Low red/far-red ratio as a signal promotes carbon assimilation of soybean seedlings by increasing the photosynthetic capacity

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Abstract

Background: Shading includes low light intensity and varying quality. However, a low red/far-red (R/Fr) ratio of light is a signal that affects plant growth in intercropping and close- planting systems. Thus, the low R/Fr ratio uncoupling from shading conditions was assessed to identify the effect of light quality on photosynthesis and CO2 assimilation. Soybean plants were grown in a growth chamber with natural solar radiation under four treatments, that is, normal (N, sunlight), N+Fr, Low (L) +Fr, and L light. Results: Results showed that low R/Fr ratio significantly increased the total biomass, leaf area, starch and sucrose contents, chlorophyll content, net photosynthetic rate, and quantum efficiency of the photosystem II compared with normal R/Fr ratio under the same light intensity condition ($P < 0.05$). Proteomic analysis of soybean leaves under different treatments was performed to quantify the changes in photosynthesis and CO2 assimilation in the chloroplast. Among the 7834 proteins quantified, 12 showed a > 1.3-fold change in abundance, of which 1 was related to porphyrin and chlorophyll metabolism, 2 were involved in photosystem I (PS I), 4 were associated with PS II, 3 proteins participated in photosynthetic electron transport, and 2 were involved in starch and sucrose metabolism. The dynamic change in these proteins indicates that photosynthesis and CO2 assimilation were maintained in the L treatment by up-regulating the component protein levels compared with those in N treatment. Although low R/Fr ratio increased the photosynthetic CO2 assimilation parameters, the differences in most protein expression levels in N+Fr and L+Fr treatments compared with those in N treatment were insignificant. Similar trends were found in gene expression through quantitative reverse transcription polymerase chain reaction excluding the gene expression of sucrose synthase possible because light environment is one of the factors affecting carbon assimilation. Conclusions: These results implied that low R/Fr ratio (high Fr light) increased the photosynthetic CO2 assimilation in the same light intensity by improving the photosynthetic efficiency of the photosystems.

Background

Light is an essential factor for crop growth and development in agricultural production [1]. Intra- or interspecies plant mutual shading often affects crop light interception [2], especially in intercropping and close planting system which are important cultivation methods in increasing resource utilization and yield [3]. Plants can perceive shading that enable them to acclimate and adjust their phenotypic and physiological characteristics to compete for limited light resource [4]. Shade often leads to elongation responses in the stem, petioles, and leaves in shade-sensitive plant species [5].

Plant shading reduces the amount of photosynthetically active radiation (PAR) and changes the spectral composition of light [6, 7]. Plants selectively absorb red (R) wavelengths through photosynthetic pigments. The far-red (Fr) spectrum is relatively enriched due to radiation reflected and transmitted by the green leaves of neighboring plants [8]. A resulting decrease in R/Fr ratio in the surrounding environment is observed. The changes in light intensity and quality under shade differ from those under low light condition [9, 10]. Low light conditions achieved using black nylon nets or fabrics do not alter the spectral composition of light, particularly the R/Fr ratio [11, 12].

Phytochromes play a key role in the perception of the R/Fr ratio signal and regulation of the plant photomorphogenesis via gene expression and physiological processes [1]. Phytochromes exist in two forms, that is, R light absorbing Pr and Fr light absorbing Pfr [1, 5]. The equilibrium between these two forms dynamically changes with the change in composition of the light spectrum within the 300–800 nm range [13]. A low R/Fr ratio is an important signal factor in shade avoidance [14]. Responses to low R/Fr ratio include increased stem elongation, decreased leaf area and branching, and changes in chlorophyll (Chl) content [8, 15, 16]. Low R/Fr ratio in normal or low light condition significantly increases the soybean biomass (dry weight) compared with normal R/Fr ratio (approximately 1.2) [6, 9].

A low R/Fr ratio indicates high enrichment of the Fr light spectrum in the plant canopy. Fr light (λ > 700 nm) makes a minimal contribution to photosynthesis according to its poor absorption by plant leaves and the low quantum yield of photosynthesis [17]. However, Zhen and van Iersel [18] reported that Fr light is required for efficient photochemistry and photosynthesis. Similarly, the Fr light of the shade is higher than that of low light, thereby increasing the net photosynthesis rate (P_n) under the same light intensity condition [11], resulting in increased the whole-plant net assimilation [6, 9]. Generally, the photosynthetic efficiency of the long wavelengths can be improved by adding short light wavelength, which is a phenomenon known as the Emerson enhancement effect [18]. The reverse effect, which is the enhancement of the quantum yield of short light wavelength by Fr light (e.g., at low R/Fr ratio) in different light intensity environments, has not received considerable attention. Some studies only indicate that Fr light can increase plant photosynthesis and biomass [6, 18]. Therefore, in uncoupling R/Fr ratio from shade, whether low R/Fr ratio (Fr light enrichment) in different light intensity regulates the photomorphogenic process needs further analysis.

Photosystems I and II (PS I and II, respectively) operate in series to carry out the primary photochemical reactions of photosynthesis [18]. These processes are involved in light absorption and energy and electron transfer, which are carried out by different related proteins [11, 21]. Proteomic analysis has been used to identify changes in plant photosynthetic proteins in salt stress [33], water deficit [22], low phosphate [23], and chlorophyll deficient [24]. We previously investigated the response of soybean photosynthetic proteins to shade condition by using isobaric tagging for the relative and absolute quantification (iTRAQ) approach [11]. However, to our best knowledge, the proteomic analysis of photosynthesis in uncoupling light intensity and R/Fr ratio from shade is important to determine the R/Fr ratio regulating the photosystems related-proteins, which affect photosynthesis and CO_2 assimilation.

Soybean (*Glycine max* (L.) Merr.) is one of the most important cultivated crops of protein and oil worldwide [25, 26], which is often used for rotation or to intercrop with other crops because it can fix atmospheric N [3]. However, soybean often suffer from intra- or interspecies mutual shading [9]. Shade increases the height but decreases the biomass, chlorophyll contents, and photosynthesis of soybean plant [2, 27]. Likewise, we previously reported the response of photosynthetic proteins to shade and low light by using iTRAQ-quantitative proteomic analysis, and found that Fr light enrichment of shade increases P_n by up-regulating the gene expression levels of differential proteins compared with low light [11]. Thus, low R/Fr ratio may be a signal in promoting CO_2 assimilation by increasing the photosynthetic

capacity. Therefore, this work aims to analyze the soybean morphology, carbohydrate, and photosynthesis in response to low R/Fr ratio in different light intensities and reveal the effect of low R/Fr ratio on soybean photosynthesis using the iTRAQ technique in different light intensities.

Results

Morphological characteristics

Low R/Fr ratio directly affected soybean growth phenotype under normal or low light intensity (Fig. 1A). The plant height of soybean in the N+Fr treatment was significantly higher than that in the N treatment under normal light intensity. By contrast, the plant height in the L+Fr treatment was decreased by 20.8% compared with that of the L treatment in 42 days after sowing (Fig. 1B). After 14 days of sowing, the soybean total biomasses in N+Fr and L+Fr treatments were significantly higher than those in N and L treatments, respectively. The total biomass under normal light (N and N+Fr treatments) were higher than that under low light (L and L+Fr treatments). At 42 days after sowing, the maximum and minimum total biomasses were 2.35 and 0.34 g $plant^{-1}$ in the N+Fr and L treatments, respectively. Similar trends on biomass were also found at 14 and 28 days after sowing under different treatments (Fig. 1C). In addition, low R/Fr ratio significantly increased the leaf area per plant at 14 and 28 days after sowing under normal or low light intensity, the change in the trends of leaf area per plant were consistent with the total biomass in different treatments (Fig. 1D).

Here Insert Figure 1

Changes in chloroplast ultrastructure, sucrose and starch content

When growing in different light environments, the changes in chloroplast ultrastructure were different, the chloroplast size in N and N+Fr treatments were larger than those in L and L+Fr treatment (Fig. 2A). Starch grain (SG) size also exhibited a similar trend. The starch contents in N+Fr and L+Fr treatments were significantly higher than those in N and L treatments, respectively. The maximum and minimum starch content were 92.36 mg/g in N+Fr treatment and 69.19 mg/g in L treatment, respectively. The sucrose content of soybean growing in N+Fr treatment was significantly higher than those in other treatments (Fig. 2B).

