levels in 2012, but there were no significant differences in 2013 (**Figure 6**).
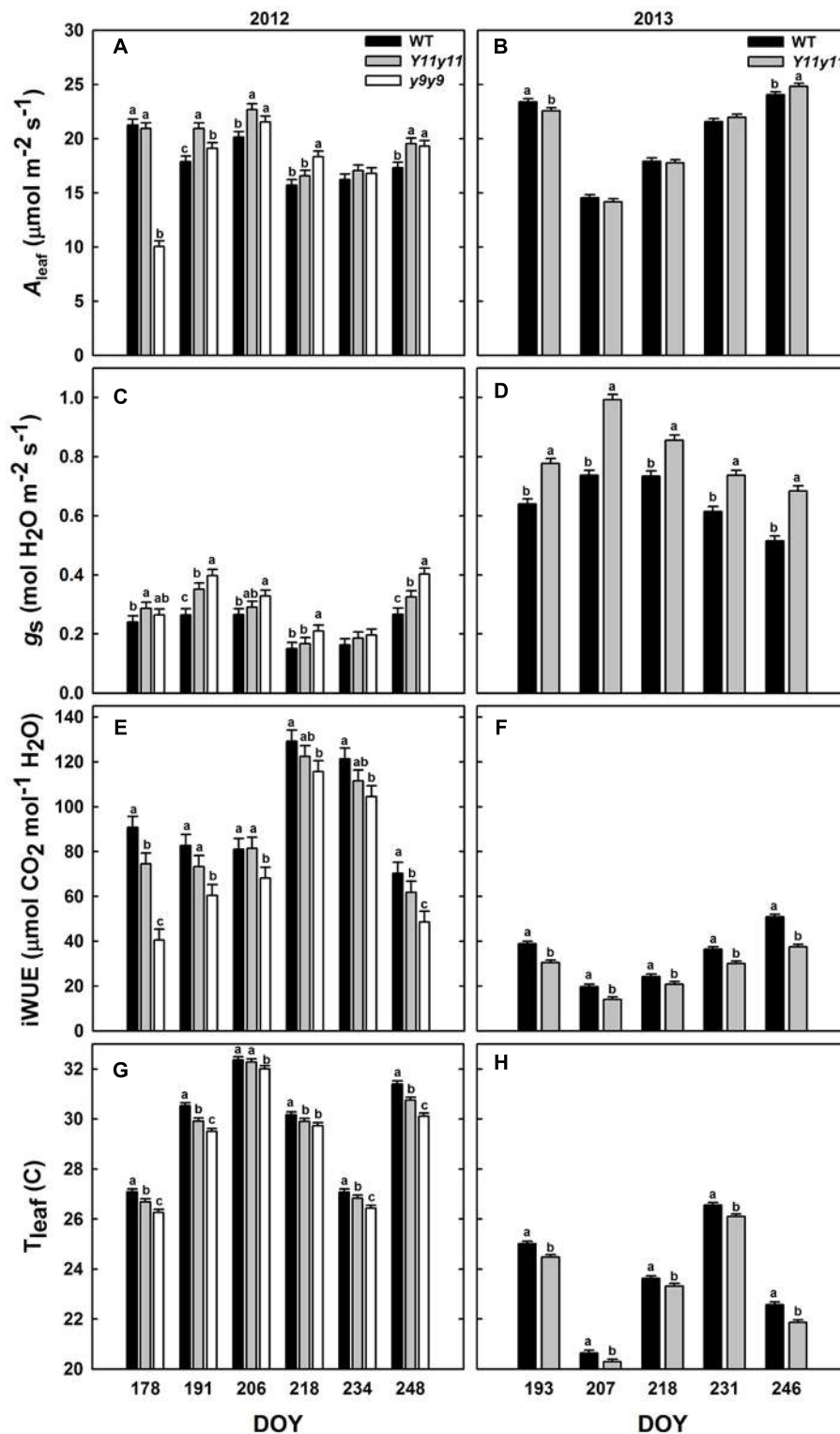
FIGURE 5 | Diurnal measurements in sun leaves from two growing seasons. Bars represent daily means of photosynthesis (A_{leaf}; **A,B**), stomatal conductance (g_s; **C,D**), intrinsic water use efficiency (iWUE; **E,F**), and leaf temperature (T_{leaf}; **G,H**) across the 2012 **(A,C,E,G)** and 2013 **(B,D,F,H)** growing seasons for WT dark green soybean (black) and two chl-deficient soybean mutants ($Y11y11$ = gray; $y9y9$ = white). Error bars represent the standard errors ($n = 3$). Letters indicate significant differences within DOY ($\alpha = 0.1$) when present.

FIGURE 6 | Light response curves from various developmental stages across two growing seasons in sun leaves. A_{leaf}, $\phi PSII$, NPQ, and ETR as a function of absorbed PPFD are shown in sun leaves during V5 of 2012 and during V5, R1/R2, and R5 of 2013. Means (solid line) and 90% confidence intervals (dashed lines) are indicated for each genotype ($n = 3$).

