



Soybean proteins GmTic110 and GmPsbP are crucial for chloroplast development and function

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ABSTRACT

We have identified a viable-yellow and a lethal-yellow chlorophyll-deficient mutant in soybean. Segregation patterns suggested single-gene recessive inheritance for each mutant. The viable- and lethal-yellow plants showed significant reduction of chlorophyll *a* and *b*. Photochemical energy conversion efficiency and photochemical reflectance index were reduced in the viable-yellow plants relative to the wild-type, whereas the lethal-yellow plants showed no electron transport activity. The viable-yellow plants displayed reduced thylakoid stacking, while the lethal-yellow plants exhibited failure of proplastid differentiation into normal chloroplasts with grana. Genetic analysis revealed recessive epistatic interaction between the viable- and the lethal-yellow genes. The viable-yellow gene was mapped to a 58 kb region on chromosome 2 that contained seven predicted genes. A frame shift mutation, due to a single base deletion in *Glyma.02g233700*, resulted in an early stop codon. *Glyma.02g233700* encodes a translocon in the inner membrane of chloroplast (GmTic110) that plays a critical role in plastid biogenesis. The lethal-yellow gene was mapped to an 83 kb region on chromosome 3 that contained 13 predicted genes. Based on the annotated functions, we sequenced three potential candidate genes. A single base insertion in the second exon of *Glyma.03G230300* resulted in a truncated protein. *Glyma.03G230300* encodes for GmPsbP, an extrinsic protein of Photosystem II that is critical for oxygen evolution during photosynthesis. *GmTic110* and *GmPsbP* displayed highly reduced expression in the viable- and lethal-yellow mutants, respectively. The yellow phenotypes in the viable- and lethal-yellow mutants were due to the loss of function of GmTic110 or GmPsbP resulting in photooxidative stress.

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1. Introduction

Chlorophyll metabolism is vital for plant growth, development and reproduction [1]. In the genomic era, new information has linked photosynthesis research with physiological, biochemical, genetic, molecular and ecological phenomena [2]. Modification and

utilization of photosynthesis proteins for increased biomass and productivity is becoming a reality [3]. Generating a deeper understanding of oxygen evolution, light harvesting complex, electron transport and energy dissipation may help us link potential photosynthesis to crop productivity [2]. As a part of light harvesting complex, chlorophylls play an important role in absorbing light energy and converting that to chemical energy during photosynthesis [4]. Chlorophyll-deficient mutants have been exploited to study chlorophyll metabolism and genetic regulation [5–11]. Identification of genes encoding for different steps of biochemical and developmental pathways is essential and chlorophyll-deficiency phenotypes present many possibilities for this identification [12–14].

In soybean, 57 chlorophyll-deficient mutants have been reported [15]. They are classified as lethal or non-lethal, of which

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25 are nuclearly inherited, the rest are cytoplasmically inherited [15]. Some of these mutant genes have been mapped to linkage groups [6,7,10,11,15–17]. Chlorophyll-deficiency mutants have been recovered in diverse experimental settings [6,8,18]. In an attempt to study genetic variation within an inbred line, single plants of the variety IAR2001BSR were planted in a wide inter-plant spacing [19]. In the progeny of IAR2001BSR, which had been exposed to a natural hail-storm, we identified a soybean line segregating for a lethal-yellow phenotype. Cross-pollinations were made using plant introduction Manchu (PI 30593) as the female parent with green plants from the segregating IAR2001BSR mutant lines. During F_{2:3} progeny-line evaluations, a viable-yellow mutant was detected in a progeny row from the cross-pollination between Manchu and IAR2001BSR.

In this study, we used a combination of physiological, cytological and genetic methods to characterize the viable- and lethal-yellow soybean mutants. The three phenotypes green, viable-yellow and lethal-yellow were easily distinguishable based on their light reflectance indices, chlorophyll abundance, electron transport activities and chloroplast structure. We identified two independent genes that control the phenotypes, showed that they displayed recessive epistatic interaction and isolated both genes using map-based cloning. The expression pattern of the characterized genes in the wildtype green, viable-yellow and the lethal-yellow mutants confirmed their role in the phenotypes.

2. Material and methods

2.1. Plant material

The soybean line IAR2001BSR was used to study genetic variability within the inbred line. Bulk seed IAR2001BSR was obtained from Dr. Silvia Cianzio, Iowa State University, Ames, IA. In 2008, 500 seeds IAR2001BSR were planted at the Bruner Farm near Ames, Iowa, in a honeycomb design [19], using an equidistant spacing of 2-m between each individual plant to minimize the unfavorable effect of selective competition. A code, termed “entry number,” was assigned to each individual plant. Field plots were damaged by natural hail in July, reducing yield in most entries. For this reason, the number of harvested plants was reduced to 171. At maturity, each plant was harvested and threshed by hand.

In 2009, progeny of single plants from the 2008 experiment were planted in a 15-m long row (50 seeds per entry). Three weeks after planting, each plot was thinned to approximately 35 plants. During the growing season, plots were evaluated based on plant color, flower color, pubescence color, and phenotypic variation within plots.

2.2. Identification of mutants in the field and strategies for genetic analysis

In a field evaluation of seedlings from the progeny of IAR2001BSR, we identified a soybean line (IAR2001BSR entry 243) segregating for lethal-yellow phenotype. This lethal-yellow mutant was assigned Genetic Type Collection number T378H. Early identification of lethal-yellow mutants in the field gave us time to make cross-pollinations for genetic analysis. Green plants from IAR2001BSR entry 243 (T378H) were tagged and used as male parents in cross-pollinations with Manchu (PI 30593). F₁ seeds were planted at the University of Puerto Rico – Iowa State University station near Isabela, Puerto Rico in October 2009. The F₁ plants were single-plant threshed and F₂ seeds from each F₁ plant from the cross combinations were planted at the Bruner Farm near Ames, IA, in May 2010. The F₂ plants were classified for plant color at the seedling stage. Green foliage F₂ plants of the segregating families were single-plant threshed and evaluated as F₂-plant progeny

rows. F_{2:3} progenies were evaluated for seedling traits in the USDA greenhouse at Iowa State University in October 2010–June 2011.

2.3. Identification of viable-yellow mutant and development of genetic material

One F_{2:3} progeny line from the cross-pollination between Manchu and IAR2001BSR entry 243, segregated 20 green, 3 viable-yellow and 9 lethal-yellow plants in the USDA greenhouse sand bench (Fig. 1). These three viable-yellow mutant plants were assigned Genetic Type Collection numbers T379, T380 and T381. Three viable-yellow mutant plants were tagged and transplanted to pots in the USDA greenhouse in June 2011. Cross-pollinations were made between Manchu as the female parent with the viable-yellow plants, KE120-1 (T379), KE120-2 (T380), and KE120-3 (T381) as male parents. F₁ seeds were planted at the University of Puerto Rico – Iowa State University station near Isabela, Puerto Rico in October 2011. The F₁ plants were single-plant threshed and 50 F₂ seeds from each F₁ plant from the cross combinations were planted in the USDA greenhouse. For genetic and molecular analyses, the remaining seeds of the selected F₂ populations were planted at the Bruner Farm near Ames, IA, in June 2012. The F_{2:3} progenies were grown and evaluated in the USDA greenhouse in November 2012 to determine the genotype of each F₂ plant by checking leaf color phenotype.

2.4. Leaf chlorophyll measurements

Leaf punches (0.7 cm²) were collected from each plant and homogenized in 1 ml of 95% ethanol using a cold mortar and pestle. The leaf homogenate was transferred to a 1.5 ml microcentrifuge tube and spun at 16,000 RPM for 30 s to pellet the solids. The supernatant was transferred to a polycarbonate spectrophotometer cuvette and absorbance was measured at 649, 654 and 665 nm using a diode array spectrophotometer (model 8453, Agilent Technologies, Santa Clara, CA, USA). All reagents and materials were chilled to 4 °C. Chlorophyll concentration was calculated using extinction coefficients [20].

2.5. Chlorophyll fluorescence

Chlorophyll fluorescence measurements were made using an integrated LED light source and pulse-modulated fluorometer (model 6400-40, LI-COR, Inc.). Leaves were dark-acclimated for 30 min prior to measurement by enclosing them in a folded patch of aluminum foil. Dark-acclimated photochemical efficiency (F_v/F_m) was calculated from measurements of F_v, F_m, and F_o to determine maximal photochemical conversion efficiency [21].

