Bioactive Constituents, Metabolites, and Functions

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Comparative analysis of phenolic compounds profile, antioxidant capacity, and expression of phenolic biosynthesis-related genes in soybean microgreens grown under different light spectra

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Abstract

The Light-emitting diode (LED)-based light sources, which can selectively and quantitatively provide different spectra, have been frequently applied to manipulate plant growth and development. In this study, the effects of different LED light spectra on the growth, phenolic compounds profile, antioxidant capacity and transcriptional changes in genes regulating phenolic biosynthesis in soybean microgreens were investigated. The results showed that light illumination decreased the seedling length and yield but increased phenolic compounds content. Blue light and ultraviolet-A (UV-A) induced significant increases in total phenolic and total flavonoid content, as compared with the white light control. Sixty-six phenolic compounds were identified in the soybean samples, of which isoflavone, phenolic acid, and flavonol were the main components. Ten phenolic compounds obtained from the orthogonal partial least squares discriminant analysis (OPLS-DA) were reflecting the effect of light spectra. The antioxidant capacity was consistent with the phenolic metabolite levels, which showed higher levels under blue light and UV-A compared with the control. The highest transcript levels of phenolic biosynthesis-related genes were observed under blue light and UV-A. The transcript levels of *GmCHI*, *GmFLS* and *GmIOMT* were also upregulated under far-red and red light. Taken together, our findings suggested that the application of LED light could pave a green and effective way to produce phenolic compounds-enriched soybean microgreens with high nutrition quality, which could stimulate further investigations for improving plant nutritional value and should have a wide impact on maintaining human

health.

Keywords: soybean microgreens; light quality; light-emitting diodes (LEDs); flavonoids; antioxidant capacity; Principal component analysis

Introduction

In recent years, there has been an increasing demand for vegetables with high levels of biologically active substances. In this context, microgreens, which are delicate, flavorful and highly nutritional, have been recognized as a new kind of "functional foods".[1-2] Microgreens indicate "tender immature vegetables produced from the seeds of vegetables (e.g. radish and celery) and herbs (e.g. basil and mustard) under light illumination, having two fully developed cotyledon leaves with or without the emergence of a rudimentary pair of first true leaves".[3] The edible portions of microgreens are mainly the hypocotyls with cotyledons and first true leaves. Common microgreens are usually grown from the seeds of mustard, broccoli, pak choi, radish, tatsoi and pea.[3-4] Nowadays, soybean (*Glycine max* L.) microgreens are growing popular due to their great taste, crisp texture, as well as their high level of phytochemicals, including but not limited to phenolics, saponin, and vitamins that are particularly beneficial for human health.[5-9]

Phenolic compounds are secondary metabolites in plants, which include several major subclasses, including flavonols, flavanones, flavones and isoflavones, phenolic acids and phenylpropanoids.[10] They play diverse roles in plant growth and development, besides, they also function in interactions between plants and environments.[11-12] On the other hand, the beneficial roles of dietary phenolic metabolites in human health, including antioxidative, anti-inflammation, anti-cancer, coronary heart disease and diabetes

prevention, and cardiovascular protection, have been supported by numerous studies.[13-16] Fruits and vegetables are the main dietary sources of phenolic compounds for humans. Meanwhile, phenolic compounds are their important indices, as they confer color, aroma and antioxidant properties.[17] Therefore, it is worth investigating how to increase the content of phenolics in our dietary vegetables.