Chl Reductions Had Little Effect on Biochemical Photosynthetic Capacity

Enhanced biochemical photosynthetic capacity, as measured by A/C_i curves, were transient in these specific mutants. Within sun leaf analyses, $Y11y11$ had higher $V_{c,max}$ compared to WT on DOY 190 in 2012 (**Figure 7A**). Conversely, $V_{c,max}$ was reduced in $y9y9$ on DOY 175 in 2012 (**Figure 7A**). This corresponded to a >85% reduction in $y9y9$ chl content (**Figure 2C**) and a significant decrease in A_{leaf} (**Figure 5A**). There were no significant differences between WT and $Y11y11$ $V_{c,max}$ in 2013 (**Figure 7B**). J_{max} was also slightly lower in $y9y9$ sun leaves on DOY 175 (**Figure 7D**). However, J_{max} was greatest in $Y11y11$ sun leaves on DOY 190 in 2012 (**Figure 7D**) and in the R1/R2 developmental stage in 2013 (**Figure 7E**). Both $V_{c,max}$ and J_{max} showed similar relationships with chl content that declined steeply at chl contents less than 100 μmol m^{-2} while declining

more gradually at chl contents greater than approximately 200 μmol m^{-2} (Supporting Information Figures S5B,C). Sun leaf $C_{i,inflection}$ differed in $y9y9$ compared to WT and $Y11y11$ both early and late in the 2012 season (**Figure 7G**) with no significant effects occurring between WT and $Y11y11$ in 2013 (Supporting Information Table S1 and **Figure 7H**). In 2013, WT and $Y11y11$ shade leaf parameters did not differ in R1/R2, but $V_{c,max}$ and J_{max} were significantly greater in WT compared to $Y11y11$ in R5 (**Figures 7C,F,I**).

R_d Was Significantly Lower in $Y11y11$ than WT during 2013 Reproductive Stages

A significant genotype effect ($p < 0.01$) on R_d was evident in sun leaves during 2013 (Supporting Information Table S1). $Y11y11$ R_d in sun leaves was 13% lower than WT across the experiment

TABLE 3 | Parameters from photosynthetic light response (A/Q) curves in two growing seasons.

		2012			2013			
		Sun			Sun		Shade	
Parameter	Stage	WT	*Y11y11*	*y9y9*	WT	*Y11y11*	WT	*Y11y11*
A_{sat}	V5	33.2b	47.4a	28.6b	42.1a	41.0a	–	–
	R1/2	–	–	–	31.6a	28.1b	27.2a	19.1a
	R5	–	–	–	29.9a	27.6a	34.5a	35.4a
	MSE		4.3			1.3		4.9
ϕCO_2	V5	0.068a	0.061a	0.036b	0.067b	0.081a	–	–
	R1/2	–	–	–	0.047a	0.043a	0.063a	0.056a
	R5	–	–	–	0.048a	0.058a	0.057a	0.064a
	MSE		0.00004			0.003		0.005

Parameters were calculated from photosynthesis versus absorbed PPFD. A_{sat} ($\mu mol\ m^{-2}\ s^{-1}$) was calculated after fitting the data to a non-rectangular hyperbola. ϕCO_2 was the slope of the linear portion at low light. A/Q curves were only conducted in vegetative (V5) growth in 2012, whereas 2013 curves were conducted in V5 and two reproductive stages: flowering (R1/2) and pod-fill (R5). Mean square error (MSE) is reported from each ANOVA. Different letters represent significant differences at $\alpha = 0.1$.

(**Table 2** and Supporting Information Figure S6A). An apparent reduction of ~12% was seen in *Y11y11* shade leaves (**Table 2** and Supporting Information Figure S6B), but shade leaf genotype effects on R_d were not significant (Supporting Information Table S1).

Mutant Canopies Absorbed Less Light Early in the Season Despite Similar LAI

Canopy LAI was only significantly affected by chl reductions in 2012 ($p < 0.05$). In 2012, WT and *y9y9* reached peak LAI on DOY 208, on which day WT LAI was significantly greater than mutant LAI by 25–30% (**Figure 8A**). *Y11y11* did not reach peak LAI until DOY 220 (**Figure 8A**). *y9y9* LAI was also significantly lower than WT by ~30% on DOY 220 and 236 (**Figure 8A**). There were no significant within-day differences between WT and *Y11y11* LAI in 2013 wide row widths (**Figure 8B**). Within-day differences between WT and *Y11y11* LAI in 2013 were only significant in the narrow row widths on DOY 221 (**Figure 8C**).

Light interception measurements in 2012 began when both the WT and *Y11y11* canopies had already reached interception fractions of ~0.8 (**Figure 8D**). The *y9y9* canopy did not reach this fraction of light interception until approximately 2 weeks later but intercepted slightly more light at the end of the season (**Figure 8D**). Light interception measurements began relatively earlier in canopy development in 2013 and showed a slight lag in light interception by the *Y11y11* canopy as compared to WT in wide row spacing (**Figure 8E**) and a substantial lag in the narrow row spacing (**Figure 8F**).