Here Insert Figure 2

Chlorophyll content, photosynthesis, and quantum yield of PS II

The Chl a, Chl b, and total Chl in normal light condition (N and N+Fr treatments) were significantly lower than those in low light (L and L+Fr treatment) (Fig. 3). The total Chl contents in N+Fr and L+Fr treatments (low R/Fr ratio) were significantly increased by 6.5% and 14.3% compared with those in N and L treatments (normal R/Fr ratio), respectively.

The light response curves of the assimilation rate vs. the photosynthetic photon quanta flux density (PPFD) of four treatments were shown in Fig. 4. The assimilation rates of the four treatments presented significant difference when PPFD was higher than 200 μ mol m^{-2} s^{-1} (Fig. 4A). The maximum values of photosynthetic rate (P_{max}) and the light saturation point (LSP) appeared in N+Fr treatment compared with those in other treatment. P_{max} and LSP decreased by 14.9 and 47.4% under N treatment with respect to the corresponding values under N+Fr treatment. Similarly, P_{max} and LSP decreased by 23.8 and 38.2% under L treatment relative to the L+Fr treatment, respectively. The minimum values of P_{max} and LSP, which appeared under L treatment, were 5.77 μ mol CO_2 m^{-2} s^{-1} in the 318.17 μ mol m^{-2} S^{-1}, respectively (Fig. 4B).

In addition, the quantum yield of PSII is the fraction of light absorbed by leaves for photochemical electron transport. In this study, the quantum yields of PSII under N and N+Fr treatments were significantly higher than that under L and L+Fr treatments. Furthermore, a reduced R/Fr ratio increased the quantum yield of PSII by 15.18% under L+Fr treatment, respectively, with respect to that under L treatment (Fig. 4C).

Here Insert Figure 3

Here Insert Figure 4

Soybean leaf proteomic analysis

The total protein of the soybean leaves was extracted from different treatments, and the protein profiles were explored using the iTRAQ technique. A total of 9890 protein groups were identified, among which 7834 proteins were quantified (Table S1). On the basis of at least > 1.3- of fold change ($P < 0.05$), among the quantified proteins, we found that 15 proteins were up-regulated, and 41 proteins were down-regulated for N+Fr vs. N; 102 proteins were up-regulated, and 548 proteins were down-regulated in L+Fr vs. N, 180 proteins were up-regulated, and 183 proteins were down-regulated in L vs. N (Fig. 5A).

The differentially accumulated proteins were classified into three groups (cellular component, molecular function, and biological process) on the basis of GO enrichment analysis (Fig. 5B). The main biological functional categories represented were metabolic, cellular, and single-organism processes. According to the molecular functional properties, these proteins were mainly classified into catalytic activity, binding, and structural molecule activity. The subcellular location- annotation information of the identified proteins indicated that chloroplast-associated proteins accounted for 32.1%, 39.1%, and 36.9% of the unique proteins in N+Fr vs. N, L+Fr vs. N, and L vs. N, respectively (Fig. 5C).

To visualize the differences in protein abundance among the N+Fr, L+Fr, L, and N treatments, we used KEGG pathways and visualized as a heat map through a two-tailed Fisher's exact test. Thirteen different functional categories were selected for analysis. As illustrated in Fig. 6, among the functional categories, C metabolism and photosynthesis-antenna proteins were related to photosynthetic CO_2 assimilation. The

proteins involved in photosynthesis in N+Fr and L+Fr treatments were down-accumulated compared with those in N treatment, whereas proteins in L treatment were up-accumulated.

Here Insert Figure 5

Here Insert Figure 6

Key protein associated with photosynthesis assimilation of soybean leaves in response to different light conditions

A total of 12 differentially expressed proteins related to photosynthetic CO_2 assimilation were detected by iTRAQ analysis under N+Fr, L+Fr, and L treatments compared with those detected in N treatment. Among these differentially expressed proteins, one protein was related to porphyrin and chlorophyll metabolism, two proteins were involved in PS I, four proteins were associated with PS II, three proteins participated in photosynthetic electron transport, and two proteins were involved in starch and sucrose metabolism (Table 1). The expression levels of nine proteins (i.e., Protochlorophyllide reductase [POR], Photosystem I subunit [PsaD], Chlorophyll a/b binding protein 1 [Lhcb 1], Lhcb 2, Lhcb 4, Lhcb 6, PetE, PetF, and Sus) were up-regulated under L treatment compared with N treatment. However, the expression levels of the two proteins (i.e., POR and Lhcb 1) in N+Fr treatment and two proteins (i.e., PsaH and PetH) in L+Fr treatment were down-regulated compared with those in N treatment.

Here Insert Table 1

qRT-PCR results confirming the differentially expressed proteins

To assess the validity of the iTRAQ data, we randomly selected six gene products, including the POR, PsaD, Lhcb 1, PetE, and Sus gene expressed levels, according to differential protein classification for RT-PCR analysis, (Fig. 7). The qRT-PCR results showed that under L treatment, significant increase in the transcript level was observed for POR, PsaD, Lhcb 1, and PetE compared with N treatment. The POR, PsaD, and PetE expression levels were up-regulated under L+Fr treatment. By contrast, the expressions levels of POR, PsaD, and Lhcb 1 were down-regulated under N+Fr treatment compared with those under N treatment. The change in the Gmss 1 was different from that of POR in N+Fr, L+Fr, and L treatments.

Here Insert Figure 7

Discussion

Light is one of important environmental factors that regulate crop growth and development [11]. Shading often occurs in high density planting and intercropping, which results in the changes in light intensity and quality above the leaf or crop canopy [7]. Light that passes through a canopy is rich in Fr light but poor in R light [29]. Chlorophyll depletes R light, whereas Fr light is predominantly reflected and transmitted by the leaf [30]. Low R/Fr ratio and light quanta are the main light properties in plant shading environment.

Therefore, a normal R/Fr ratio (approximately 1.2-1.3) and low R/Fr ratio (< 0.42) in different light intensities can meet the objectives of this study (Table 2 and Fig. 8).

Plants that can detect a low R/Fr ratio in different light intensities will initiate a series of morphological changes and consequently express shade avoidance characteristics such as increased stem elongation and decreased root biomass [31]. A low R/Fr ratio also significantly increased the stem elongation of soybean under normal light intensity, while opposite trend was found under low light intensity (Fig. 1A and B). Light intensity regulates plant height compared with R/Fr ratio under low light condition [9]. However, a low R/Fr ratio significantly increased the total biomasses and leaf area of soybean under the same light intensity condition (Fig. 1C). Similarly, the whole plant biomass of geranium and snapdragon increases with additional Fr radiation (low R/Fr ratio) [6].

Photosynthesis is the basis for material accumulation, which is dependent on both light quantity and quality [18]. Low R/Fr ratio (high Fr light) improved photosynthesis under normal or low light intensity condition (Fig. 4) [18]. Similar to the reverse of Emerson enhancement effect [32], the photosynthetic efficiency of short wavelengths may be improved by increasing long wavelength. Generally, PSI tends to be under-excited relative to PSII under shorter wavelength light, this limits the overall rate of photochemistry [36, 37]. However, long and short wavelength lights can complement each other, i.e. one light over-excites PSI and the other light over-excites PSII [33]. Thus, the P_{max} values in N+Fr and L+Fr treatments (high enrichment of Fr light) were significantly higher than those in N and L treatments, respectively (Fig. 4B), similar trends were found in the quantum yield of PSII (Fig. 4C). Although low light intensity significantly decreased the Chl content (Chl a and b) compared with normal light condition, a low R/Fr ratio significantly increased the Chl content per unit area under the same light intensity conditions [16]. The change in the trends of leaf area, Chl content, P_n, and quantum yield of PSII was consistent with the total biomass in different treatments (Fig. 1, 3–4), thereby indicating that low R/Fr ratio might improve the photosynthetic capacity of soybean in the same light intensity.