Phenolic compounds are biosynthesized by activities of the enzymes of the general phenylpropanoid pathway, in which *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *flavanone* 3-*hydroxylase* (*F3H*), *flavanone* 3-*hydroxylase* (*F3'H*), *flavonol synthase* (*FLS*), *isoflavone synthase* (*IFS*) and *isoflavone 6-O-methyltransferase* (*IOMT*) are key genes. The first committed step in the biosynthesis of phenolic compounds from the general phenylpropanoid pathway is catalyzed by CHS. The structural genes, such as *CHI*, *F3H*, *F3'H*, *FLS*, *IFS,* and *IOMT*, are responsible for the synthesis of phenolic compounds, such as flavanone and flavonols.[18] The biosynthesis of phenolic compounds in plants is regulated by a lot of environmental factors, of which light regime (e.g. light intensity, photoperiod, and light quality) is one of the most important ones.[19] It has been well documented that light signals are perceived through distinct photoreceptors, including phytochromes, cryptochromes, phototropins and UV RESPONSE LOCUS 8 (UVR8),[20,21] and light play fundamental roles in the regulation of phenolic compounds biosynthesis. For example, it was reported that flavonoids content was positively correlated with the proportions of far-red light and negatively correlated with the UV-A

and red/far-red ratio in tobacco leaves.[22] The mRNA transcription level of *FtPAL* and *FtF3'H* and the phenolic compounds content of buckwheat sprouts were reported to be increased under white and blue light, as compared with red light.[23] In addition, the total phenolic compounds content in rice leaves under different light treatments followed the orders of blue>white>red>green>dark.[24] The transcriptional regulatory networks mediate light signal transduction by coordinating activation and inhibition of specific downstream genes and have a key role in light-regulated phenolic biosynthesis. For example, UVR8 could interact with COP1 to prevent it from targeting HY5 for destruction and allow HY5 to activate UV-B response genes (e.g. *CHS* and *FLS*) to promote flavonoid biosynthesis in apple.[25]

The microgreens are commonly cultivated in controlled environments with artificial lights, among which LED lights have been paid special attention in the past two decades. LED lights are regarded as innovative light sources for vegetable production in controlled environment (e.g. greenhouse and closed-type plant factory), because of their unique advantages including the flexible light spectral composition, small size, long lifetime, cool emitting temperature, as well as energy-saving properties.[26] The launch of LEDs now enables us to manipulate light quality efficiently. Several studies have reported that light treatment could affect the accumulation of flavonoids in germinated soybean. However, conflict and less comparable results were obtained in the previous studies. For instance, it was reported that the content of total phenolic, saponin and

103 isoflavone of soybean sprouts were significantly increased after exposure to light.[5, 27-28]
104 On the contrary, Lee et al.[9] reported that light exposure showed little effect on the
105 accumulation of the total isoflavone in soybean sprouts. Recently, ultraviolet light-
106 induced changes in phenolic compounds accumulation in germinated soybean have been
107 reported in continuance, and the results have been associated with the increase in those
108 compounds. For instance, ultraviolet B (UV-B) light was shown to significantly increase
109 isoflavone content in soybean sprouts.[7, 29-31] Similarly, UV-A was reported to induce
110 anthocyanin biosynthesis in hypocotyls of soybean sprouts.[32] Besides, the antioxidant
111 capacity of germinated soybean was also reported to be subjected by light treatments
112 (such as fluorescent light and UV-B).[8, 27, 31] However, to the best of our knowledge, the
113 systematic profiling analyses of phenolic compounds under different light spectra in
114 soybean microgreens are still limited.
115
116 In the present work, LED lights were applied to precisely modulate spectral distribution
117 and light intensity. The soybean microgreens were cultured in the dark and under white,
118 far-red, red, green, blue and UV-A LED lights to systematically analyze the effect of
119 light spectra on phenolic compounds profile and antioxidant capacity of soybean
120 microgreens. To better understand the regulation of phenolic biosynthesis under
121 different light spectra, the transcript levels of genes related to phenolic biosynthesis, as
122 well as the correlations between metabolism and transcription in soybean microgreens
123 were also investigated.