*A*can Was Similar in WT and *Y11y11* Plots during the 2013 Season

A_{can} was calculated for each genotype based on CO_2 drawdown rates that accounted for soil respiration and leaf area within an enclosed chamber. The WT and *Y11y11* canopies had similar A_{can} on a leaf area basis ($p = 0.41$; **Figure 9**). A significant DOY effect ($p < 0.0001$) most likely occurred because average
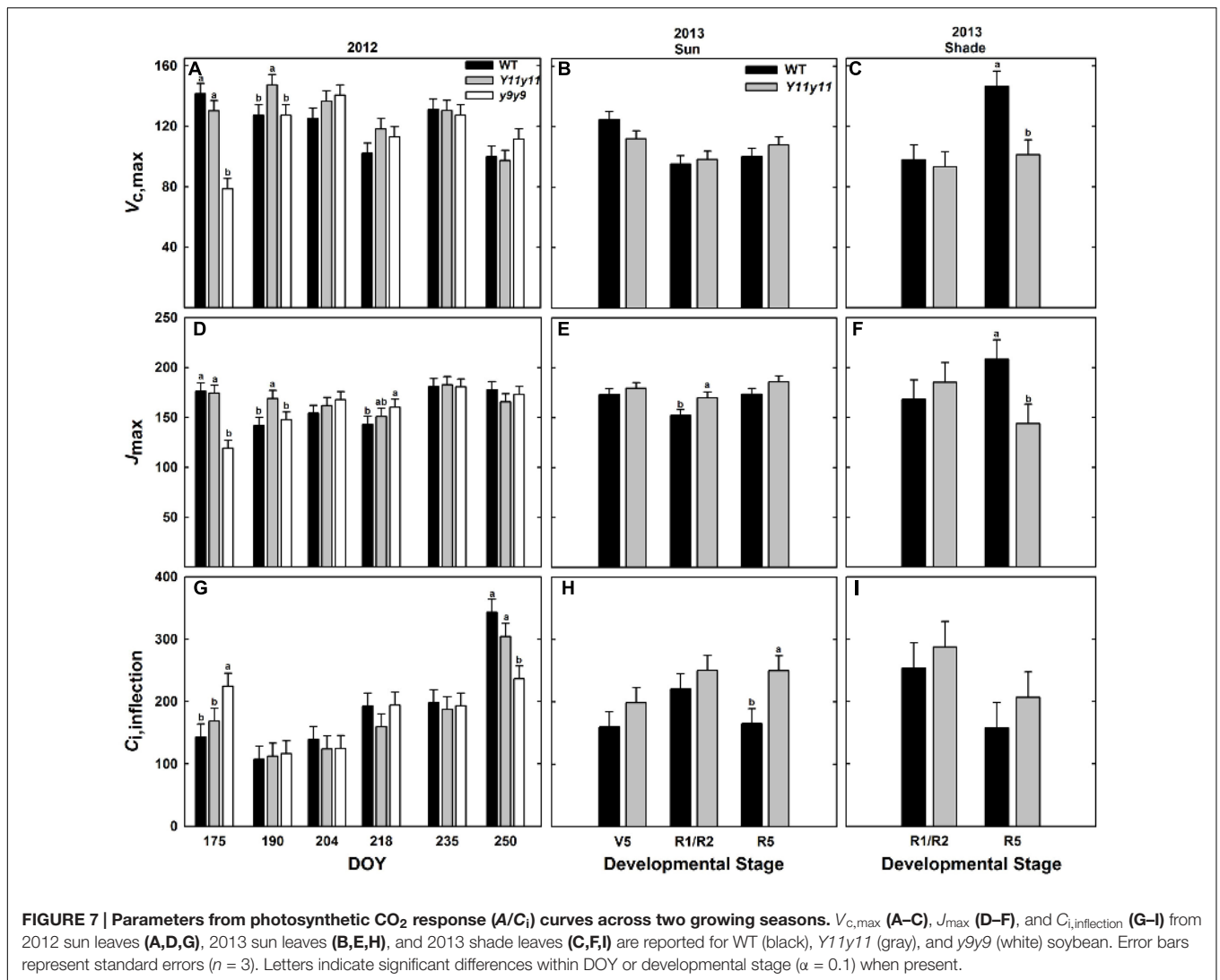
photosynthetic rates on a leaf area basis decreased drastically after canopy closure (**Figures 8B,E**) as the area of shaded leaves increased in proportion to fully sunlit leaves in both genotypes (data not shown).