2.6. Leaf spectral reflectance

To measure spectral reflectance of leaves, we used a spectral analysis system (model UniSpec-SC VIS/NIR, Amesbury, MA, USA) with a 256-element photodiode array having a spectral range of 350–1100 nm, with a sampling interval of 3.3 nm and a spectral resolution of 10 nm. A bifurcated fiber optic delivers light from an internal 7.0 W halogen lamp via one branch and receives reflected light via the other. Leaves were placed into a leaf clip that holds the fiberoptic at 60° and maintains a 2.3 mm diameter field of view (FOV). Sample reflectance was measured and referenced to the reflectance of a white standard (barium sulfate). Ten scans were averaged per recorded spectrum. Dark scans and white reference scans were performed frequently to detect instrument drift on the spectra. We calculated the photochemical reflectance index (PRI) from reflectance at 531 and 570 nm [22].

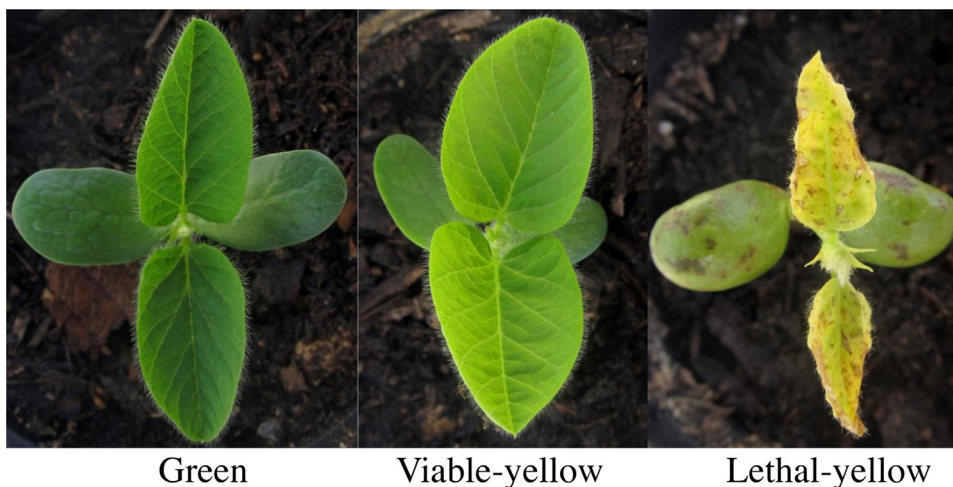


Fig. 1. Phenotypes of wildtype green, viable- and lethal-yellow mutant plants. The plants were grown two weeks at 25 °C under 18 h light ($100 \mu\text{E m}^{-2} \text{s}^{-1}$).

2.7. Electron microscopy

Soybean leaves were immersed in a Silgard® dish with 2.5% glutaraldehyde fixative in 50 mM Na-cacodylate buffer, pH 7.2, containing 5 mM CaCl_2 [23] and cut into 1 mm $\times$ 4 mm strips. Strips were weighted with stainless-steel screens and fixed for 2 h at room temperature with gentle rocking. Fixative was aspirated and samples were washed 3x with cacodylate- CaCl_2 buffer over 1 h with rocking. Samples were post-fixed for 2 h in sealed vials with 1% osmium tetroxide containing 0.8% potassium ferricyanide in distilled water, rinsed 3x in distilled water, then dehydrated in graded ethanol solutions to 100% and infiltrated with graded mixtures of Spurr's epoxy embedding medium over 2 days and polymerized at 70 °C overnight. Thin sections (ca. 60 nm) of leaf sample cross-sections were mounted on glow-discharged grids and examined with a Hitachi H-600 transmission EM at 75 kV. Toluidine-blue-stained, 500- μm thick sections of the same material were used for light microscopy to assess chloroplast numbers. Chloroplasts or plastids were counted on enlargements of whole sections using a grid that covered about 2/3 of the palisade-layer cells just below the leaf epithelium.

2.8. DNA isolation and bulked segregant analysis (BSA)

Plant DNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) method as described previously [24]. BSA was used to identify molecular markers closely linked to the viable- and lethal-yellow genes [25]. Two DNA bulks were established based on $F_{2:3}$ phenotypic data for each mutant. One bulk was generated by pooling DNA from 10 homozygous dominant (green phenotype) F_2 individuals and the second was generated by pooling DNA from 10 homozygous recessive (viable- or lethal-yellow phenotype) F_2 individuals. These bulks were diluted to a final concentration of 50 ng/ μl .

2.9. Molecular analysis

Sequences of Simple Sequence Repeat (SSR) primers were designed based on information from Soybase (<http://soybase.org/resources/ssr/php>) [26,27]. SSR markers were amplified by the polymerase chain reaction (PCR) in a 10 μl reaction mix that contained 40–50 ng of genomic DNA, 1 $\times$ PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 0.25 μM of each primer, 200 μM of each dNTP, 2.0 mM MgCl_2 , and 0.25 units of Biolase DNA polymerase (Bioline USA Inc, Taunton, MA). The PCR program employed was 2 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 1 min at 72 °C;

and a final extension of 72 °C for 10 min. The PCR products were separated on a 4% agarose gel at 150 V for 2 h in $0.5 \times$ TBE buffer.

The putative closely linked SSR markers identified in the BSA analysis were used to screen the entire F_2 mapping population. Map positions of the locus and the SSR markers in the final map were calculated with the program Mapmaker 2.0 [28], with a minimum LOD value of 3.0 and a maximum recombination value of 40%. Linkage calculations were done using the Kosambi mapping function [29]. The “MapChart” software was used to generate maps [30].

The putative candidate genes were amplified using a long range PCR kit following manufacturer's instructions (QIAGEN Inc., Valencia, CA, USA). PCR products were run on 0.8% agarose gels. Desired bands were excised and purified using a gel fragment extraction kit (VWR, Radnor, PA, USA). DNA was sequenced using Sanger sequencing at Functional Biosciences, Inc. in Madison, Wisconsin. Sequence alignments were performed using Vector NTI express (Life Technologies Corporation, Grand Island, NY, USA).

2.10. Expression analyses of Glyma.02G233700 and Glyma.03G230300

Tissues were harvested from young leaves of 15-day old soybean plants. Expression analyses were performed on three biological replicates. Tissues were frozen in liquid nitrogen and total RNA was isolated using the TRIzol® reagent following manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA was treated with DNase to remove residual DNA contamination using DNase I following manufacturer's instructions (Thermo Scientific, Waltham, MA, USA).

Quantitative Reverse Transcription-PCR (qRT-PCR) experiments for all samples were conducted with a BioRad CFX96 System using iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad Laboratories, Hercules, CA, USA). *Glyma.02G233700* was amplified using primers *Glyma.02G233700* 1F (5'GAGATTGTCTATATATCCGAGTTTG3') and *Glyma.02G233700* 1R (5'TCCATGTGCATAGCAGCA3'). *Glyma.03G230300* was amplified using primers *Glyma.02G230300* 2F (5'CTTGCTGAGAACTTGTAGAGAT3') and *Glyma.02G230300* 2R (5' TCCAGGAAGTCTTTGGT3'). The soybean *Actin 1* gene (*Glyma.19G000900*), that displays constitutive expression, was used as a reference in the qRT-PCR analyses. PCR primers for the *Actin 1* gene were *Actin 1F* (5'CAGGAATGGTTAAGGCAGGA3') and *Actin 1R* (5'CCATACCAACCATCACACCA3'). qRT-PCR reactions were performed in total volume of 10 μl that contained 5 μl of 2x one-step SYBR® Green Reaction mix, 0.125 μl iScript™ Reverse Transcriptase, 0.75 μM of each the primer and 100 ng total RNA.

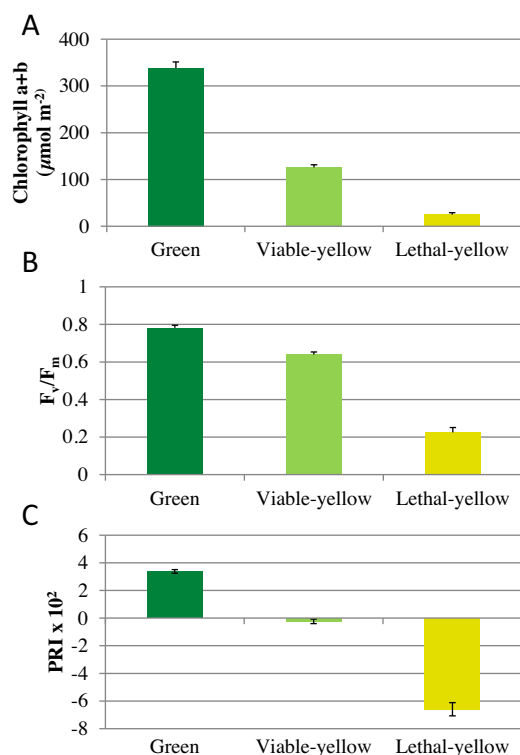


Fig. 2. Comparison of photosynthetic parameters for wildtype green, viable- and lethal-yellow mutant plants. A) Total chlorophyll content in wildtype green, viable- and lethal-yellow mutant plants. B) Chlorophyll fluorescence (F_v/F_m) used to measure photochemical conversion efficiency in the dark-acclimated wildtype green, viable- and lethal-yellow mutant plants. C) Photochemical reflectance index (PRI) of wildtype green, viable- and lethal-yellow mutant plants. Error bars represent standard error.