124

125 Materials and methods

126 Plant materials and growth conditions

127 Seeds of soybean (*Glycine max* L., cv. 'Dongnong 690') were surface sterilized by 0.5%
128 sodium hypochlorite for 30 min and then washed with distilled water. Sterilized seeds
129 were soaked in distilled water for 6 h to accelerate the germination. Then approximately
130 80 of germinated seeds were sown evenly in a plastic tray (20 × 14 × 5 cm) with two
131 layers of filter papers. One tray represented as one replicate, and three replicates for each
132 treatment were used. Prior to the light treatment, the germinated seeds were cultured in
133 the dark for 2 days, and then they were exposed to different light spectra as supplied by
134 LED lamps (Heliospectra RX30, Göteborg, Sweden) and grown-up to harvest time. In
135 this study, there are seven treatments with different light conditions. The germinated
136 seeds constantly grown in the dark was referred as dark treatment (D), while those grown
137 under the broad wavelength of white LED light was referred as white light (W). Other
138 different light spectra and their peak spectra were as follow: (1) far-red light (FR), 735
139 nm; (2) red light (R), 660 nm; (3) green light (G), 520 nm; (4) blue light (B), 450 nm;
140 and (5) UV-A light (UV-A), 380 nm. The spectral distribution of the LED lamps was
141 shown in Supplemental Fig. S1. Plants were grown under 30 μmol m^{-2} s^{-1} of light
142 intensity (photosynthetic photon flux density, PPFD) with a photoperiod of 12 h/12 h
143 (light/dark). The light intensity was monitored by a spectroradiometer (Avaspec-2048-
144 CL, Avantes, Apeldoorn, Netherlands) and maintained by adjusting the distance between

the LED lamps and the canopies of microgreens. Throughout the study, the temperature and relative humidity were consistently maintained at 25 ℃ and 80%, respectively. The microgreens were watered as needed and the trays under the same LED light treatment were randomly arranged every day to maintain the uniformity of the light environment. To investigate the effect of LED light spectra on morphological and physiological changes, the plant samples were collected at 6 days after sowing. The fresh samples were used for the morphological analysis, the lyophilized shoots samples were used for the phytochemical content and antioxidant capacity analysis, and part of the fresh samples was frozen in the liquid nitrogen and stored at -80 °C for the analysis of gene transcript levels. For each treatment, three replicates were used. The ungerminated soaked seeds (SS) were recognized as raw seeds and second control in this study.

Measurement of growth parameters

For the analysis of the growth, the fresh weight was determined by measuring the weight of 10 seedlings and was expressed as g per 10 seedlings. The yield was expressed as the edible microgreens weight germinated from 100 g of dry soybean seeds.[33] All growth parameters were performed in triplicate.

Determination of total phenolic content and total flavonoid content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method as described previously.[34] Briefly, the freeze-dried shoot samples (1 g) were extracted with 10 mL of 50% methanol, the supernatant was collected after centrifugation and analyzed spectrophotometrically using the Folin-Ciocalteu reagent. Garlic acid was used as a

166 standard and the results were expressed as mg of garlic acid equivalents (GAE) per g dry
167 weight (DW). Total flavonoid content (TFC) was determined according to the methods
168 reported by Sharma et al.[35] Briefly, the freeze-dried shoot samples (1 g) were extracted
169 with 10 mL of 95% methanol, the supernatant was collected. Then 1 mL of the
170 supernatant was mixed with 1 mL of aluminum trichloride solution, and diluted to 25
171 mL with ethanol. The absorbance was measured at 510 nm, with rutin as the standard
172 for the calibration curve. The results were expressed as mg of rutin equivalents (RE) per
173 g DW.

174 **Determination of phenolic compounds profile by high-performance liquid**
175 **chromatography-mass spectrometric (HPLC-MS)**

176 The phenolic profile in shoots of soybean microgreens was determined according to Xie
177 et al.[36], with minor modifications. The 500 mg of freeze-dried sample was extracted with
178 6 mL of 80% methanol in an ultrasonic bath for 30 min. Then, 4-methylumbelliferone
179 was then added as an internal standard. After centrifuging (10,000 g for 20 min), the
180 supernatant was collected and vacuum-dried (Concentrator plus, Eppendorf, Germany),
181 and dissolved in 500 μL of 80% methanol. Then the supernatants were filtered through
182 a 0.22–μm membrane filter and collected for further analysis.