The starch and sucrose contents in leaf reflect the photosynthetic CO_2 assimilation ability. In this study, a low R/Fr ratio (high enrichment of Fr light) under N+Fr and L+Fr treatments can significantly increase the starch and sucrose content with respect to the corresponding values under N and L treatments, respectively (Fig. 2B). When shorter wavelength light is supplemented with far-red light that preferentially excites PSI, the excitation balance between the two photosystems can improve the overall rate of photochemistry and subsequently CO_2 assimilation [33]. Similarly, the change in trends of starch and sucrose content in different treatments were similar to P_n, quantum yield of PSII, and total biomass (Fig. 1, 2, 4). In addition, the promotive effect of additional Fr radiation on photosynthesis and whole-plant net assimilation was previously reported in lettuce and geranium [12, 18]. Together, these results suggested that the high enrichment of Fr light possibly contributed to increased starch and sucrose contents by improving photosynthetic capacity of soybean leaves.

Proteins play important regulatory functions when plants suffer from different abiotic stresses. Proteomics is an approach for the systematic identification of all proteins expressed in a cell or tissue

[34]. In this study, we mainly focused on the differentially expressed proteins related to photosynthesis (Table 1). The POR is the key functional protein in regulating chlorophyll metabolism [35, 36]. The POR protein expression was up-regulated under the L treatment relative to that under the N treatment, whereas an opposite trend was found in N+Fr treatment (Table 1). POR expression significantly increased with the decrease in irradiance [37]. Our previous research also found that maize shade or low light can improve the POR protein expression [11]. The qRT-PCR analysis results confirmed the observed response of POR to different light intensities and qualities (Fig. 7). POR is a negative control protein of light-dependent response, especially for light intensity [38]. Although low R/Fr ratio in the same light intensity condition can improve the Chl contents per unit leaf area (Fig. 3), opposite trends were found in the POR protein and gene expression levels. (Table 1; Fig. 7). This result was related to the unit (mg cm^{-2}) of Chl contents [11, 39].

Photosystems can be classified into PS I and PS II, which are the functional and structural units of protein complexes involved in photosynthesis [40]. The PsaD subunit of PS I is a peripheral protein that provides a docking site for ferredoxin and interacts with other PS I subunits [41]. PsaD is highly sensitive to the light environment, and high light intensity significantly decreases the PsaD content [42]. Similarly, low light up-regulated the PsaD protein and gene expression levels because the high protein expression of PS II can improve the electron transport, which increases the PsaD protein expressions under low light condition [11]. The low R/Fr ratio (Fr light enhancement) may reverse the PsaD protein expression under low light condition (Table 2). This finding was similar to the results showing Fr light can improve the efficient photochemistry and photosynthesis [18].

The light-harvesting antenna (LHCII) is the major pigment- protein complex associated with PS II, thereby forming the PS II-LHCII supercomplex in which several LHCIIs surround the reaction center core complex [43]. The Lhcb levels decrease as light intensity increase with a significant trend for Lhcb 1, Lhcb 2, and Lhcb 4 [5, 6, 44]. Our results agreed with these data (Table 1). Similarly, the protein Lhcb 1 and Lhcb 2 expression levels decreased at excessive irradiance [43]. However, Lhcb 1, Lhcb 2, Lhcb 4, and Lhcb 6 levels remained unchanged under L+Fr treatment (low R/Fr ratio) compared with those under N treatment. Similar result also was found in the gene expression of Lhcb 1 (Fig. 7). These results indicated that increasing the Fr light wavelength (low R/Fr ratio) can improve the photosynthetic efficiency under short wavelength. Ahmadova and Mamedov [45] indicated that the low energy of the Fr photons plays an important role in the photochemical progress.

PetE is a low molecular weight protein that is involved in the linear and cyclic electron transport in oxygenic photosynthetic organisms; it catalyzes the electron transfer from the membrane-bound Cyt b6/f complex to P700 [46]. PetH is a redox partner protein of PetF, which is a light-dependent electron transfer protein [47]. These proteins are highly sensitive to the light environment. Low light condition can up-regulate PetE and PetF expression (Table 1, Fig. 7). The changes in PetE and PetF protein trends were similar to those of Lhcb 1, Lhcb 2, Lhcb 4, and Lhcb 6 proteins under low light condition. This phenomenon may have occurred because the degree of increased thylakoid stacking improved the light capture and electron transport by up-regulating relative proteins in low light compared with those in

normal light (Table 1) [28]. However, the low R/Fr ratio (N+Fr and L+Fr treatments) barely affected the PetE and PetF expression level compared with normal R/Fr ratio (normal light). This result was similar to the findings of our previous research [11]. The change in PetH expression was different from that of PetF under different treatments (Table 1), because PetH is rate-controlling protein in the protein–protein interaction reaction between PetH and PetF as the electron transfer partner (Okada, 2009). This finding may explain why the chlorophyll a/b binding proteins up-regulated in low light but P_n decreased compared with other treatments (Fig. 4).

Leaf starch and sucrose metabolism progress was also affected by light environment (Fig. 2) [48]. The SS expression levels were significantly up-regulated under L and L+Fr treatment (Table1). However, an opposite trend was found in the gene expression pattern of Gmss1 under the L and L+Fr treatment (Fig. 7). Similarly, the sucrose level in low light condition decreased first due to decrease in the activities of sucrose synthesizing enzymes (i.e., SPS and SS-s) [49]. These results may be related to the different gene and protein expression levels for the same protein under different treatments.

Conclusions

We provided an overview of the characteristic proteomic dynamics of photosynthesis and CO_2 assimilation in the chloroplast of soybean under different light environments by using the iTRAQ technique. A total of 12 differentially expressed proteins showed a >1.3-fold change in abundance. Most of these proteins were up-regulated under L treatment, but they did not significantly change under N+Fr and L+Fr treatments compared with under N treatment. Simultaneously, a low R/Fr ratio (low or normal light intensity) can improve the leaf area, photosynthesis, quantum yield of PSII, and total biomass in soybean. These results suggested that low R/Fr ratio (high enrichment of Fr light) in the same light intensity can increase the photosynthetic CO_2 assimilation by improving the photosynthetic capacity.

Methods

Plant materials and treatment design

Nandou 12, which is a major soybean cultivar that is closely planted or intercropped with other crops in agriculture production in Southwestern China, was selected as the experimental material. Soybean seeds were provided by the Nanchong Institute of Agricultural Sciences, Sichuan Province, China. Seeds were soaked in wet filter paper for 1 day at 30 ℃. The germinated seeds were planted in containers (40 cm in length, 20 cm in width, and 15 cm in height) filled with humidified organic soil with a seedling spacing of 10 cm.

The experiment was divided into two parts. One part was used for morphology measurement. The containers including germinated seeds were directly placed under different light environment treatments, and each treatment included three containers.

The other part was used for physiological and proteomic analyses. Avoiding the differences in soybean growth period under different light conditions, when the soybean growth stage was the development of the first trifoliolate leaf (before the second trifoliolate leaf appeared) under normal light condition, the seedlings were then divided into four groups under different light environment treatments. After 15 days of treatment, the second trifoliolate leaf was sampled around 10 am to measure its physiological parameters and analyze differentially expressed proteins. The plants were grown in growth chamber with natural solar radiation. The soybean seedlings were watered every 2 days with 0.2% Hoagland's solution[50]. The temperature was maintained at 25 ℃ for 12 h at daytime and at 20 ℃ for 12 h at night. Relative humidity was approximately 60%.

According to our previous report [9], black nylon net was used to adjust the light intensity (PAR) and Fr light-emitting diode (LED) (36 W, light peaking at 735 nm) light sources were used to adjust the R/Fr ratio in 50 cm height of soybean canopy. The PAR and spectral irradiance of soybean canopy were measured at noon on a sunny day, every measurement was replicated five times. The following four treatments were used (Table 2 and Fig. 8): normal light, normal light plus Fr light, low light, and low light plus far-red light. The PAR and spectral irradiance were measured using LI-190SA quantum sensors (LI-COR Inc., Lincoln, NE, USA) and a fiber-optic spectrometer (AvaSpec-2048; Avantes, Netherlands) placed at 10 cm above the soybean canopy, respectively [7] [9].

Here Insert Table 2

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Morphological characteristics

The plant height from the soil surface to the growing point of soybean. The biomass and leaf area of five soybean seedlings were measured every 14 days after 14 days of sowing under four treatments. Leaves were scanned using a flatbed scanner (CanoScan LiDE 200, Canon Inc., Japan), and the leaf area (cm2) was measured by Image J 1.45 s. Biomass samples were over-dried at 105 ℃ for 0.5 h to destroy the tissues and then dried at 80 ℃ for 72 h to a constant weight [9].