The PCR conditions were as follows: 50°C for 10 min, 95°C for 1 min, then 40 cycles of 95°C for 10 s, 57°C for 30 s, and 68°C for 30 s. Cycle threshold values of each gene were compared to the reference gene (*Actin 1*) and the relative expression levels were determined. Wildtype green plant was used as a control and its expression was compared with the mutants to check for upregulation or downregulation. The amplification specificity was checked by melting curve analysis of the PCR products performed by ramping the temperature to 95°C for 10 s and back to 65°C for 5 s followed by incremental increases of 0.5°C/cycle up to 95°C.

Publicly available RNAseq dataset for soybean genes was used to compare expression of *Glyma.02G233700* and *Glyma.03G230300* during different developmental stages (http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Gmax) [31].

2.11. Statement of data and seed availability

Seed of the yellow mutants were submitted to U.S. National Plant Germplasm System (Soybean) and are available under genetic type collection numbers T378H (Lethal-yellow), T379 (Viable-yellow KE120-1), T380 (Viable-yellow KE120-2) and T381 (Viable-yellow KE120-3). Supplementary Fig. S1 in the online version at DOI: [10.1016/j.plantsci.2016.07.006](https://doi.org/10.1016/j.plantsci.2016.07.006) contains light microscope images of leaf cross sections. Supplementary Fig. S2 in the online version at DOI: [10.1016/j.plantsci.2016.07.006](https://doi.org/10.1016/j.plantsci.2016.07.006) shows graphical representation of *Glyma.02G233700*. Supplementary Fig. S3 in the online version at DOI: [10.1016/j.plantsci.2016.07.006](https://doi.org/10.1016/j.plantsci.2016.07.006) shows sequence comparison of viable-yellow candidate gene *Glyma.02G233700*. Supplementary Fig. S4 in the online version at DOI: [10.1016/j.plantsci.2016.07.006](https://doi.org/10.1016/j.plantsci.2016.07.006) shows sequence comparison of lethal-yellow candidate genes *Glyma.03G230000*

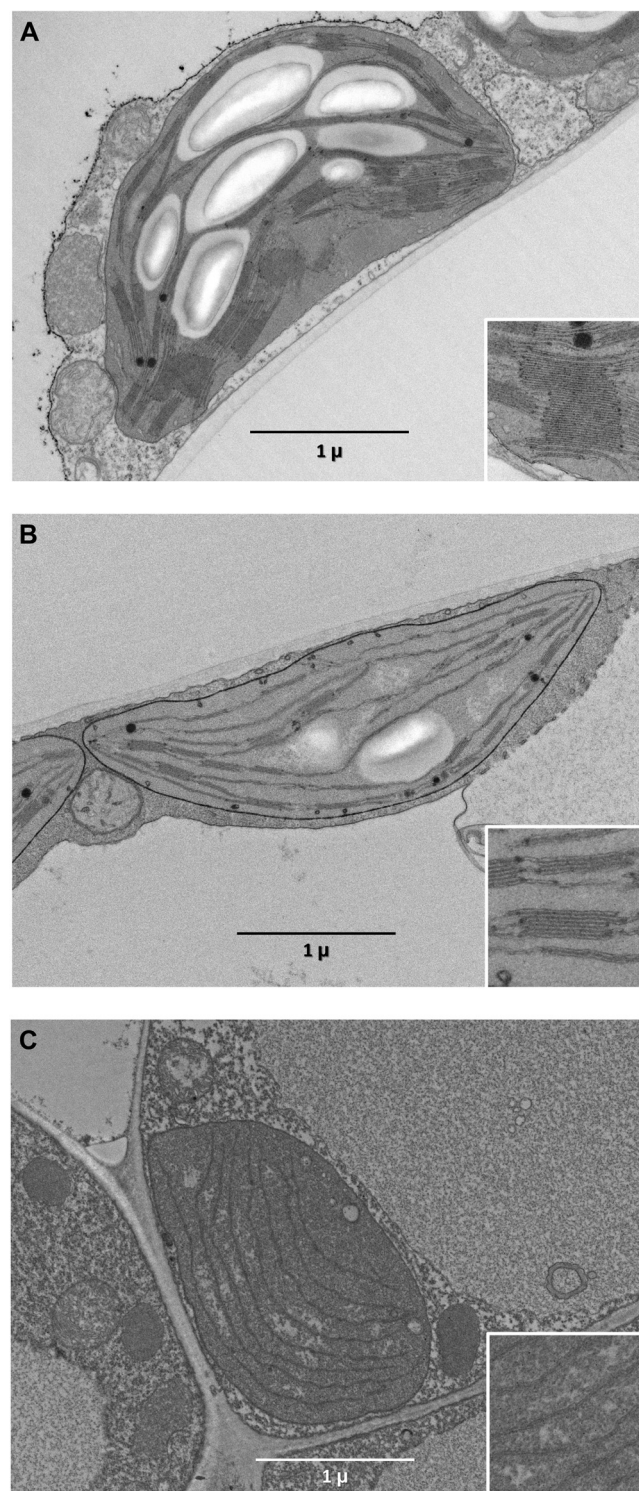


Fig. 3. Electron micrographs of chloroplasts of wildtype green, viable- and lethal-yellow plants. A) Wildtype green (*YvYv YlYl*). Palisade mesophyll chloroplast, showing numerous, well-developed grana with ordered, stacked thylakoids, dense stroma, a few prominent pigment granules between lamellae and large starch grains. Inset: typical granum of 14 thylakoids. B) Viable-yellow mutant (*yvYv YlYl*). Palisade mesophyll chloroplast, showing long lamellae with recognizable but less well-developed grana containing fewer thylakoids per granum and fewer starch grains than wildtype green. Nucleoids and pigment granules can be seen in the less-dense stroma. Inset: five thylakoids in a granum, the maximum seen in viable-yellow. C, Lethal-yellow mutant (*yvYv ylyl*). Leaf palisade mesophyll cell showing small proplastid-like organelles with only a few lamellae, no thylakoids or grana and no starch grains. Note unassociated lamellae and nucleoids (clear areas). Calibration = 1 μm.

Table 1
Number of green and lethal-yellow F₂ and F_{2:3} soybean plants from the cross-pollination between Manchou and heterozygous lethal-yellow plant (IAR2001BSR) based on single-gene hypothesis.

Population	No. F ₂ plants				No. F _{2:3} families				No. plants in segregating F _{2:3} families			
	Green	Lethal-Yellow	χ^2 (3:1)	P	All Green	Segregating	χ^2 (1:2)	P	Green	Lethal-Yellow	χ^2 (3:1)	P
'Manchu' × Lethal-yellow (T378H)	133	8	28.09	<0.01	37	73	0.004	0.84	1982	569	9.88	<0.01

Chi-square values calculated to test goodness of fit to a 3:1 or 1:2 ratio.
P = probability of a greater value of chi-square.

Table 2
Number of green, viable-yellow and lethal-yellow F₂ plants from the cross-pollination between 'Manchu' and KE120-1, KE120-2, and KE120-3 (viable-yellow mutants).

Cross-combination	No. of F ₂ plants			χ^2 (3:1)	P	χ^2 (9:3:4)	P
	Green	Viable-yellow	Lethal-yellow				
'Manchu' × KE120-1-1 (T379)	27	8	4			4.63	0.1
'Manchu' × KE120-1-2 (T379)	40	8	0	1.77	0.18		
'Manchu' × KE120-2 (T380)	41	9	0	1.31	0.25		
'Manchu' × KE120-3 (T381)	30	7	1			11.09	0.004

'Manchu' was used as female parent. KE120-1 through 3 are the three viable-yellow plants. There were two cross-pollinations with KE120-1.
Chi-square values calculated to test goodness of fit to a 3:1 or 9:3:4 ratio.
P = probability of a greater value of chi-square.

Table 3
Segregation pattern, chi-square, and P value for the population line of soybean viable-yellow.