183 Mass spectrometry analysis of the extracts was performed using a Waters G2-XS Q-TOF
184 system (Waters Corp., USA). Chromatographic separation was performed on an
185 ACQUITY UPLC BEH C18 column (2.1×100 mm, 1.7 μm particle size, Waters, USA).
186 Mobile phase A consisted of 0.1% formic acid in the water, and mobile phase B consisted

of 0.1% formic acid in methanol. The gradient used was as follows: starting at 5% B (0–0.5 min), increasing to 95% B (0.5–11 min), and maintaining at 95% B (11–13 min), returning to 5% B and re-equilibrating (13-15 min). The injection volume was 2 μL and the flow rate of 0.4 mL min^{-1}. Mass spectrometry was performed using electrospray source in positive ion mode with MSe acquisition mode, with a selected mass range of 100–1000 m/z. The lock mass option was enabled using leucine-enkephalin (m/z 556.2771) for recalibration. The ionisation parameters were the following: the capillary voltage was 2.5 kV, sample cone was 40 V, source temperature was 120°C, and desolvation gas temperature was 400°C. Data acquisition and processing were performed using Masslynx 4.1.

RNA extraction and qRT- PCR

Total RNA was extracted from cotyledon and hypocotyl tissues using Trizol reagent (Invitrogen, Gaithersburg, USA) according to the manufacturer's instructions. The concentration of RNA samples was measured using NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Wilmington, DE, USA). The RNA samples with A260/A280 ration between 1.8 and 2.0 were used. First-strand cDNA was synthesized using the PrimeScriptTMRT reagent kit with a gDNA eraser (TaKaRa, Dalian, China). Triplicate quantitative assays of RNA extract from three independent extracts were performed. The qRT-PCR reactions were performed using an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) with SYBR® Premix Ex Taq TM (TAKARA: RR420A). The PCR cycling conditions were 1 cycle of 95 °C for 30 s,

followed by 40 cycles of 95 °C for 3 s, 60 °C for 30 s. The expression of the corresponding genes was calculated using the $2^{-\Delta\Delta CT}$ method with the soybean housekeeping gene *Actin* (J01298) as a reference. The white light treatment (W) was regarded as control group. The primers were designed by Primer Premier 6 software (Premier Biosoft International, USA), and primer sequences were shown in Supplemental **Table S1**.

Antioxidant capacity assay

The 2, 2-diphenyl-1-picrylhydrazyl free radical (DPPH) scavenging capacity assay, the ferric reducing antioxidant power (FRAP) assay and the ABTS scavenging activity assay were used to evaluate the antioxidant capacity in soybean microgreens. The sample extractions of microgreens were prepared in the same way according to the determination of phenolic profile, as described above. DPPH scavenging capacity assay was determined according to Yang et al.[37]. Briefly, 200 μL of diluted crude extracts or standards were mixed with 3.8 mL of 60 μmol L^{-1} DPPH solution. After the incubation in the dark at room temperature for 60 min, the absorbance at 515 nm was measured. The DPPH scavenging capacity was calculated as follows: DPPH scavenging activity (%) = 1 − (absorbance of sample/absorbance of control) × 100%. A series of standard (Trolox) solutions were used for the calibration curve. The result was expressed as μmol Trolox g^{-1} DW.

The FRAP assay was determined according to Kosewski et al.[38], with minor modifications. Briefly, 200 μL of crude extracts were mixed with 2.8 mL of ferric-TPTZ

reagent and incubated at 37 ℃ for 10 min. Then the absorbance at 595 nm was measured. The results were calculated into μM Fe^{2+} on the calibration curve and expressed as μM Fe^{2+} 100 g^{-1} DW.

ABTS scavenging activity assay was performed according to Wang et al.[33]. For the reaction, 100 μL of diluted crude extracts or standard solutions were mixed with 3 mL of the ABTS solution, the mixtures were incubated in the dark at room temperature for 30 minutes. Then the absorbance was measured at 734 nm. A series of standard (Trolox) solutions were used for the calibration curve. The final ABTS values were expressed as μmol Trolox g^{-1} DW.