Effects of Chl Reductions Were More Pronounced on ε$_i$ Compared to ε$_c$ or ε$_p$

ε$_i$, ε$_c$, and ε$_p$ were calculated for WT, *Y11y11*, and *y9y9* in 2012 and WT and *Y11y11* in 2013, which also incorporated a row spacing treatment. In 2012, a significant decrease occurred in *y9y9* ε$_i$ compared to WT and *Y11y11*, and ε$_i$ was significantly lower in narrow rows of *Y11y11* compared to WT in 2013 (**Tables 4, 5**). In calculating ε$_c$, biomass was corrected for leaf, stem, and pod energy content. Pod energy, measured in 2013 only, differed between WT and *Y11y11* during late R5 (Supporting Information Table S2) and was 1 MJ kg^{-1} lower during R8 than the reported value from Amthor et al. (1994; Supporting Information Table S2). Although differences in ε$_c$ were not resolvable at $\alpha = 0.1$ (**Table 4**), the percent reduction between WT and *Y11y11* ε$_c$ was less in the wide row spacing experiment of 2013 (3%) compared to 2012 (10%), and *Y11y11* ε$_c$ was almost 10% greater than WT ε$_c$ in the narrow row spacing of 2013 (**Table 5**). ε$_p$ was not significantly affected by genotype in either year or row spacing treatment (**Tables 4, 5**).

Reducing Chl Content Did Not Improve Yield in the Mutants

Overall yields were generally lower in 2012 compared to 2013 (**Table 5**), even though S_t was 30% greater in 2012 (**Table 1**). The lower yield is most likely due to a severe drought that occurred early in the 2012 growing season (**Figure 1C**). WT yield was significantly greater than *y9y9* yield in 2012 and *Y11y11* yield in 2013 wide row spacing (**Table 4**). Measured yields were not significantly different in the narrow row spacing of 2013 (**Table 5**). In addition, seed mass per 100 seeds was significantly reduced in *y9y9* during 2012 and *Y11y11* during 2013 (**Table 5**).

FIGURE 7 | Parameters from photosynthetic CO₂ response (A/Cᵢ) curves across two growing seasons. $V_{c,max}$ **(A–C)**, J_{max} **(D–F)**, and $C_{i,inflection}$ **(G–I)** from 2012 sun leaves **(A,D,G)**, 2013 sun leaves **(B,E,H)**, and 2013 shade leaves **(C,F,I)** are reported for WT (black), *Y11y11* (gray), and *y9y9* (white) soybean. Error bars represent standard errors ($n = 3$). Letters indicate significant differences within DOY or developmental stage ($\alpha = 0.1$) when present.
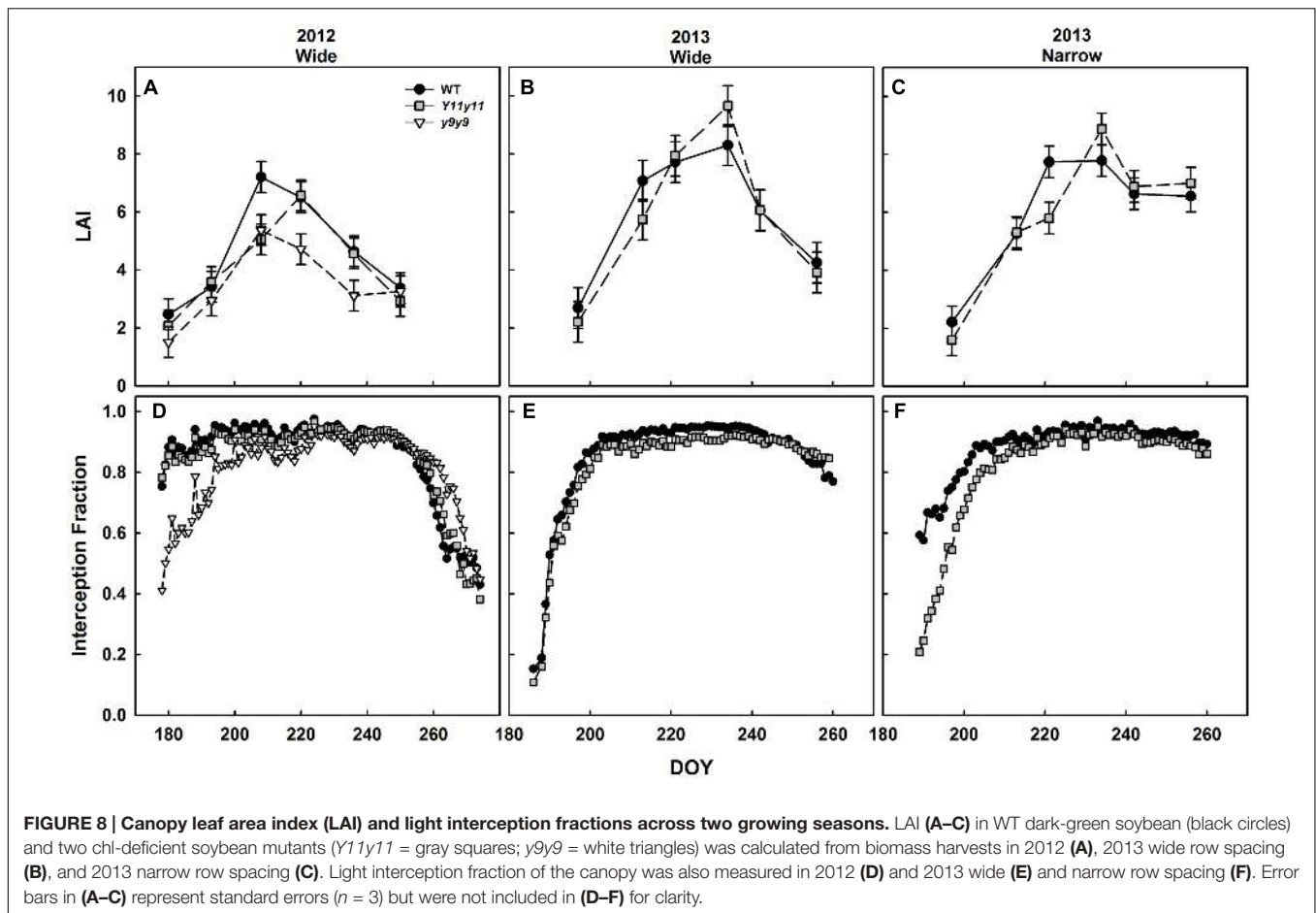
DISCUSSION