Chloroplast ultrastructure

As described by Yang et al. [11], the segments (2 mm × 2 mm) of the second trifoliolate leaf were fixed at 4 ℃ in 3% glutaraldehyde, and treated with 1% osmium tetroxide. Then, the fixed segments were dehydrated in a graded acetone series and embedded in Epon812. The semithin sections were stained and cut with a diamond knife. Then, the sections were stained with acetate and lead citrate, and examined using a transmission electron microscope (TEM; HITACHI, H-600IV, Japan).

Measurements of sucrose and starch

Leaf samples were over- dried at 105 ℃ for 0.5 h to destroy the tissues and then dried at 80 ℃ for 24 h to a constant weight. According to the methods of Rosa et al (2009) with some changes [51], soluble sugars were extracted from 0.5 g dried samples by homogenization in 5 ml of 80 % (v/v) ethanol. After heating the homogenate in a water bath, the insoluble fraction was removed by centrifugation at 3500 g for 10 min. The precipitate was homogenized and centrifuged again. Supernatants were pooled and then diluted up to 25 ml with 80 % ethanol. For starch extraction, the residues left in the centrifuge tubes after sugar extraction were added with 2 ml of water. Then, the tubes were placed in boiling water bath for 15 min. After cooling, 2 ml of 9.2 M perchloric acid (PCA) was added. After stirring for 15 min, the supernatants were collected after centrifuging the contents at 3500 g for 10 min. The residues were re-extracted two times with 2 ml of 4.6M PCA. After centrifugation, the supernatants were combined, volumes were made to 50 ml with water [51]. Sucrose and starch were determined colorimetrically using the phenol-sulphuric acid method, as described by [52].

Photosynthesis, photosynthetic pigment concentration, and quantum yield of PS II

As described by Yang et al. [2], the second trifoliolate leaf was selected to measure photosynthetic characteristics using a Li-6400 portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA), environment temperature 25 ℃ and a CO_2 concentration of 400 μmol mol^{-1} from 9:00 to 11:00. Eleven light intensity levels (0, 20, 50, 100, 150, 200, 400, 600, 800, 1000, and 1200 μmol m^{-2} s^{-1}) were imposed. On a light response curve, PPFD was located on the horizontal axis and P_n was on the vertical axis (P_n-PPFD curve). The P_{max} and LSP were then estimated using the method proposed by Yang et al. [11]. In addition, the quantum efficiency of the photosystem II was calculated according to the method by Yang et al. [9].

Then, four 15 mm diameter disks were removed from the center of each middle leaf and then cut into pieces with the size of approximately 3 mm. These pieces were placed in 10 ml of 80% acetone in the dark at 20 ℃ for 24 h. The photosynthetic pigment concentrations were calculated according to the method of Sims and Gamon [39]. Triplicates were prepared for each treatment.

Protein extraction, digestion, and iTRAQ labeling

After 15 days of treatment, the second trifoliolate leaves were ground in liquid nitrogen. As described by Yang et al. [11], the cell powder was transferred into centrifuge tube and sonicated in a lysis buffer and 1% protease inhibitor cocktail on ice by using a high-intensity ultrasonic processor (Scientz). The remaining debris were removed by centrifugation. Proteins were precipitated with cold 15% TCA for 2 h at -20 ℃. After centrifugation at 4℃, the supernatant was discarded, and the remaining precipitate was washed three times with cold acetone. The protein was then redissolved in buffer, and protein concentration was determined. The protein solution was reduced and alkylated for 45 min with 20 mM

IAA at room temperature in the dark. The protein sample was diluted by adding 100 mM TEAB. Finally, trypsin was added at a trypsin-to-protein for digestion. Approximately 100 μg of protein for each sample was digested with trypsin for the subsequent experiments. For iTRAQ lambelling, Peptides were desalted with Strata X C18 SPE column (Phenomenex), vacuum-dried, reconstituted in 0.5 M TEAB, and then processed according to the manufacturer's protocol for the 4-plex iTRAQ kit.

HPLC fractionation and LC-tandem mass spectrometry (MS/MS) analysis

As our previous report [11], the sample was divided into fractions through high pH reverse-phase HPLC by using Agilent 300 Extend C_{18} column. After the peptides were dried through vacuum centrifugation, and then were dissolved in 0.1% FA and directly loaded onto a reverse-phase analytical column (Acclaim PepMap RSLC, Thermo Scientific). Gradient elution was performed at a constant flow rate of 350 nl/min on an EASY-nLC 1000 UPLC system. The resulting peptides were then analyzed using an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Fisher Scientific).

Database search and analysis

MS/MS data were processed using MaxQuant with integrated Andromeda search engine (v.1.5.2.8). As described by Yang et al. [11], iTRAQ 8-plex was selected as the quantification method, and the default values of all the other parameters in MaxQuant were selected. A 1.3- or 0.77-fold cut-off with a $P < 0.05$ was used to identify the up-regulated or down-regulated protein expression, respectively. Proteins were functionally annotated using Gene Ontology (GO) annotation[24]. The differentially accumulated proteins were also assigned to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [53].

Real-time quantitative polymerase chain reaction (qRT-PCR) verification

qRT-PCR assay was used to confirm the reliability of the proteomics. RNA isolation was performed following the methods of Yuan et al. [54]. The β-tubulin gene was used as the reference control in the present study. RT-PCR was performed on a CFX96 system machine (Bio-Rad, USA). All primers are listed in Table S2.

Statistical analysis

Significance was determined by one-way analysis of variance on SPSS software (version 16.0). Data were presented as mean ± standard deviation from three independent biological replicates. Statistical significance was considered at $P < 0.05$.

Abbreviations

iTRAQ: Isobaric tags for relative and absolute quantification

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Not applicable

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

FY, QL and YC performed the experiment; LF, XWu and YF performed some experiments; FY, MAR, XW, TY, WL, JL, JB, KS and WY conceived the original research plans; FY and WY designed the experiments, analyzed the data, and wrote the article. All authors read and approved the final manuscript.

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Tables

Table 1. Differentially expressed proteins associated with soybean photosynthetic CO_2 assimilation under different light environments. "/" indicates insignificant accumulation. N, N+Fr, L+Fr, and L denote normal light (normal PAR and normal R/Fr ratio), normal light plus far-red light (normal PAR and low R/Fr ratio), low light plus far-red light (low PAR and low R/Fr ratio), and low light (low PAR and normal R/Fr ratio), respectively.

Accession no.	Description	N+Fr vs. N	L+Fr vs. N	L vs. N
Porphyrin and chorophyll metabolism				
A0A0R4J3L3	Protochlorophyllide reductase (POR)	0.75	/	2.72
Photosystem I				
A5Z2K3	Photosystem I subunit (PsaD)	/	/	1.38
Photosystem II				
A0A0R4J5I3	Chlorophyll a/b binding protein 1 (Lhcb 1)	0.37	/	3.80
Q93YG3	Chlorophyll a/b binding protein 2 (Lhcb 2)	/	/	1.52
I1JLH0	Chlorophyll a/b binding protein 4 (Lhcb 4)	/	/	1.44
I1KR46	Chlorophyll a/b binding protein 6 (Lhcb 6)	/	/	1.44
Photosynthetic electron transport				
C6SVR0	Plastocyanin (PetE)	/	/	1.46
C6T1J0	Ferredoxin-1 (PetF)	/	/	1.38
I1JCG8	Ferredoxin-NADP reductase (PetH)	/	0.44	/
Starch and sucrose metabolism				
I1MBQ9	Sucrose synthase (SS)	/	1.42	1.92
I1KAT2	Starch synthase (GlgA)	/	0.60	/

Table 2. Light intensity and red/ far-red ratio of soybean canopy in different treatments. N, N+Fr, L+Fr, and L denote normal light (normal PAR and normal R/Fr ratio), normal light plus far-red light (normal PAR and low R/Fr ratio), low light plus far-red light (low PAR and low R/Fr ratio), and low light (low PAR and normal R/Fr ratio), respectively. The different letters in each table row are significantly different (P=0.05).