Cross-combination	No. of F ₂ plants				No. of F _{2:3} families				No. of plants in segregating F _{2:3} families			
	Green	Yellow	χ^2 (3:1)	P	All Green	Segregating	χ^2 (1:2)	P	Green	Yellow	χ^2 (3:1)	P
'Manchu' × viable-yellow (KE120-1-2; T379)	129	38	0.45	1	45	82	0.25	0.6	1129	346	1.87	0.17

Chi-square values calculated to test goodness of fit to a 3:1 or 1:2 ratio.
P = probability of a greater value of chi-square.

and *Glyma.03G230100*. Supplementary Fig. S5 in the online version at DOI: [10.1016/j.plantsci.2016.07.006](https://doi.org/10.1016/j.plantsci.2016.07.006) shows graphical representation of *Glyma.03G230300*. Supplementary Fig. S6 in the online version at DOI: [10.1016/j.plantsci.2016.07.006](https://doi.org/10.1016/j.plantsci.2016.07.006) shows sequence comparison of lethal-yellow candidate gene *Glyma.03G230300*.

3. Results

3.1. Identification and inheritance of the lethal-yellow mutant

In the summer of 2009, 171 entries of IAR2001BSR were evaluated for flower color, plant color, pubescence color and phenotypic variation within plots. During evaluation of early stages of development, one entry, IAR2001BSR entry 243, segregated for the lethal-yellow phenotype (T378H). Mutant plants displayed distinct phenotype with yellow leaves, drastic reduction in growth rate and died within three weeks from germination (Fig. 1). The segregation pattern showed 33 green and 14 lethal-yellow plants, suggesting clear single-gene recessive inheritance for IAR2001BSR entry 243 (χ^2 (3:1), P value 0.45).

Green plants (heterozygous; T378H) from the entry segregating for green and lethal-yellow plants were used as the male parent and crossed with Manchou as the female parent. The F₂ segregation ratios deviated significantly from the expected 3 green: 1 lethal-yellow ratio. Data are shown for one F₂ population from the cross between Manchou and IAR2001BSR (heterozygous lethal-yellow) (Table 1). F_{2:3} progeny-line evaluation showed the expected 1:2 ratio for non-segregating: segregating lethal-yellow (Table 1).

3.2. Identification and inheritance of the viable-yellow mutant

Among F_{2:3} progeny lines of the cross between Manchou and IAR2001BSR (T378H; heterozygous lethal-yellow), one segregated 20 green, 3 viable-yellow, and 9 lethal-yellow plants. The viable-yellow plants depicted pale-green colored leaves with slight reduction in growth rate, but survived until maturity (Fig. 1).

Manchu (female parent) was crossed with identified viable-yellow mutants, KE120-1 (T379), KE120-2 (T380), and KE120-3 (T381). All F₁ plants were green. The F₂ populations segregated into two phenotypic classes (green: viable-yellow: lethal-yellow plants and green: viable-yellow plants) (Table 2). For the genetic and molecular analyses of the viable-yellow mutant, 167 F₂ individuals from the cross between Manchou and KE120-1-2 (T379) were analyzed. The segregation patterns in F₂ and F_{2:3} indicated single-gene recessive inheritance (Table 3). Two of the cross combinations {Manchu × KE120-1-1 (T379) and Manchou × KE120-3 (T381)} showed three phenotypic classes (green: viable-yellow: lethal-yellow) in F₂ that suggested epistatic interaction involving two genes (Table 2).

3.3. Physiological analyses of mutant phenotypes

The total chlorophyll (a+b) content on the first unifoliate leaves of three week old plants showed substantial differences between the three lines. Green, viable- and lethal-yellow plants showed chlorophyll content of 338.50 $\mu\text{mol}/\text{m}^2$, 125.74 $\mu\text{mol}/\text{m}^2$ and 24.84 $\mu\text{mol}/\text{m}^2$, respectively, representing a reduction of chlorophylls compared to green, by 62.88% in viable-yellow and 92.66% in the lethal-yellow mutant (Fig. 2A). We did not find any significant differences in chlorophyll a:b ratio between the different phenotypes (data not shown).

We measured maximum photochemical conversion efficiency, defined as F_v/F_m in the dark acclimated state, to assess whether these lines were susceptible to photoinhibition or other malfunctions of thylakoid processes. Wildtype F_v/F_m values above 0.76 were consistent with healthy leaves. The viable-yellow mutants exhibited depressed F_v/F_m values below 0.65, which indicated photosystem II damage resulting from over excitation of electron transport during growth or structural deficiencies (Fig. 2B). Extremely low F_v/F_m values in the lethal-yellow phenotype suggest that there is little to no electron transport activity present in these samples (Fig. 2B).

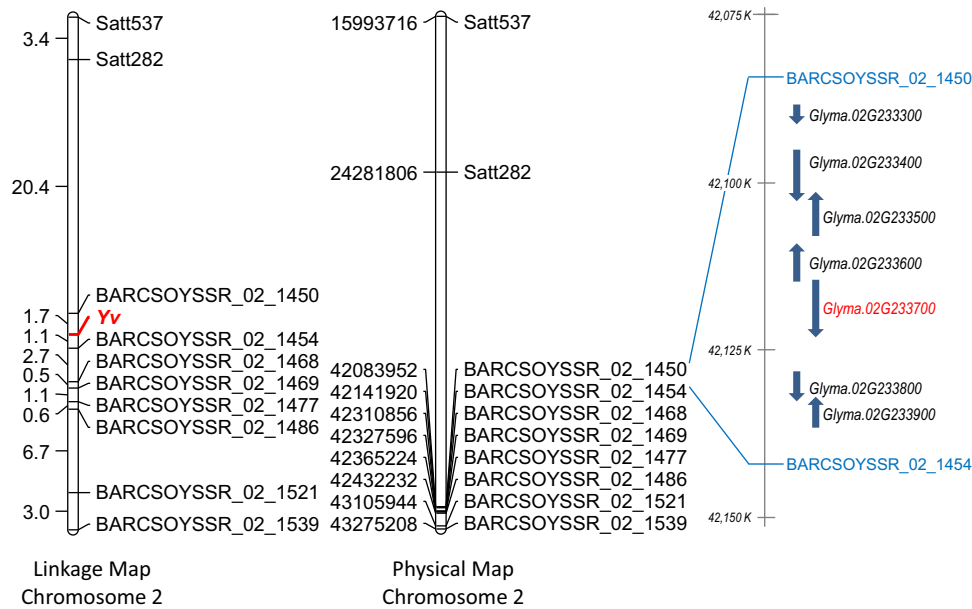


Fig. 4. Genetic linkage map and sequence-based physical map of soybean chromosome 2 showing locations of SSR markers close to the viable-yellow (*Yv*) gene. Genetic distances are shown in centiMorgans (cM) and physical distances are shown in base pairs (bp).

We used leaf spectral reflectance as a rapid screening protocol to compare mutant and wildtype lines. Photochemical reflectance index (PRI) is a wavelength ratio calculated from spectral reflectance that correlates with the epoxidation state of xanthophyll cycle pigments and photosynthetic light use efficiency [32]. Comparisons of PRI at a single time point are significantly correlated with the carotenoid/chlorophyll ratios between soybean lines, with positive numbers in wildtype lines indicating the ratio $> 0.030 \text{ mol mol}^{-1}$ of healthy leaves (Fig. 2C). Values near zero (-0.002) were observed for the viable-yellow mutant. Values below -0.060 in the lethal-yellow lines are consistent with the bright yellow coloration and nonfunctional electron transport of these leaves (Fig. 2C).

3.4. Cytological analysis of the chloroplasts

Light and transmission electron microscopic examinations revealed that the chloroplasts of both the viable- and lethal-yellow plants were clearly abnormal compared to the green plants. Green plants displayed palisade mesophyll chloroplasts with a well-defined thylakoid system with long, parallel lamellae, grana commonly with 10–14 thylakoids, dense stroma and abundant starch grains (Fig. 3A). Chloroplasts of the viable-yellow mutant, however, were thinner at mid-girth than green chloroplasts, had at most 4–5 stacked thylakoids per granum, a less dense stroma and fewer starch grains (Fig. 3B). The lethal-yellow plants exhibited poorly differentiated palisade mesophyll cells containing what appeared to be early stages of developing chloroplasts. Lethal-yellow plastids had round and pyriform morphologies about $1.5\text{--}2 \mu\text{m}$ mid-plastid diameter and a dense stroma. Pyriform plastids contained 6–10 mostly-parallel lamellae (Fig. 3C), whereas the smaller, round organelles contained 2–4 concentric lamellae.