Reducing chl content in soybean was hypothesized to lead to an improved distribution of light in the canopy, resulting in benefits to leaf and canopy photosynthesis and therefore yield (Zhu et al., 2010; Ort et al., 2011; Drewry et al., 2014). This hypothesis was tested on two chl-deficient mutants that reportedly performed well in optimal field conditions in a previous study (Pettigrew et al., 1989). As the 1st year of the field study revealed severe limitations in the *y9y9* mutant, the 2nd year focused on comparison of the *Y11y11* mutant to the WT and by adding a narrow row spacing treatment that modeling predicted would advantage the light-green phenotype due to earlier canopy closure. Despite transient benefits to mutant leaf-level photosynthesis, no increases in canopy-level processes were evident during either growing season, both of which experienced drought conditions. While the results of this study confirmed neither our hypothesis nor the earlier published work in which the *Y11y11* mutant significantly outyielded the parental Clark

cultivar, they do indicate that soybean, and likely many other crop plants, significantly overinvest in chl since a >50% chl reduction had little negative impact on biomass accumulation or yield. In addition, the small negative effects reduced chl did have on yield in our study were likely due to pleiotropic effects of the mutation. This outcome suggests that more sophisticated approaches for chl reduction, such as developmentally timed transgenic technology, may promote an opportunity to reinvest nitrogen and energy resources saved in chl reduction into increasing the biochemical photosynthetic capacity, leading to increased yield.

Although the *Y11y11* mutant demonstrated the potential to surpass WT in light use efficiency and A_{sat}, the improvements were transient. The increases in light use efficiency corresponded with a narrow range of chl reductions (**Table 3** and **Figures 2C,D, 6**), which may support the hypothesis that an optimal chl content exists and is lower than current levels (Ort et al., 2011). Benefits to A_{leaf} were seen only when chl content in *Y11y11* was reduced to 30–40% of WT (**Figures 2C,D**), which corresponded to a 22% reduction in leaf$_{abs}$ (**Figure 3**)

FIGURE 8 | Canopy leaf area index (LAI) and light interception fractions across two growing seasons. LAI **(A–C)** in WT dark-green soybean (black circles) and two chl-deficient soybean mutants (*Y11y11* = gray squares; *y9y9* = white triangles) was calculated from biomass harvests in 2012 **(A)**, 2013 wide row spacing **(B)**, and 2013 narrow row spacing **(C)**. Light interception fraction of the canopy was also measured in 2012 **(D)** and 2013 wide **(E)** and narrow row spacing **(F)**. Error bars in **(A–C)** represent standard errors (*n* = 3) but were not included in **(D–F)** for clarity.
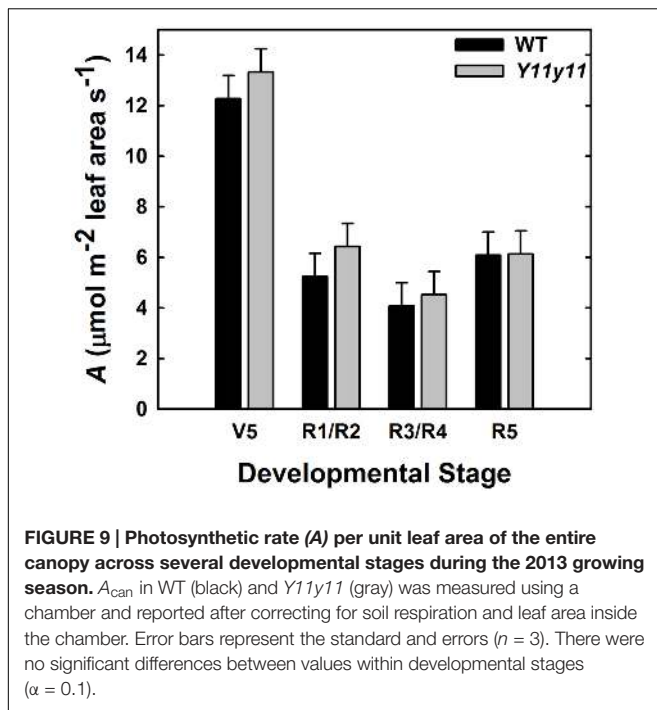
that is less than the reduction predicted by Beer's law (Slattery et al., 2016). As chl content increased in the mutant throughout the growing season (**Figures 2C,D**) and the percent difference between mutant and WT leaf$_{abs}$ decreased (**Figure 3**), benefits at the leaf level became too small to resolve (**Figure 6**). In other species, chl contents correlated with increased A_{leaf} ranged from 30% in tobacco (Edwards et al., 1993) to 60% in maize (Edwards et al., 1993) and cowpea (Habash et al., 1994) as compared to the dark-green WT, suggesting that optimal chl content may be species specific.