Treatment	N	N+Fr	L+Fr	L
PAR ($mol \cdot m^{-2} \cdot s^{-1}$)	566.50±2.24a	566.57±1.84a	64.22±0.68b	63.33±0.14b
R/Fr ratio	1.33±0.081a	0.42±0.05b	0.08±0.012c	1.26±0.027a

Figures

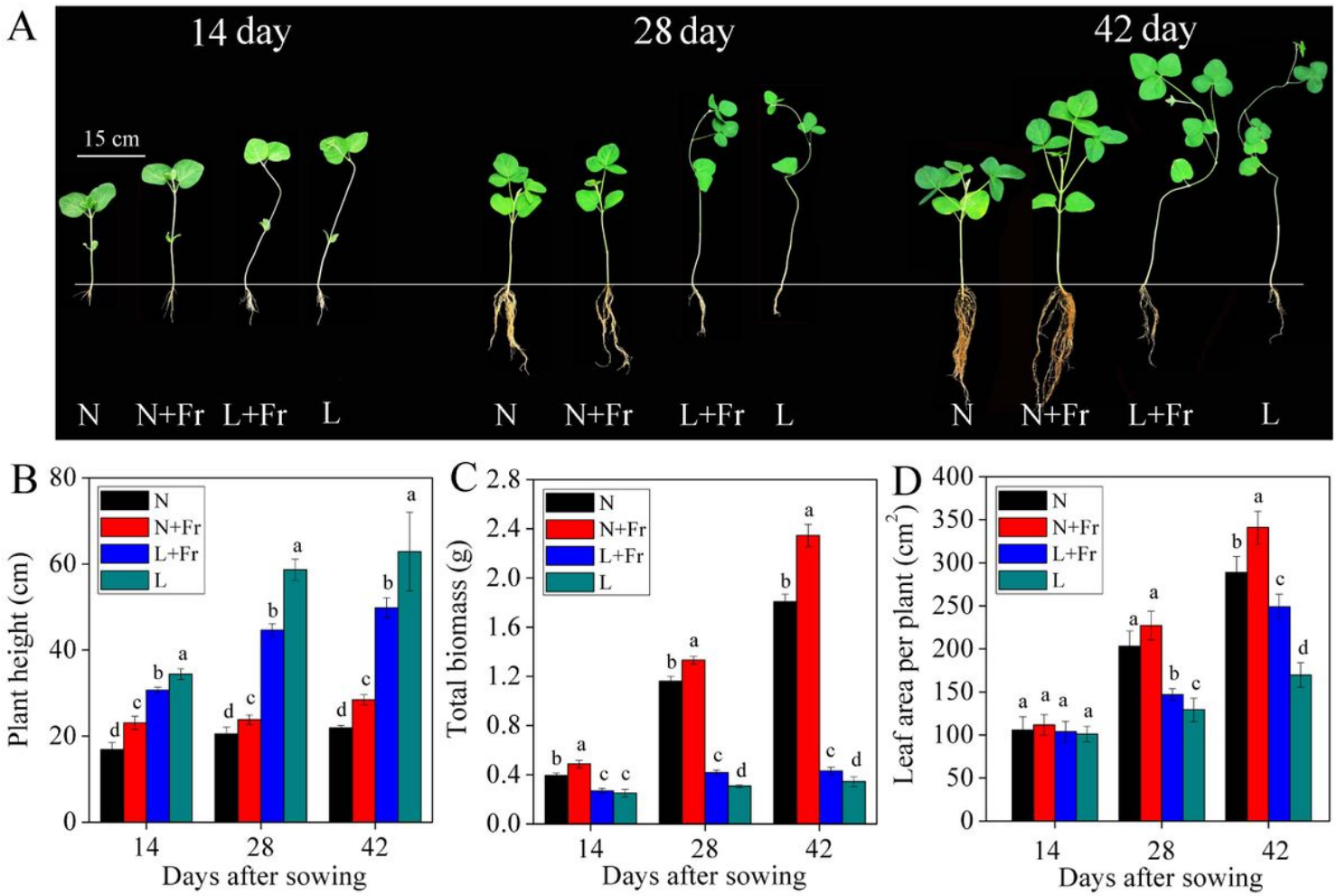

Figure 1

Soybean growth performance (A), plant height (B), total biomass (C), and leaf area (D) under different treatments. N, N+Fr, L+Fr, and L denote normal light (normal PAR and normal R/Fr ratio), normal light plus far-red light (normal PAR and low R/Fr ratio), low light plus far-red light (low PAR and low R/Fr ratio), and low light (low PAR and normal R/Fr ratio), respectively. Each value was expressed as the mean ± SD. The means for each treatment without common letters were significantly different at P=0.05 according to Duncan's multiple range test.

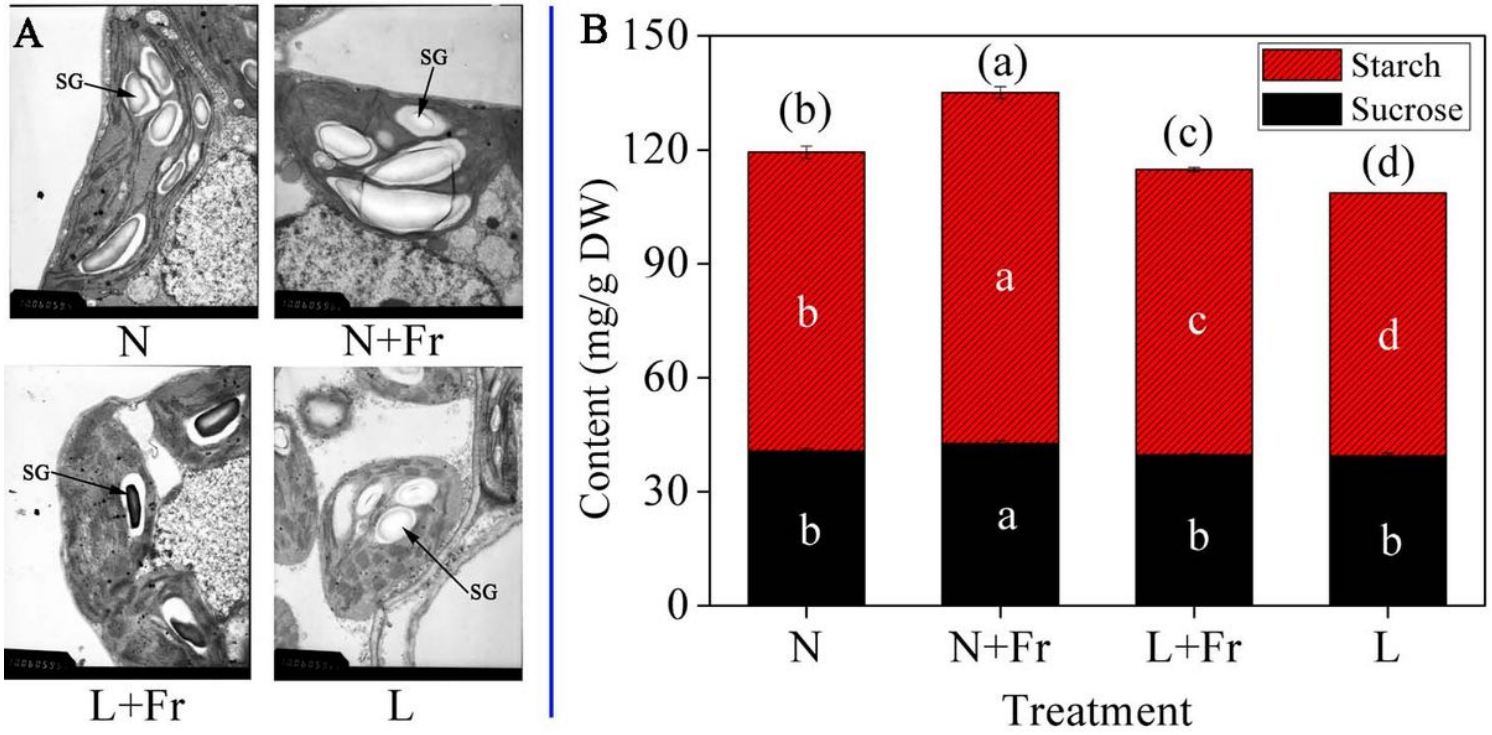

Figure 2

Soybean chloroplast ultrastructure (A), starch and sucrose contents (B) under different treatments. N, N+Fr, L+Fr, and L denote normal light (normal PAR and normal R/Fr ratio), normal light plus far-red light (normal PAR and low R/Fr ratio), low light plus far-red light (low PAR and low R/Fr ratio), and low light (low PAR and normal R/Fr ratio), respectively. SG stands for starch grain. Data are expressed as the means ± SD of triplicates. Means followed by different letters are significantly different at P=0.05.