Data analysis

Statistical data analysis was processed by one-way analysis of variance (ANOVA) and Duncan's multiple range test at the confidence level of $p < 0.05$, using the SPSS 19.0 (SPSS Inc., Chicago, USA). All data are reported as the mean of three replicates ± standard error.

To evaluate the effect of light spectra on the phenolic compounds profile, the dataset of phenolic metabolites was submitted to OPLS-DA conducted by SIMCA-P (ver 16.0, MKS Umetrics, Malmo, Sweden), using different light treatments as predicted qualitative *Y* variables and metabolites as *X* variables. The principal component analysis (PCA) was initially used to obtain an overview of the trends. The quality of PCA was evaluated by the parameters of R^2 (cum) and Q^2 (cum), which representing the goodness of fit and predictability, respectively. For the supervised OPLS-DA, the goodness of the

model was appreciated using the determination coefficient R^2X and R^2Y, and the predictive power was quantified by the cross-validated determination coefficient Q^2. The ruggedness of the OPLS-DA model was investigated using permutation tests that performed with 200 times. The volcano plot was performed to identify the best discriminating metabolites (biomarkers) which could discriminate different light treatments using the correlation coefficient (Corr.Coeff)/p(corr) between sample score and variable value and the variable importance for the projection (VIP) values (VIP $>$ 1). The S plot was performed to identify the biomarkers that make the difference between UV-A and white light control.

Results

Effect of light spectra on the growth of soybean microgreens

Overall, the fresh weight of soybean microgreens was significantly decreased after exposure to light, as compared with the microgreens grown in the dark. The fresh weight under far-red light was significantly decreased by 16.30% in comparison with those grown under white light control. The fresh weight under other light spectra exhibited no significant changes (**Fig.1A**). The effect of light spectra on the yield of microgreens was similar to that on the fresh weight. The yield under light conditions was significantly reduced when compared with the darkness. However, the yield did not differ between different light spectra, except that under far-red light it was significantly lower than that under red light. The yield reached the lowest value under far-red light, while the highest

271 value under red light, which was 15.61% higher and 12.24% lower than that of white
272 light control, respectively (**Fig.1B**).

273 **Effect of light spectra on the TPC and TFC of soybean microgreens**

274 The TPC and TFC of soybean microgreens at 1, 2, 4 and 6 days after sowing were
275 determined (**Fig. 2**). The results showed that the TPC and TFC markedly increased with
276 the culture time. From the second day after sowing, the content of TPC and TFC of the
277 microgreens was significantly higher than that of the SS, regardless of whether the light
278 was present or not. Compared with soaked seeds, the TPC and TFC of microgreens at 2
279 days after sowing were significantly increased by 24.61% and 25.57%, respectively.
280 The TPC and TFC progressively increased during seedling growth, whether in the dark
281 or under the light. In the dark, the TPC and TFC of microgreens at 6 days after sowing
282 reached the highest values, which were 65.86% and 163.01% higher than that of the
283 soaked seeds, respectively. After exposure to light, the TPC and TFC were differently
284 altered by light spectra. In general, light illumination was beneficial for the increase of
285 TPC. When compared with the white light control, the TPC of microgreens at 4 days
286 after sowing were significantly increased by 12.10% and 13.53% under blue light and
287 UV-A, respectively. Similarly, the TPC of microgreens at 6 days after sowing were
288 significantly increased by 12.73% and 27.65% under blue light and UV-A, respectively
289 (**Fig. 3A**). No significant difference was observed between the white light control and
290 other monochromatic light. At 4 days after sowing, the TFC of microgreens grown under
291 monochromatic light was significantly decreased when compared with the white light

292 control. However, no significant difference was observed between the monochromatic
293 light treatments, except red light, which was significantly higher than other
294 monochromatic light treatments. The TFC was significantly decreased by 30.66% under
295 green light, while significantly increased under both blue (22.76%) and UV-A light
296 (21.71%) at 6 days after sowing (**Fig. 3B**).