Improved leaf light use efficiency is the anticipated result of improved light distribution within the low chl leaf. In dark-green leaves, the majority of light absorption occurs in the uppermost adaxial chloroplasts, causing light limitation in lower chloroplasts (Vogelmann and Evans, 2002; Evans and Vogelmann, 2003; Slattery et al., 2016). This has been shown to create a within-leaf gradient in photosynthesis (Evans and Vogelmann, 2003) as well as photoinhibition (Oguchi et al., 2011) that decreases with depth in the leaf and limits overall photosynthetic efficiency. Light sheet microscopy analyses revealed more gradual PPFD attenuation in light-green soybean leaves compared to dark-green leaves. This resulted in significantly more red and blue light reaching the spongy mesophyll chloroplasts of the light-green leaves, which correlated with greater photosynthetic light

use efficiency (Slattery et al., 2016). Thus, if chloroplasts within the leaf mimic leaves within a canopy, then decreasing chl content may have ameliorated the large disparity of light availability in the lower leaf to increase photosynthetic light use efficiency.

However, other attributes of the chl mutants may have also played roles in altering photosynthetic performance. SLW was reduced in both mutants (**Figures 2A,B**). Changes in SLW are due to changes in either leaf thickness or leaf density, both of which can affect photosynthesis (Niinemets, 1999). Although leaf thickness was not directly measured in this study, leaf thickness did not change in a previous study on the same soybean genotypes, even when SLW was significantly different (Slattery et al., 2016). Therefore, changes in SLW were almost certainly due to reduced leaf density in the chl mutants, which can increase the proportion of intercellular space and thus improve CO_2 diffusion throughout the leaf (Niinemets, 1999). In addition, chl deficiency in these mutants was accompanied by increased g_s, which would increase carbon supply to the inner leaf and further confound deciphering the chl reduction impact on A_{leaf}. A similar increase in g_s was seen in rice chl mutants that exhibited greater rates of A_{leaf} in non-limiting environmental conditions (Gu et al., 2017). Those authors hypothesized that the increase in g_s may have been due to changes in vein

FIGURE 9 | Photosynthetic rate (A) per unit leaf area of the entire canopy across several developmental stages during the 2013 growing season. A_{can} in WT (black) and *Y11y11* (gray) was measured using a chamber and reported after correcting for soil respiration and leaf area inside the chamber. Error bars represent the standard and errors (n = 3). There were no significant differences between values within developmental stages (α = 0.1).

structure in the rice mutants (Gu et al., 2017). More analyses are needed to separate the effects of reduced chl, increased g_s, and altered leaf anatomy on A_{leaf} improvements or the lack thereof.

A decline in photosynthetic efficiency and capacity in *y9y9* was correlated with severe reductions in chl. Despite greater g_s and lower SLW, which was evident in *Y11y11* as well, the *y9y9* mutant displayed reduced A_{sat} and light use efficiency compared to the WT (**Figures 4A**, **5A**, **6**, **7A** and **Table 3**) when chl was reduced by more than 85% compared to the WT (**Figure 2C**). Similar responses to severe reductions in chl content (>80%) seen in *y9y9* have been reported in *Y11y11* when grown in controlled environment chambers at lower than field light levels (Xu et al., 1993; Slattery et al., 2016). These two mutants are characterized by higher chl a/b ratios due to greatly reduced PSII-associated light harvesting complexes (LHCII) and to some extent PSI-associated light harvesting complexes (LHCI; Ghirardi and Melis, 1988). Theoretically, severe deficiencies in LHCII could have negative effects at the leaf level, including greater levels of photoinhibition (Leverenz et al., 1992; Xu et al., 1993), reduced photoprotective capacity (Ort, 2001), and lowered connectivity among PSII centers and therefore lower quantum yield at low light (Allen and Forsberg, 2001). In this study, extreme reductions in chl coupled with large reductions in carotenoids (>40%; **Table 2**) were correlated with impaired photoprotective mechanisms such as *NPQ* (**Figure 6**). ϕCO_2 was also significantly reduced in *y9y9* (**Tables 2**, **3**). Thus, these data suggest improving leaf photosynthetic efficiency through lowering chl a and chl b concentrations proportionally, but not to an extent that impairs photosynthetic and photoprotective capacity.