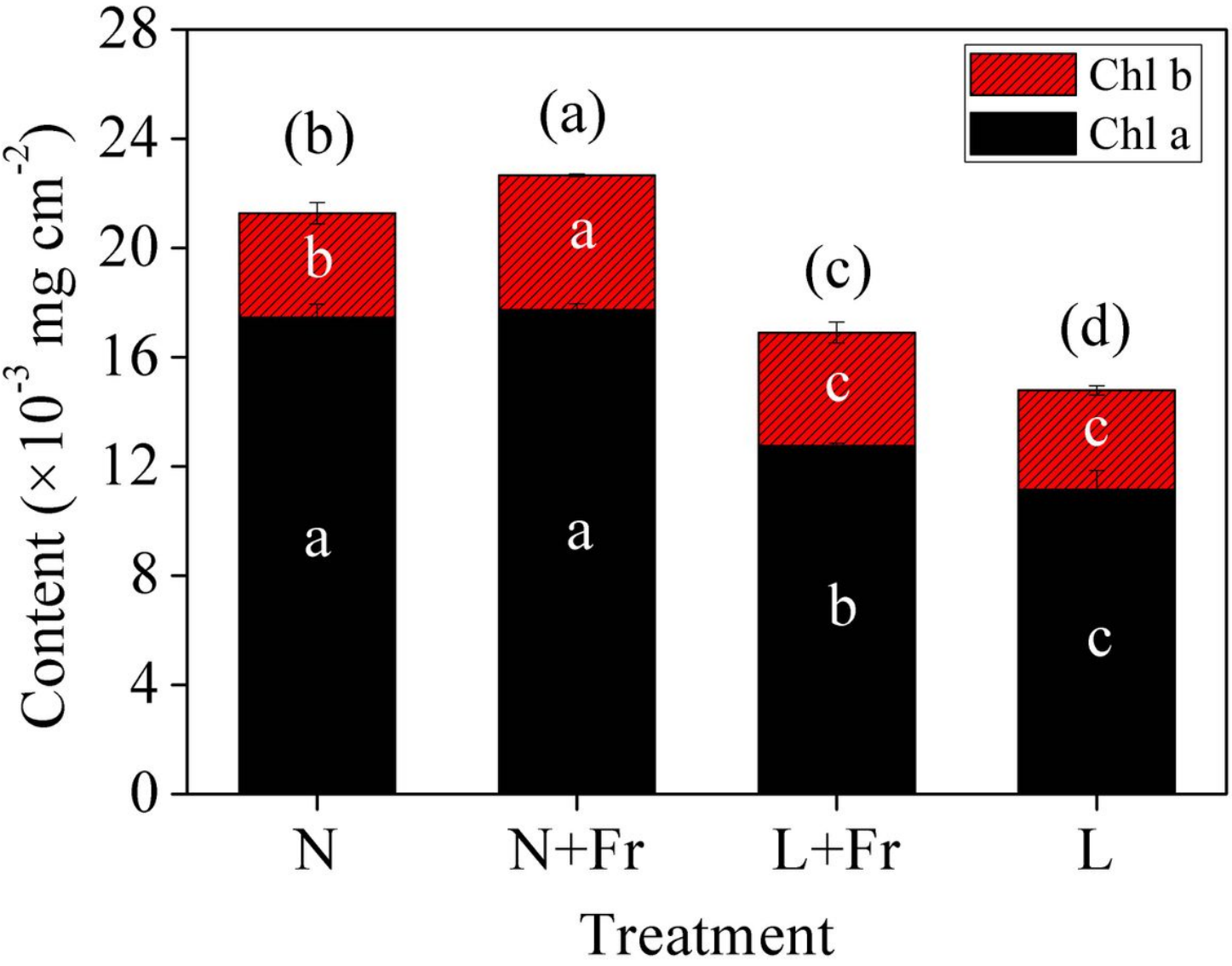

Figure 3

Chlorophyll (Chl) content of soybean leaves under different treatments. N, N+Fr, L+Fr, and L denote normal light (normal PAR and normal R/Fr ratio), normal light plus far-red light (normal PAR and low R/Fr ratio), low light plus far-red light (low PAR and low R/Fr ratio), and low light (low PAR and normal R/Fr ratio), respectively. Data are expressed as the means ± SD of triplicates. Means followed by different letters are significantly different at P=0.05.

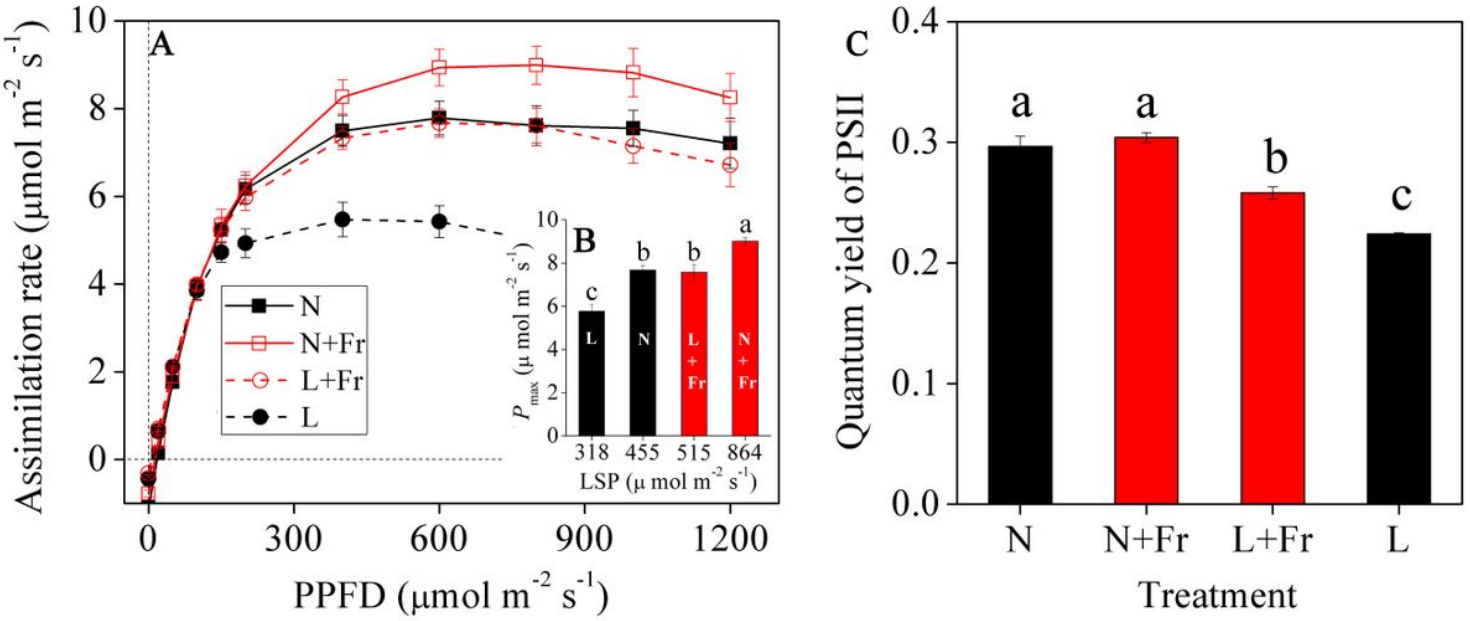

Figure 4

Light response curves of net photosynthetic rate (A), light saturation point (B), and quantum yield of PSII (C) of soybean leaves under different treatments. N, N+Fr, L+Fr, and L denote normal light (normal PAR and normal R/Fr ratio), normal light plus far-red light (normal PAR and low R/Fr ratio), low light plus far-red light (low PAR and low R/Fr ratio), and low light (low PAR and normal R/Fr ratio), respectively. Pmax and LSP represent the maximum photosynthetic rate and the light saturation point. Data are expressed as the means ± SD of triplicates. Means followed by different letters are significantly different at P=0.05.