297 **Identification and comparative analysis of phenolic compounds profile**

298 The impact of light spectra on the phenolic compounds profile was investigated by using
299 HPLC-MS. Sixty-six major phenolic compounds were determined qualitatively and
300 quantitatively according to their retention time (RT), molecular weight, formula, and
301 mass error. These compounds have been classified into six subgroups, including
302 isoflavone, flavone, flavanone, flavan-3-ol, flavonol and phenolic acid (**Table S2**). In
303 both soaked seeds and soybean microgreens, isoflavone was the most prevalent
304 compound, which accounted for 24%-28% over total concentration. In soaked seeds, the
305 most abundant compound was isoflavone (accounting for 28% over total concentration),
306 followed by flavonol (23%), flavone (20%) and phenolic acid (13%). After germination,
307 the phenolic compounds profile was largely altered. In general, the proportion of flavone
308 and flavan-3-ol decreased, the proportion of phenolic acid increased, while others
309 remained the same. Compared with the microgreens grown in the dark, the proportion
310 of flavone in microgreens grown under light increased. Compared with the white light
311 control, the proportion of flavanone in microgreens grown under monochromatic light
312 increased, especially under red and green light (**Fig. 3**).

A heat map was used to better visualize the changes in relative phenolic compounds levels of microgreens grown under different light conditions, as well as the soaked seeds (**Fig. 4**). In the soaked seeds, it was shown that the relative isoflavone abundance was higher than those in the microgreens, while the relative phenolic acid abundance was lower than those in the microgreens, which generally agreed with the previous results (**Fig. 3)**. In the microgreens, the enrichment of phenolic compounds was observed. Metabolites isoflavone (e.g. Daidzein 7-O-glucuronide, Daidzin, Dihydrodaidzein 7-O-glucuronide and Glycitein 7-O-glucuronide), flavone (e.g. Chrysoeriol 7-O-(6"-malonyl-glucoside) and Luteolin 7-O-malonyl-glucoside), flavanone (e.g. Naringenin 4'-O-glucuronide, Naringenin 7-O-glucoside and Narirutin), flavan-3-ol (e.g. (-)-Epicatechin 3-O-gallate), flavonol (e.g. Kaempferol 3-O-(6"-malonyl-glucoside) and Kaempferol 3-O-xylosyl-glucoside) and phenolic acid (e.g. Syringic acid) were abundant under white light, as compared with the dark. When compared with the white light control, monochromatic light tailored the composition of phenolic compounds to different extents. For example, 6,7,3',4'-Tetrahydroxyisoflavone abundance was increased under far-red, red, green, blue light and UV-A, among which blue light and UV-A had the most significant effect. On the other hand, the phenolic compounds were also found decreased under monochromatic light. For example, daidzin abundance was reduced under all monochromatic light treatments, especially under blue light. It was also noticed that some metabolites detected under white light control were absent under monochromatic lights. For instance, Kaempferol 3-O-sophoroside was absent under red and green light,

334 and Apigenin 7-O-glucoside was absent under far-red light and UV-A.

335 **Phenolic compounds discrimination by OPLS-DA modeling**

336 To better illustrate the effect of light spectra on the accumulation profile of phenolic
337 compounds, OPLS-DA was performed using the metabolites dataset. The important
338 phenolic compounds that discriminate the effect of light treatments were identified (**Fig.**
339 **5**). For the PCA analysis, the clusters were not well gathered and the predictive reliability
340 was not satisfactory (R^2X=0.491, Q^2=0.152) (**Fig. 5A**). Therefore, the supervised OPLS-
341 DA analysis was performed. The OPLS-DA score plot results were satisfactory with an
342 excellent determination coefficient (R^2X = 0.972, R^2Y=0.843, Q^2 = 0.681) (**Fig. 5B**),
343 showing improved model predictability and good ability to explain the variation between
344 the light treatments. The different groups were separated into three clusters, namely the
345 white light cluster, UV-A cluster, and the rest cluster contained the rest five-light
346 treatments (including the dark treatment). The differences between the white light
347 control and monochromatic light treatments were discriminated along the horizontal axis,
348 while the differences between the UV-A and other light treatments were discriminated
349 along the vertical axis. Besides, when comparing light spectra, it was found that the
350 shorter the light wavelength has larger the t2 values (**Fig. 5B**). In permutation testing,
351 the values of R^2 (=0.29) and Q^2 (=-0.832) indicated the ruggedness of the model (**Fig.5C**).
352 The volcano plot was made to screen out the most altered phenolic compounds as
353 affected by the light spectra, using a VIP $\geqslant$ 1. The results showed that 10 out of 66
354 phenolic metabolites were found to have both optimal loading score and variable