When chloroplasts (green or viable-yellow) or plastids (lethal-yellow) were counted in plastic sections with light microscopy, the numbers per area of palisade mesophyll layer were comparable for green and lethal-yellow (Supplementary Fig. S1 in the online version at DOI: [10.1016/j.plantsci.2016.07.006](https://doi.org/10.1016/j.plantsci.2016.07.006)). There were significantly fewer chloroplasts in the viable-yellow leaf. The overall morphology of the palisade mesophyll cells of the viable-yellow mutant leaves had the same columnar morphology of those in the

green plant, whereas the palisade mesophyll cells in the lethal-yellow mutant were smaller or distorted, indistinguishable in section.

3.5. Genetic mapping of the viable-yellow gene

To determine the genetic location of the viable-yellow gene, the 800 SSR markers from all 20 soybean molecular linkage groups (MLG) were tested on the green and the viable-yellow bulks. BARCSOYSSR.02.1477 showed polymorphism between the bulks, which suggested that the viable-yellow mutant was located on chromosome 2 (MLG D1b). Markers in the vicinity of BARCSOYSSR.02.1477 were analyzed for polymorphism between the parents and nine additional markers (Satt537, Satt282, BARCSOYSSR.02.1450, BARCSOYSSR.02.1454, BARCSOYSSR.02.1468, BARCSOYSSR.02.1469, BARCSOYSSR.02.1486, BARCSOYSSR.02.1521, and BARCSOYSSR.02.1539) showed polymorphism. These 10 polymorphic markers were tested on the F_2 population to develop a genetic linkage map (Fig. 4). The viable-yellow gene was flanked by BARCSOYSSR.02.1450 and BARCSOYSSR.02.1454 at a distance of 1.7 cM and 1.1 cM, respectively (Fig. 4).

3.6. Isolation of the viable-yellow gene

Comparison of the genetic linkage map and sequence-based physical map revealed that the viable-yellow region between BARCSOYSSR.02.1450 and BARCSOYSSR.02.1454 markers was $\sim 58 \text{ kb}$ and there were 7 predicted genes in this region (Fig. 4; Table 4). The analysis of molecular and cellular functions of these predicted genes resulted in the identification of *Glyma.02G233700* as a putative candidate gene due to its possible involvement in photosynthesis (Table 4). The *Glyma.02G233700* protein shows homology to a translocon protein in the inner envelope membrane of chloroplasts (Tic110) in pea and Arabidopsis [33,34]. To confirm if *Glyma.02G233700* is the viable-yellow candidate gene we sequenced both the viable-yellow plant and the wildtype green plant. *Glyma.02G233700* is a 7280 bp gene that contains 15 exons (Supplementary Fig. S2 in the online version at DOI: [10.1016/j.plantsci.2016.07.006](https://doi.org/10.1016/j.plantsci.2016.07.006)). The 3364 nucleotide transcript codes for a

Table 4

Predicted genes present within the viable-yellow genomic region.

Gene	Start Position (bp)	End Position (bp)	Predicted Protein/Function
<i>Glyma.02G233300</i>	42088692	42090406	No functional annotation
<i>Glyma.02G233400</i>	42095461	42101804	No functional annotation
<i>Glyma.02G233500</i>	42102582	42107758	tRNA Pseudouridine synthase/ The intramolecular conversion of uridine to pseudouridine within an RNA molecule
<i>Glyma.02G233600</i>	42110262	42114645	tRNA Pseudouridine synthase/ The intramolecular conversion of uridine to pseudouridine within an RNA molecule
<i>Glyma.02G233700</i>	42114851	42122130	Translocon at the inner envelope membrane of chloroplasts (TIC 110)
<i>Glyma.02G233800</i>	42128487	42131570	Peroxidase/Peroxidase activity
<i>Glyma.02G233900</i>	42133240	42136315	Peroxidase/Peroxidase activity

Glyma.02G233700

Green	MNPSTLTTPSHTRPPLLSPFHTRRRRFKVSLPRCSSSSAASSPPPPPPPPPPQRPCKDLK	60
<i>Yv_mutant</i>	MNPSTLTTPSHTRPPLLSPFHTRRRRFKVSLPRCSSSSAASSPPPPPPPPPPQRPCKDLK	60

Green	GIDVLVDKLSPPARLATS AVIVAGAAAAGYGLGSRFGGSRYAALGGAVALGAAGGAAAYA	120
<i>Yv_mutant</i>	GIDVLVDKLSPPARLATS AVIVAGAAAAGYGLGSRFGGSRYAALGGAVALGA PAVLRRTL	120

Green	LNAAAPQVAAVNLHNYVAAFDDPSKLKKEEIEAIAISKYGVSKQDEAFKTEICHIYSEFVS	180
<i>Yv_mutant</i>	-----	120
Green	SVLPPGGEELKGDEVDRIVSFKNLSGIDDPDAAAMHMEIGRKFFRQRLEVGDADADVEQR	240
<i>Yv_mutant</i>	-----	120
Green	RAFQKLIYVSNLVFGDASSFLLPWKRVPKVTDSQIEVAVRDNAQRLFASKLKSVMGRDIDA	300
<i>Yv_mutant</i>	-----	120
Green	EKLVALRKEQQLCRLSDELAENLFRDHTRKLVEENISEANRILKSRTKAVPGATQAI AEL	360
<i>Yv_mutant</i>	-----	120
Green	DKVLA FNLLISFKNHPDVRFARGVGPI SLVGGEYDGDRIEDLKLLYRAYVSDALSGG	420
<i>Yv_mutant</i>	-----	120
Green	RMEDDKLAALNQLRNIFGLGKREAE AISLDVTSKVYRKRLAQAAADGELEMADSKAAFLQ	480
<i>Yv_mutant</i>	-----	120
Green	NLCDELHFD PQKASELHEEIYRQKLQRCVADGELNEEDVAALLRMRVMLCIPQQIVEAAH	540
<i>Yv_mutant</i>	-----	120
Green	SDICGSLFEKVVEKAIASGVGDYDAEIQKSVRKAAGHLRLTREVAMSIASKAVRKIFINY	600
<i>Yv_mutant</i>	-----	120
Green	IKRARAAGNRTESAKELKKMIAFNLT LVVTNLVEDIKGESTDISSEEPVKEDITQT DDEEW	660
<i>Yv_mutant</i>	-----	120
Green	ESLQTLKKIRPNKELTEKLGKPGQTEITLKDDLPERDRTDLYKTYLLYCLTGEVTRVPFG	720
<i>Yv_mutant</i>	-----	120
Green	AQITTKKDDSEYLLNQLGGIGLSSQEI VEVHRLAEQAFRQQA EVILADGQLTKARVE	780
<i>Yv_mutant</i>	-----	120
Green	QLNNLQKQVGLPQEYAKI IKSITTTKMAAAIETAVTQGRNLNMKQIRELKEADVLD SMV	840
<i>Yv_mutant</i>	-----	120
Green	SENLR ETLFKKTVD DIFSSGTGEFDT EEVYEKIPSDLNINKEKARGVVHELAKGRLSNSL	900
<i>Yv_mutant</i>	-----	120
Green	IQAVSLLRQRNQGVVSS LNDLLACDKAVPSQPVSWEVPEELSDLYTIYLSNPTPENLS	960
<i>Yv_mutant</i>	-----	120
Green	RLQYLLGINDSTAAALREIGDRLLNTTAE EEFVVF	995
<i>Yv_mutant</i>	-----	120

Fig. 5. The amino acid sequence comparison of Glyma.02G233700 in the wildtype green plant and the viable-yellow mutant plant. The wildtype gene contains 995 amino acids, but the mutant only contains 120 amino acids. The difference is due to a deletion of a base pair in the first exon of the gene resulting in an early stop codon. Green, wildtype green plant; *Yv_mutant*, viable-yellow mutant plant.

995 amino acid polypeptide. A single base pair deletion was identified in the first exon of *Glyma.02G233700* in the viable-yellow plant, but not in wildtype green plant (Supplementary Fig. S3 in the online version at DOI: [10.1016/j.plantsci.2016.07.006](https://doi.org/10.1016/j.plantsci.2016.07.006)). The deletion of guanine at the 465th position caused a frame shift and generated a premature stop codon that resulted in a truncated protein (Fig. 5). We named the *Glyma.02G233700* gene as *GmTic110*. These results support *GmTic110* as the potential candidate gene for the viable-yellow mutation in soybean.