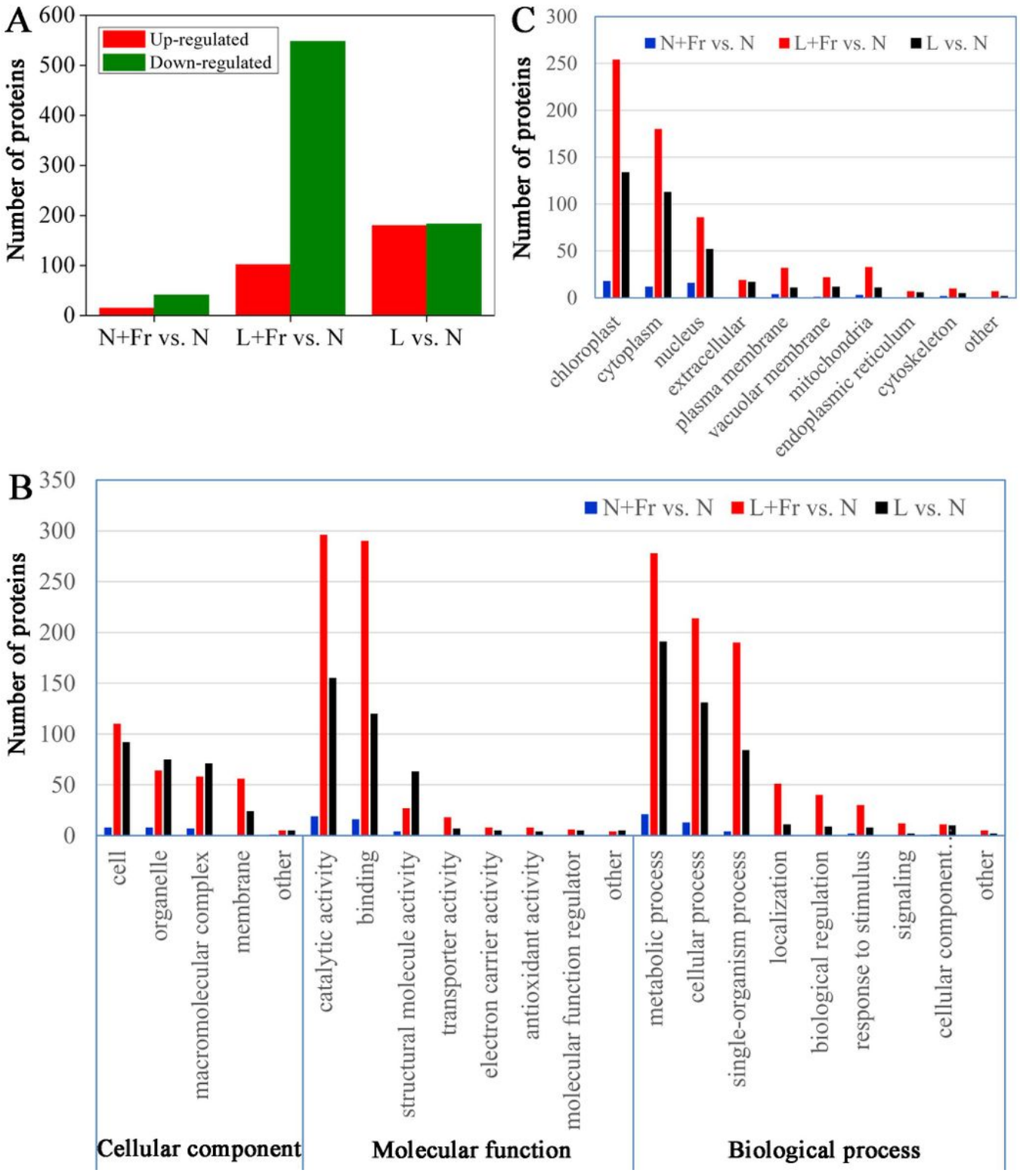

Figure 5

Differential protein expression analyses of soybean leaves under different light environments. (A) Histogram of the up- and down-regulated protein number under normal light plus far-red light (N+Fr), low light plus far-red light (L+Fr), and L conditions compared with those under N condition. (B) GO classification of differentially accumulated proteins. (C) The subcellular classification of the differentially accumulated protein number under N+Fr, L+Fr, and L treatments compared with that under N treatment.

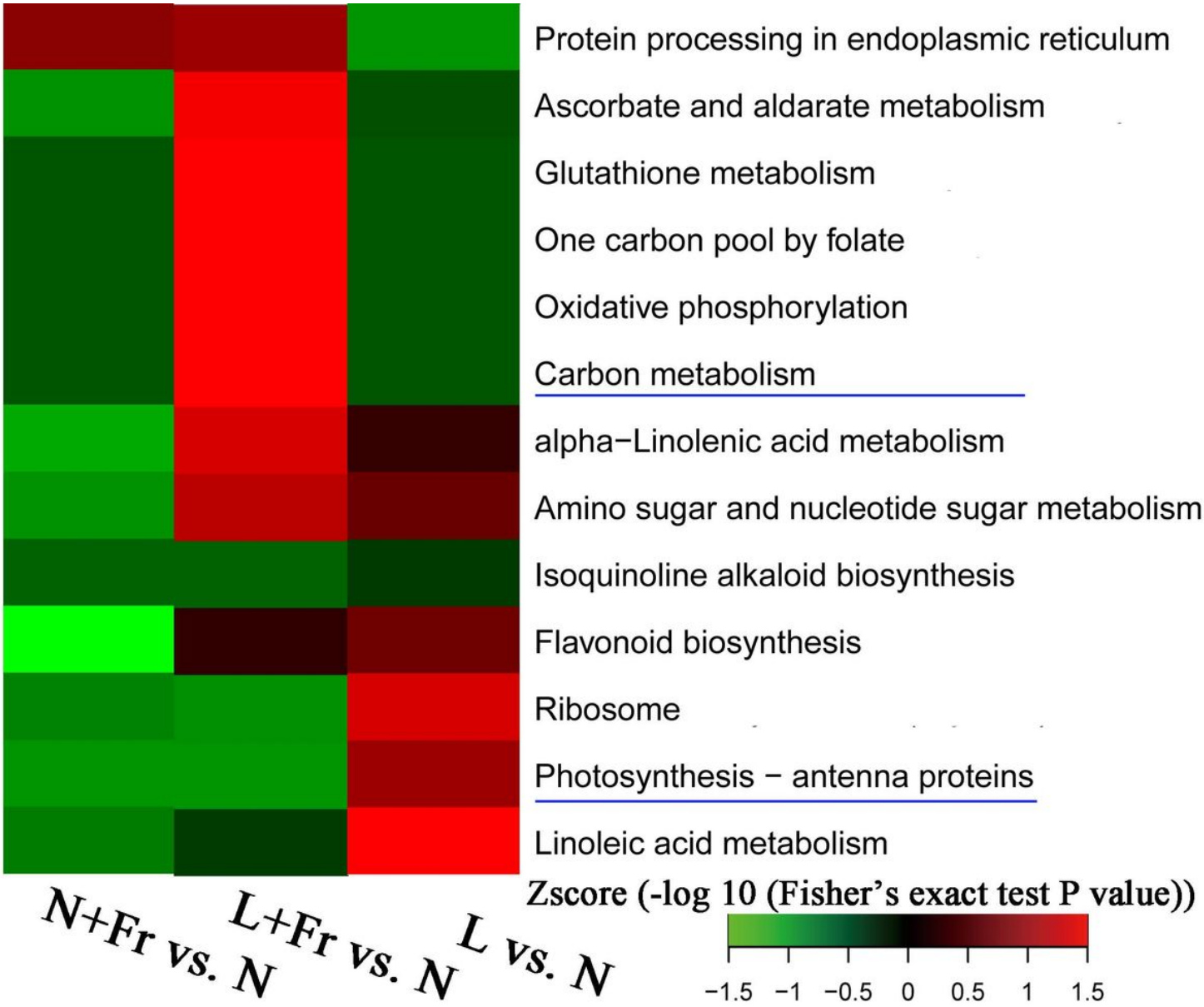

Figure 6

KEGG pathway-based enrichment analysis of differentially accumulated proteins. N, N+Fr, L+Fr, and L denote normal light (normal PAR and normal R/Fr ratio), normal light plus far-red light (normal PAR and low R/Fr ratio), low light plus far-red light (low PAR and low R/Fr ratio), and low light (low PAR and normal R/Fr ratio), respectively. Red colors indicate up-accumulated proteins and green colors indicate down-accumulated proteins in the N+Fr, L+Fr, and L treatments compared with the N treatment.