Despite transient leaf-level improvements in photosynthesis and photosynthetic efficiency, chl reductions only significantly affected the canopy parameter of ε_i. Although no improvements in A_{can} or ε_c were evident in the chl-deficient soybean, the fact that there was also no decline in these parameters with a >50% reduction in chl suggests that soybean significantly overinvest in chl. This is consistent with a recent study that showed soybean also overinvest in LAI to the detriment of canopy productivity (Srinivasan et al., 2016). However, less pigment led to significant depressions in ε_i in *y9y9* in 2012 and *Y11y11* in 2013 narrow row widths (**Tables 4**, **5**), mostly due to reduced light interception by the mutants early in the season, even when LAI was similar (**Figure 8**). The mutant chl content in this study was the lowest early in the season (**Figures 2C,D**), which would be expected to both increase transmission to the soil and increase the proportion of reflectance not reabsorbed by upper canopy leaves during early growth. As LAI increased, chl content also increased in the mutants, which in turn would be expected to limit light penetration to deeper layers in the canopy. An ideal situation for maximizing light absorption early in the season would be normal chl content until the canopy has closed (Melis, 2009). This would suppress weed vigor through increased competitiveness, which is reasoned to be why plants evolved with much higher chl content than is needed to maximize photosynthesis (Donald, 1968). As LAI increases, decreasing chl biosynthesis in new leaves would alleviate oversaturation in times of high light and allow greater transmittance to the lower canopy. However, light reflection and thus loss of canopy absorbance will also increase at the top of the canopy; thus, a more light-use efficient canopy may not necessarily lead to an increase in A_{can} from chl reductions alone.

Reduced pigmentation was hypothesized to benefit leaf water use along with canopy ε_c, but the greater g_s that accompanied chl reductions in these mutants likely prevented these effects. Increased albedo was associated with lower T_{leaf} in the mutants as expected (**Figures 5G,H**), but cooler T_{leaf} may have been, at least in part, an effect of greater g_s and therefore cooling via transpiration in the mutants (**Figures 5E,F**). Greater g_s was most likely a result of the mutations causing chl deficiency. A recent study identified a mutation in the magnesium chelatase subunit-I gene (CHLI) as the cause of the light-green phenotype of *Y11y11* (Campbell et al., 2015). In *A. thaliana*, both CHLI and CHLH mutants have demonstrated ABA-insensitivity (Tsuzuki et al., 2011; Du et al., 2012) that is independent of chl biosynthesis (Du et al., 2012). Whether the greater g_s in *y9y9* that occurred in this study is also due to ABA-insensitivity is unknown since the mutation has not yet been identified, but greater g_s has been reported in *y9y9* compared to WT regardless of water stress (Luquez et al., 1997). The increase in g_s resulted in lower mutant iWUE (**Figures 5C,D**) and integrated canopy WUE (Supporting Information Figure S4). Lower canopy WUE in the mutants would likely result in greater soil moisture depletion (Hussain et al., 2013) and therefore greater susceptibility to drought, which occurred during both of the growing seasons (**Figures 1C,D**). Drought stress significantly reduces ε_c (Slattery et al., 2013), and the greater susceptibility to drought in the mutants may have dampened even small benefits of reduced chl on ε_c and yield.

TABLE 4 | ANOVA results of genotype effects on canopy parameters across two different years and row spacing treatment levels in 2013.

	2012		2013			
	0.38 m row space		0.38 m row space		0.19 m row space	
Parameter	F-value	P-value	F-value	P-value	F-value	P-value
ε_i	8.81	<0.05	4.56	0.17	16.6	<0.1
ε_c	0.87	0.43	0.47	0.50	0.79	0.38
ε_p	2.72	0.18	1.85	0.31	4.40	0.17
Seed yield	8.77	<0.05	38.8	<0.05	1.15	0.40
Seed mass	2.78	0.18	784	<0.01	9.41	<0.1

Effects of genotype on interception efficiency (ε_i), conversion efficiency (ε_c), partition efficiency (ε_p), seed yield, and seed mass are indicated.

TABLE 5 | Parameter estimates of canopy level processes related to the Monteith equation (Monteith, 1977) and yield.