3.7. Genetic mapping of the lethal-yellow gene

To determine the genetic location of the lethal-yellow gene, 800 SSR markers covering all 20 soybean chromosomes were used on the green and the lethal-yellow bulks. Satt022 showed polymorphism between the bulks, which suggested that the lethal-yellow mutant gene was located on chromosome 3 (MLGN). Analysis of markers that were close to Satt022 showed that 11 were polymorphic between the parents. The polymorphic markers

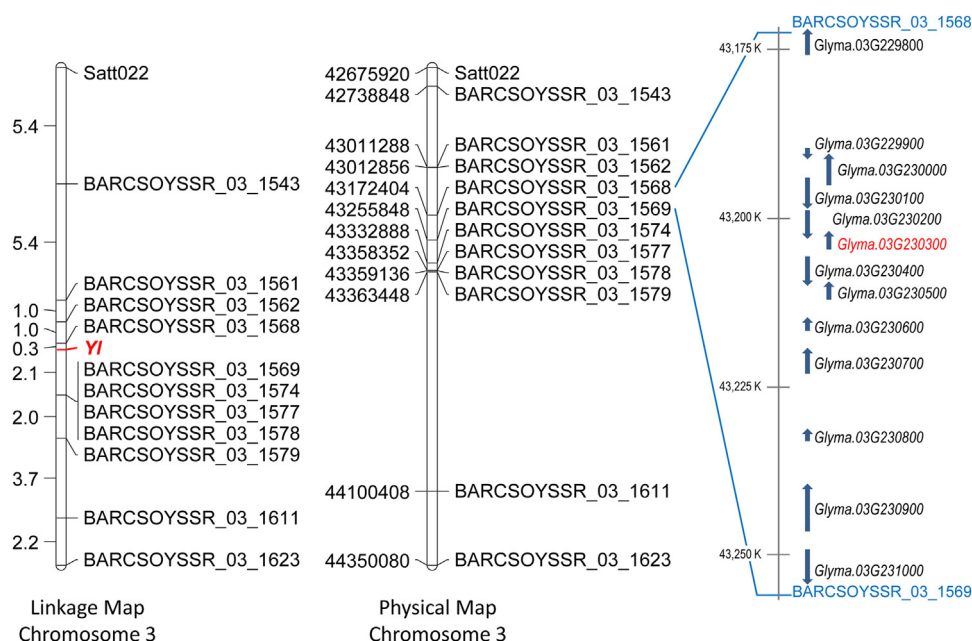


Fig. 6. Genetic linkage map and sequence-based physical map of soybean chromosome 3 showing locations of SSR markers close to the lethal-yellow (*Yl*) gene. Genetic distances are shown in centiMorgans (cM) and physical distances are shown in base pairs (bp).

were BARCSOYSSR.03.1543, BARCSOYSSR.03.1561, BARCSOYSSR.03.1562, BARCSOYSSR.03.1568, BARCSOYSSR.03.1569, BARCSOYSSR.03.1574, BARCSOYSSR.03.1577, BARCSOYSSR.03.1578, BARCSOYSSR.03.1579, BARCSOYSSR.03.1611, and BARCSOYSSR.03.1623. Polymorphic markers were tested on the F_2 generation. Analysis of the marker data showed that the lethal-yellow gene was flanked by BARCSOYSSR.03.1568 and BARCSOYSSR.03.1569 at a distance of 0.3 cM and 2.1 cM, respectively (Fig. 6).

3.8. Isolation of the lethal-yellow gene

SSR markers of the lethal-yellow region were physically mapped using the soybean genome sequence (<http://www.phytozome.net/>) [35]. The lethal-yellow region between the BARCSOYSSR.03.1568 and BARCSOYSSR.03.1569 markers was ~83 kb (Fig. 6). Using this information, we located 13 predicted genes in this region (Fig. 6; Table 5). Of these, *Glyma.03G230000*, *Glyma.03G230100*, and *Glyma.03G230300* were annotated to code for proteins potentially involved in photosynthesis (Table 5). Sequence comparisons of the *Glyma.03G230000* and *Glyma.03G230100* genes showed no differences between the green and lethal-yellow individuals in the exons (Supplementary Fig. S4 in the online version at DOI: [10.1016/j.plantsci.2016.07.006](https://doi.org/10.1016/j.plantsci.2016.07.006)). Sequencing of *Glyma.03G230300* revealed that it is a small gene with a genomic sequence of 1895 bp that encodes a 277 amino acid protein (Supplementary Fig. S5 in the online version at DOI: [10.1016/j.plantsci.2016.07.006](https://doi.org/10.1016/j.plantsci.2016.07.006)). Sequence comparisons showed a single base insertion in the second exon of the gene in the lethal-yellow individuals compared to the green individuals (Supplementary Fig. S6 in the online version at DOI: [10.1016/j.plantsci.2016.07.006](https://doi.org/10.1016/j.plantsci.2016.07.006)). As a result of this insertion, there was a frame shift that caused an early stop codon in the gene, resulting into a truncated protein lacking 144 amino acids at the carboxyl end (Fig. 7). *Glyma.03G230300* was annotated to be the PsbP protein that is critical for the regulation and stabilization of Photosystem II and plays an important role in Ca^{2+} and Cl^- retention [36,37]. In soybean, there was no other protein that showed such high homology to *Glyma.03G230300*. The next closest homolog, *Glyma.19G227400*, displayed 57.8% identity. We named the *Glyma.03G230300* gene as *GmPsbP*.

3.9. Expression analyses of *GmTic110* and *GmPsbP* in the wildtype (green) and the mutants

Quantitative RT-PCR analyses were conducted for *GmTic110* and *GmPsbP* on RNA isolated from leaves of the green, viable-yellow and lethal-yellow plants. *GmTic110* showed high expression levels in the green and lethal-yellow plants and minimal expression in the viable-yellow mutant (Fig. 8A). On the other hand, *GmPsbP* showed high expression in green and viable-yellow, but only nominal expression in the lethal-yellow mutant (Fig. 8B). These analyses confirmed that the frame shift mutations in *GmTic110* and *GmPsbP* resulted in the viable-yellow and lethal-yellow phenotypes in the mutants, respectively.

3.10. Expression of *GmTic110* and *GmPsbP* in different tissues

RNA-seq data comparison of *GmTic110* and *GmPsbP* in different soybean tissues revealed that both of the genes were expressed in most tissues (Fig. 9). For *GmPsbP* minimum expression was present in flowers; for *GmTic110*, however, lowest expression was observed in roots. As expected for the genes involved in photosynthesis, both of the genes showed the highest level of expression in leaves (Fig. 9). Co-expression of *GmPsbP* and *GmTic110* in different soybean tissues showed a correlation of 0.79, suggesting involvement of both genes in the same metabolic pathway (Fig. 9).

4. Discussion

4.1. The viable- and lethal-yellow mutants show monogenic inheritance

During field evaluation at an early stage of development, we found mutant lines segregating for lethal-yellow phenotype in IAR2001BSR entry 243. In its original background of IAR2001BSR, the lethal-yellow was inherited as a single-recessive gene. In the F_2 generation, from the cross-pollination between Manchou and IAR2001BSR, the expected ratio deviated significantly from 3 green plants: 1 lethal-yellow plant. However, $F_{2:3}$ segregations confirmed the expected single-recessive gene inheritance of the mutant phe-

Table 5

Predicted genes present within the lethal-yellow genomic region. The genes predicted to be involved in photosynthesis are present in bold font.

Gene	Start Position (bp)	End Position (bp)	Predicted Protein/Function
<i>Glyma.03G229800</i>	43172457	43175687	Glycosyl transferase/Transferring glycosyl groups
<i>Glyma.03G229900</i>	43189661	43190489	No functional annotation
<i>Glyma.03G230000</i>	43190797	43194898	NAD dependent epimerase/Dehydratase
<i>Glyma.03G230100</i>	43193894	43197583	Uncharacterized membrane protein
<i>Glyma.03G230200</i>	43198874	43202160	Low density lipoprotein receptor related
<i>Glyma.03G230300</i>	43202604	43204498	Photosystem II reaction center PsbP family protein/Photosynthesis
<i>Glyma.03G230400</i>	43205651	43209277	Plant neutral invertase
<i>Glyma.03G230500</i>	43209994	43211988	RNA polymerase associated protein RTF1 homolog/(involved in regulation of TATA box-binding protein)
<i>Glyma.03G230600</i>	43215435	43216516	Protein of unknown function
<i>Glyma.03G230700</i>	43219838	43222853	Importin alpha/Directed movement of a protein from the cytoplasm to the nucleus
<i>Glyma.03G230800</i>	43231839	43232824	Transcription factor with the basic helix-loop-helix (bHLH) domain
<i>Glyma.03G230900</i>	43239772	43246618	Haloacid dehalogenase-like hydrolase
<i>Glyma.03G231000</i>	43249214	43253614	Galactose oxidase

Glyma.03G230300

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Green      MANAIIMPGLLRPTQTTPFRLPNHHSFHSPRCSLSLTPQSAKAFVPRRKALSLILS 60
Yl_mutant  MANAIIMPGLLRPTQTTPFRLPNHHSFHSPRCSLSLTPQSAKAFVPRRKALSLILS 60
          *****