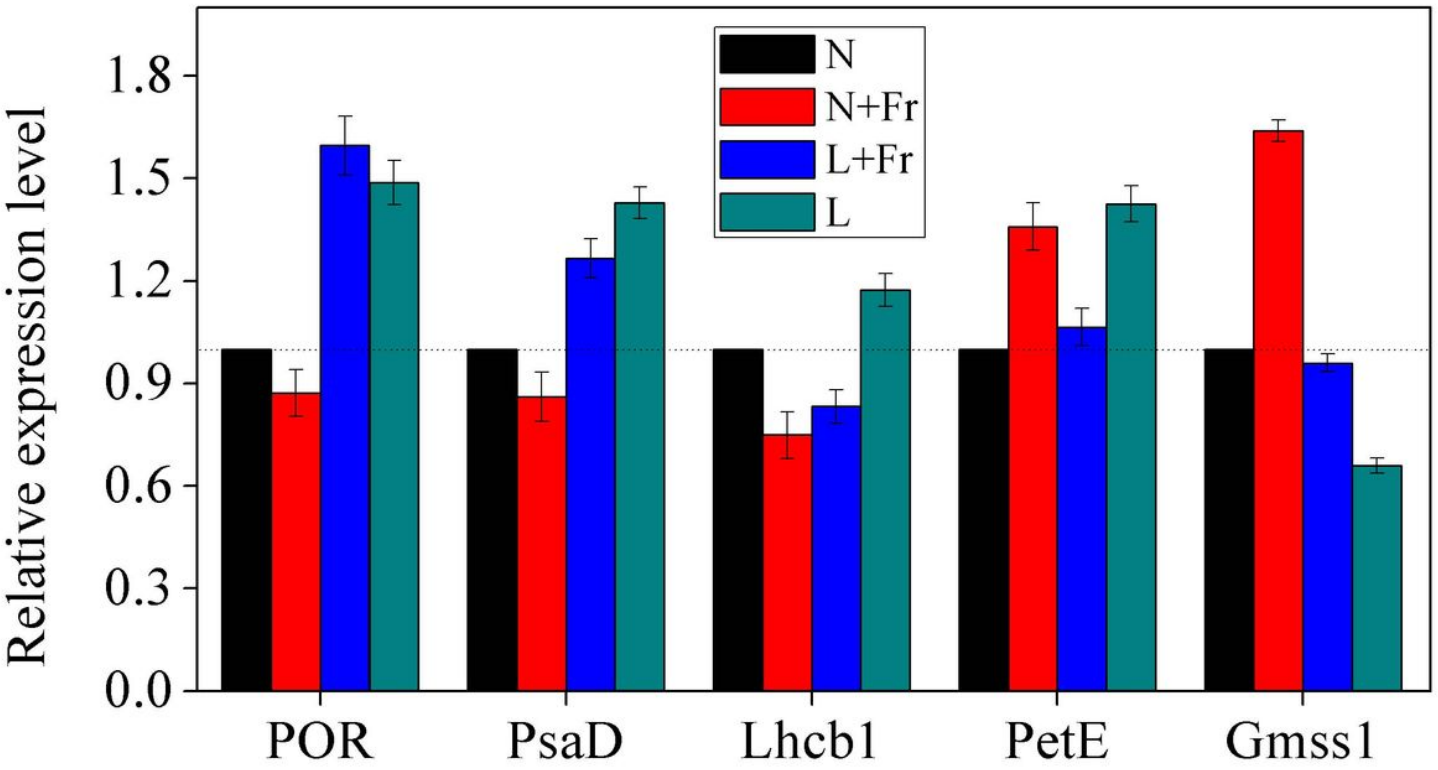

Figure 7

Quantitative RT- PCR validations of the genes related to the differentially expressed proteins in N+Fr, L+Fr, and L treatments compared with those in N treatment. POR, PsaD, Lhcb 1, and PetE represent protochlorophyllide reductase, photosystem I subunit, chlorophyll a/b binding protein 1, and plastocyanin, respectively. Values are expressed as mean ± SD (n=3).

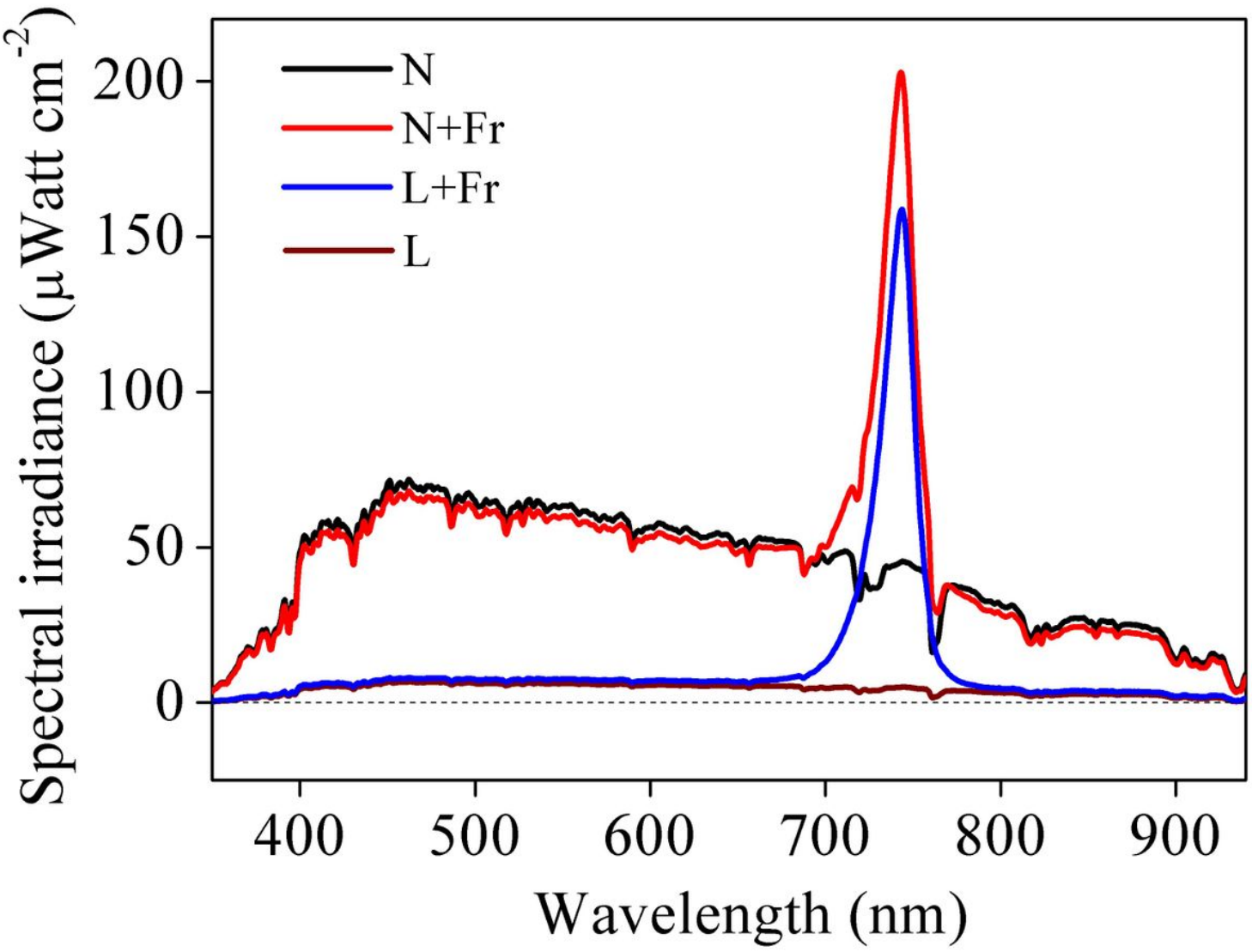

Figure 8

Spectral irradiance distribution of soybean canopy in different treatments from 350 - 950 nm. N, N+Fr, L+Fr, and L represent normal light, normal light plus far-red light, low light plus far-red light, and low light, respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xlsx
- TableS2.docx