	2012			2013			
	0.38 m row space			0.38 m row space		0.19 m row space	
Parameter	WT	Y11y11	y9y9	WT	Y11y11	WT	Y11y11
Incident PAR (MJ m^{-2})	883	883	883	642	642	642	642
ε_i	0.868a	0.860a	0.801b	0.882a	0.857a	0.878a	0.807b
	(0.034)	(0.034)	(0.034)	(0.008)	(0.008)	(0.012)	(0.012)
ε_c	0.0226a	0.0204a	0.0198a	0.0272a	0.0263a	0.0319a	0.0330a
	(0.002) 12	(0.001) 14	(0.002) 14	(0.003) 18	(0.003) 18	(0.002) 18	(0.004) 18
ε_p	0.476a	0.490a	0.518a	0.416a	0.421a	0.495a	0.542a
	(0.017)	(0.017)	(0.017)	(0.021)	(0.021)	(0.033)	(0.033)
Seed yield (g m^{-2})	184.4a	157.2ab	136.7b	324.6a	223.5b	281.1a	216.1a
	(7.8)	(7.8)	(7.8)	(11.5)	(11.5)	(42.9)	(42.9)
Seed mass (g (100 seeds)$^{-1}$)	15.5a	14.4ab	14.2b	15.0a	13.0b	15.5a	13.2b
	(0.40)	(0.40)	(0.40)	(0.38)	(0.38)	(0.55)	(0.55)

Estimates are across two growing seasons and two different row spacing treatment levels in 2013. Incident photosynthetically active radiation (PAR) is reported for the duration of the measurements. Interception efficiency (ε_i), conversion efficiency (ε_c), partition efficiency (ε_p) are reported along with measured seed yield and mass per 100 seeds. Values within experiments with different letters represent significant differences at $p < 0.1$. The values in parentheses represent standard error of the regression slope for ε_c and mean square error from ANOVA analyses in all other parameters. Sample size for determining ε_c is indicated next to the standard errors and was $n = 3$ for all other parameters.

In the previous study where A_{can} and yields were greater in $Y11y11$ compared to WT, water was not limiting due to irrigation (Pettigrew et al., 1989), suggesting that these specific mutations causing chl deficiency may limit productivity in times of even moderate water stress. Although rice chl mutants with greater g_s resulted in higher yields when grown at a higher planting density, the authors do not report any water limitations. Further assessments of chl-deficient crops may need to incorporate tests of efficiency and productivity in non-optimal field conditions while maintaining or improving WUE.

The results of this study suggest that soybean overinvest in chl; thus, chl reduction represents an opportunity to reinvest nitrogen from pigment-proteins into rate limiting photosynthetic enzymes that could increase photosynthetic capacity (i.e., $V_{c,max}$ and J_{max}). However, nitrogen reallocation was not realized to any significant extent in these mutants. For example, a study by Evans and Poorter (2001) showed that 12.9% of leaf organic nitrogen was associated with pigment-protein complexes and 21.6% was associated with Rubisco in plants grown at high light. Therefore, if pigment-proteins were reduced by 50% and all of the nitrogen associated with pigment-proteins was reallocated to

Rubisco, carboxylation capacity could potentially increase by up to 30%. However, across both seasons, $Y11y11$ $V_{c,max}$ increased <5% compared to WT (**Table 2**). J_{max} only increased by 3% in 2012 and 7% in 2013 in $Y11y11$ compared to WT (**Table 2**). Therefore, further intervention would be required to redirect nitrogen savings from reduced investment in pigment-proteins in low-chl plants to the most beneficial targets for increasing photosynthesis (Zhu et al., 2007).

CONCLUSION

This study demonstrated early season benefits of reduced chl content on leaf photosynthetic efficiency and capacity. However, the effects of reduced chl were confounded with leaf properties and greater g_s in this study and a similar study in rice. Further analyses will be needed to parse out the impacts of chl reductions on leaf structure, g_s, and A_{leaf}. The mutants used in this study, although relatively robust in optimal field conditions, captured less light early in the growing season and used water less efficiently, which may have impaired the effects of

reduced chl during the drought conditions experienced in both growing seasons. Despite the pleiotropic effects of the mutations, limitations to biomass accumulation and yield were minimal, signifying an overinvestment in chl in dark-green soybean.

As evident from these results, the methods of obtaining and maintaining optimal chl concentrations require further consideration. Optimizing chl concentration within the canopy and throughout the season is required to reduce wasted light early in the season via transmission to the soil and later in the season as reflected light from the top of the canopy while maintaining a more even light distribution within the canopy. Additionally, it is crucial to identify and utilize targets that eliminate the pleiotropic effects, such as greater water loss and photooxidative effects, that can accompany many chl biosynthesis mutations. Lastly, nitrogen reinvestment will need optimization to maximize nitrogen use efficiency in low-chl plants. This suggests that directed approaches, such as transgenic technology, are required for greater benefits of reduced chl on A_{can}, ε_c, and yield.

AUTHOR CONTRIBUTIONS

All authors participated conceiving and designing the research. RS, AV, and CB performed the research. RS and AV analyzed the data. RS and DO wrote the article, which was reviewed by all other authors.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00549/full#supplementary-material

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