Green      SYFLSEAGLALAQQSPVFREYIDAFDGYSFQYPGSWIQVRGAGADIFFRDPFVLDENLSL 120
Yl_mutant  SYFLSEAGLALAQQSPVFREYIDAFDGYSFQYPGSWIQVRGAGADIFFRDPFVLDENLSL 120
          *****

Green      EISSPSSSQYKSVEDLGPPQEAGKVLKQYLTEFMSTRLGVRRESNILSTSSRVADDGRL 180
Yl_mutant  EISSPSSFPVQEC----- 133
          *****

Green      YYQVEVNIKSYANNNELAVMPEARVVRLEWDRRYLSVLGVENNQLYELRLQVPENVFSEE 240
Yl_mutant  -----

Green      ENDLRRIMDSFRVNKIAA 258
Yl_mutant  -----

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Fig. 7. The amino acid sequence comparison of Glyma.03G230300 in the wildtype green plant and the lethal-yellow mutant plant. The wildtype gene contains 258 amino acids, but the mutant only contains 133 amino acids. The difference is due to an insertion in the second exon of the gene resulting in an early stop codon. Green, wildtype green plant; Yl_mutant, lethal-yellow mutant plant.

notype. Deviation in the F₂ population might have occurred due to lower percent germination of the lethal-yellow genotypes. Fewer than expected lethal-yellow plants would give a distorted ratio.

Viable-yellow plants observed in the F_{2:3} progeny row from the cross between Manchu and IAR2001BSR were crossed with Manchu as female parents. No variegated (chimeric) plants were observed in the F₁ or F₂ generations. All F₁ plants had green foliage, which suggested nuclear gene inheritance. F₂ populations gave two phenotypic classes, green: viable-yellow: lethal-yellow plants and green: viable-yellow plants (Table 2). At least two cross combinations {Manchu × KE120-1-2 (T379) and Manchu × KE120-2 (T380)} showed single-gene inheritance (Table 2). These results were validated when remnant seeds of the selected F₂ population (Manchu × KE120-1-2) were planted in the field for genetic and molecular analyses (Table 3). The F_{2:3} family segregation was also consistent with single-gene inheritance (Table 3).

4.2. The viable- and lethal-yellow genes are epistatic to each other

Two of the cross combinations {Manchu × KE120-1-1 (T379) and Manchu × KE120-3 (T381)} showed three phenotypic classes (green: viable-yellow: lethal-yellow) in F₂ that suggested epistatic

interaction involving two genes (Table 2). Based on the segregation patterns of the viable- and lethal-yellow phenotypes, a relationship between two genes (Yl and Yv) was determined. A homozygous recessive mutation in one gene (ylyl) resulted in a lethal-yellow phenotype irrespective of the second gene (Fig. 10). A homozygous recessive mutation in the second gene (yyvv) in the presence of normal Yl resulted in a viable-yellow phenotype (Fig. 10). Based on this model, a homozygous recessive mutation in the Yl gene (YvYv ylyl) in the IAR2001BSR line resulted in the lethal-yellow phenotype. When lethal-yellow (in heterozygous form, YvYv Ylyl) was crossed with Manchu (YvYv YlYl), it showed monogenic inheritance (Fig. 10; Table 1). In one of the F₂ plants (YvYv Ylyl) a second mutation occurred leading to a heterozygous genotype for both genes (Yvyv Ylyl). The F_{2:3} family from this plant segregated 20 green (YvYl): 3 viable-yellow (yyvvYl): 9 lethal-yellow (YvYl or yyvylyl) (Fig. 10). When three viable-yellow plants {KE120-1 (yyvv Ylyl), KE120-2 (yyvv YlYl) and KE120-3 (yyvv Ylyl)} were crossed with Manchu (YvYv YlYl), 3 green: 1 viable-yellow or 9 green: 3 viable-yellow: 4 lethal-yellow ratios were observed (Fig. 10; Table 2). Low P-values for the chi-square test for 9:3:4 ratios can be explained by poor germination of seeds from lethal-yellow plants (Table 2). For all the crosses shown in Table 2, 50 F₂ seeds were germinated. For the two crosses that segregated into green, viable-yellow and

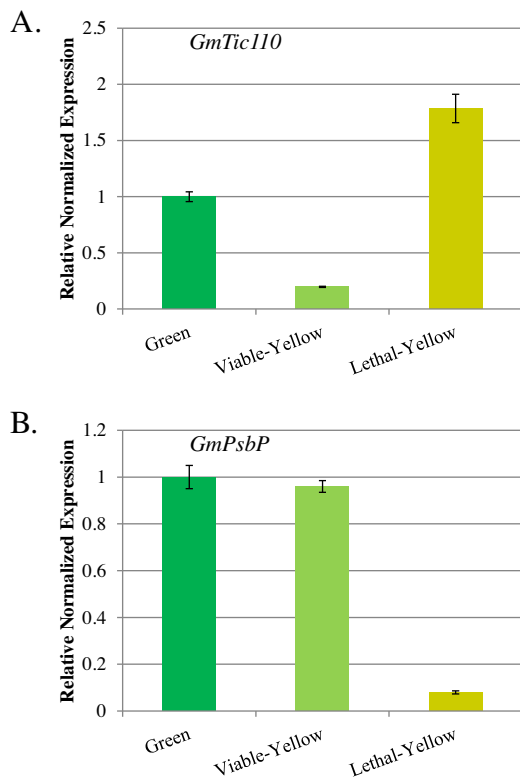


Fig. 8. Expression analyses of *GmTic110* and *GmPsbP* in wildtype green, viable- and lethal-yellow plants. A, Quantitative RT-PCR showing minimal expression of *GmTic110* in the viable-yellow mutant. B, Quantitative RT-PCR showing nominal expression of *GmPsbP* in the lethal-yellow mutant. Error bars represent standard error.

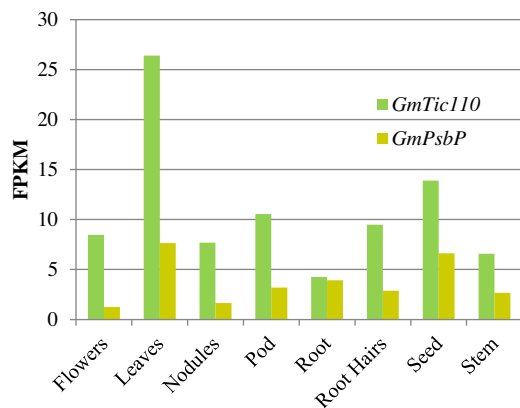


Fig. 9. RNAseq based expression comparison of *GmTic110* and *GmPsbP* in different plant tissues (<http://phytozome.jgi.doe.gov/pz/portal.html#info?alias=Org.Gmax>). Both the genes show highest expression in leaves. FPKM, Fragments per kilobase of transcript per million mapped reads. Error bars represent standard error.

lethal-yellow, germination percentage was significantly lower as compared to the other two crosses that segregated for green and viable-yellow (Table 2). This further supports the role of the low germination rates of lethal-yellow plants in distorted segregation ratios.

The genetic mechanism leading to the appearance of these chlorophyll-deficient mutants is unknown. Our results, however, showed that two mutations for different traits (viable- and lethal-yellow) may indicate involvement of two genes in the same chlorophyll biosynthesis pathway. A homozygous recessive mutation in the *Yl* gene possibly results in plants that are yellow in color, but are unable to survive. A homozygous recessive mutation in the

Yv gene may result in plants that are yellow, but are viable. Both normal genes will result in wildtype green plants.

4.3. The viable-yellow mutant is defective in the *GmTic110* protein

Fine mapping of the viable-yellow mutant led to identification of *Glyma.02G233700* as a candidate gene (Fig. 4). Sequence comparison of *Glyma.02G233700* between wildtype green and the viable-yellow mutant revealed a frame shift in the mutant due to a single base deletion (Fig. 5). *Glyma.02G233700* is annotated to encode for *GmTic110* protein. Expression analysis using qRT-PCR indicated drastically-reduced expression of *GmTic110* in the viable-yellow mutant compared to the wildtype, confirming its involvement in the viable-yellow phenotype (Fig. 8A). Tic complexes are implicated in several import processes, including the import of thousands of nucleus-encoded proteins synthesized in the cytosol [38]. Tic110 forms a prominent protein channel and plays a critical role in plastid biogenesis and plant viability [34,39]. Heterozygous mutants for *tic110* in Arabidopsis exhibited pale phenotype, however, homozygous mutants were lethal [34]. In soybean, viable-yellow mutants were viable in a homozygous recessive condition, perhaps due to the paleopolyploid nature of the soybean genome. Soybean has another gene (*Glyma.14G201500*) that is 96.4% identical to *Glyma.02G233700*.

4.4. The lethal-yellow phenotype is due to a mutation in PSII extrinsic protein, *GmPsbP*

We developed a genetic linkage map and compared that with the sequence based physical map to identify three candidate genes for the lethal-yellow mutant (Fig. 6). Sequencing results displayed a single base insertion in the mutant resulting in early stop codon in *Glyma.03G230300* (Fig. 7). *Glyma.03G230300* that encodes for *GmPsbP*, showed more than 12-fold lower expression in the lethal-yellow mutant compared to the wildtype green plant, endorsing implication of *GmPsbP* in the lethal-yellow phenotype (Fig. 8B). PsbP is one of three extrinsic proteins involved in oxygen evolution in photosystem II (PSII) and is required for photoautotrophy and maintains calcium and chloride at the photoreactive site during water oxidation [36,40,41]. In tobacco, RNA interference for PsbP resulted in pale-green phenotype with reduced growth rate and decreased chlorophyll fluorescence [37]. *psbP* mutants in *Chlamydomonas reinhardtii* showed reduced oxygen-evolving activity [42]. Higher expression of *GmTic110* in the lethal-yellow mutant as compared to the wildtype may possibly be a result of stress generated on the lethal-yellow mutant due to the lack of *GmPsbP* (Fig. 8).

4.5. The yellow phenotype results from plants response to photooxidative stress

Energy absorbed by the leaves of a plant excites the electrons in the chlorophyll and is used in PSII to convert light to chemical reducing power for photosynthesis. A plant's ability to use the available light is dependent on the ability of chlorophyll to convert the energy absorbed. Unconverted light energy absorbed by the plant can cause oxidative damage to the thylakoid processes if it is unused by electron sinks. This damage is known as photoinhibition.

The reduced photochemical efficiency and yellow color of the viable-yellow mutant plants (Figs. 2B and 2C) is consistent with plants under photoinhibitory light stress. The retarded chloroplast development resulting from reduced protein import capability due to the mutant *tic110* gene is responsible for slowed metabolism in the chloroplasts, which reduces electron sinks. Wildtype soybean will exhibit the same symptoms under chilling stress [43], which drastically slows carbon metabolism in the chloroplast

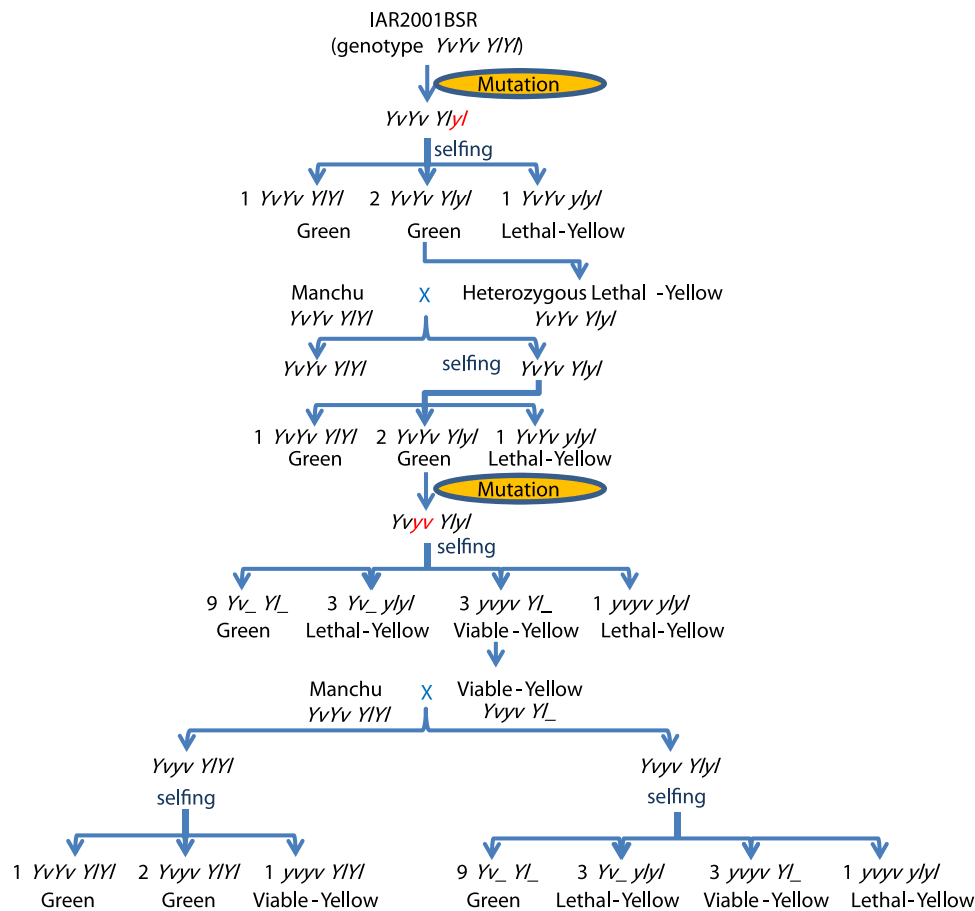


Fig. 10. Inheritance of viable- and lethal-yellow mutant genes in soybean. Genes show recessive epistatic interaction (9 green: 3 viable-yellow: 4 lethal-yellow).

thereby reducing demand for reductant generated by thylakoid processes [44]. We observed that the yellow phenotype is indistinguishable from the wildtype plants when grown in the University of Wisconsin-Stevens Point greenhouse during typically cloudy winter conditions. In the summer field conditions, however, the phenotypic differences were pronounced, supporting the conclusion that light stress is the primary mechanism causing the yellowing in these plants.

With an incomplete electron transport chain of the lethal-yellow mutant, what little pigment resides in the thylakoids is likely to be detrimental to the leaf. Nearly all absorbed light energy is likely to generate reactive oxygen species, thereby triggering a photooxidative stress response. The accumulation of yellow xanthophyll pigments and necrotic lesions observed on almost all lethal mutant plant leaves is consistent with this mechanism (Fig. 1).

4.6. TEM data supports involvement of the viable- and lethal-yellow genes in photosynthesis

The electron microscopic examination of the viable- and lethal-yellow mutants shows the strong influences of these two genes on normal chloroplast development. Consistent with a previous report, in the lethal-yellow mutant, chloroplast maturation in the palisade mesophyll layer has been completely arrested [45], whereas chloroplast development is partially rescued in viable-yellow mutants. The viable-yellow mutants show thinner chloroplasts with significantly fewer thylakoids than the wildtype. This observation is consistent with a mutation in *GmTic110* reducing chloroplast protein import, as cytoplasmic proteins comprise more than 70% of those found in thylakoid membranes. The result-

ing reduction in the electron transport would likely contribute to the observed reduction in chloroplast starch accumulation (Fig. 3B).

PSII complexes are found in the stacked thylakoid regions of normal chloroplasts. Lethal-yellow mutants, lacking the PsbP protein of PSII, are most likely unable to assemble functional PSII complexes. Since PSII resides in the stacked thylakoid regions of wildtype plants, the complete lack of stacked thylakoids is consistent with this inference.

5. Conclusion

We identified two chlorophyll deficient mutants and used them to characterize two important proteins involved in chloroplast development and function. The soybean *tic110* mutant has slowed chloroplast metabolism because of its reduced capacity for protein import. The thinner thylakoid membranes and smaller starch grains support this conclusion. Lowered metabolism also makes these plants more susceptible to photoinhibition and thus the yellow phenotype appears most prominently when plants are grown under intense light conditions. In addition to its utility for studying the inheritance of nuclear-encoded traits, this line could serve as a platform for research into photoinhibitory photooxidative stress on chloroplast function.

The mutant *psbP* gene of the lethal-yellow mutant can assist in studying the structure of PSII and the role that extrinsic proteins play in stabilizing the oxygen evolving complex. We note that *psbP* mutants in *Chlamydomonas reinhardtii* do not produce lethal mutants, so comparisons between the extrinsic proteins in these *psbP* mutants might provide insight into the ultrastructure and function of the oxygen evolving complex.

List of author contributions

D.S., S.G. and R.G.P. conceived the original research plans; D.S., E.S., T.B., S.G. and R.G.P. supervised the experiments; D.S., S.S., E.S., S.G. and R.G.P. designed the experiments; D.S., S.S., T.A., A.N., C.J., S.A., J.B., K.E., and B.D. performed most of the experiments and analyzed the data; and; D.S. wrote the article with contributions from all the authors.

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