355 importance: C5 (6"-O-Malonyldaidzin) C6 (6"-O-Malonylgenistin), C11 (Daidzin), C12
356 (Dihydrodaidzein 7-O-glucuronide), C17 (Apigenin 6-C-glucoside), C19 (Apigenin 7-
357 O-glucoside), C20 (Chrysin), C29 (Naringenin 4'-O-glucuronide) and C37 (Galangin)
358 (**Fig. 5D**). Among the ten identified phenolic metabolites, four of them belong to
359 isoflavone (C5, C6, C11, and C12), four of them belong to flavone (C17, C18, C19, and
360 C20), one of them belongs to flavanone, and the rest one belongs to flavonol.
361 The relative abundance of 6"-O-Malonylgenistin in the soaked seeds was found
362 significantly higher than that in the soybean microgreens. The concentration of 6"-O-
363 Malonylgenistin in the microgreens was significantly increased by light exposure,
364 among which monochromatic lights (especially UV-A) showed further promotion effect,
365 as compared with the white light control. The metabolic changes of Dihydrodaidzein 7-
366 O-glucuronide and Naringenin 4'-O-glucuronide showed a similar variation trend under
367 different light treatments, with the highest amount under white light and only a relatively
368 small amount (100-fold lower) under the monochromic lights, as well as in the dark. The
369 Daidzin concentration showed the highest amount under white light, while a
370 significantly lower amount under blue light (decreased by 58.73%). Compared with the
371 white light control, the concentration of 6"-O-Malonyldaidzin and Apigenin 7-O-
372 apiosyl-glucoside was significantly increased by monochromatic lights, and was more
373 pronounced under UV-A. The concentration of Apigenin 6-C-glucoside was
374 significantly decreased by FR (decreased by 7.99%) when compared with the white light
375 control. The concentration of Apigenin 7-O-glucoside was significantly increased by red

376 (33.81%), green (35.19%) and blue light (19.23%). The concentration of Chrysin and
377 Galangin was significantly increased by monochromatic lights among which far-red and
378 red light exhibited the highest promotion effects (**Fig. S2**).
379 To better evaluate the phenolic metabolite difference between UV-A and the white light
380 control, OPLS-DA was constructed ($R^2X = 0.813$, $Q^2 = 0.140$) (**Fig.5E**). It is showed
381 that UV-A and white light control were clearly separated. S-plot was further used to
382 screen the key phenolic metabolites that contributed to the differentiation between UV-
383 A and white light control. In total, nine compounds (VIP $\geqslant$ 1), including C5, C6, C11,
384 and C12, etc., were identified (**Fig.5F**).
385 **Effect of light spectra on the antioxidant capacity**
386 The antioxidant capacity of soaked seeds and soybean microgreens were evaluated by
387 three in vitro assays, DPPH, FRAP, and ABTS (**Fig. 6**). Generally, these three
388 quantitation methods showed similar results that the antioxidant capacity increased
389 significantly after seed germination. As shown in the seedling of microgreens, light
390 illumination could significantly increase the antioxidant capacity, when compared with
391 the darkness. Under light conditions, compared with the white light control, UV-A was
392 found to have the highest antioxidant capacity. For example, the DPPH value and FRAP
393 value under UV-A were 1.32 and 1.25-fold higher than that of the white light control,
394 respectively. Moreover, blue light also showed a significant increase in the FRAP value,
395 which was 1.16-fold higher than that of the white light control. The antioxidant capacity
396 was significantly decreased under far-red, red and green light conditions, as compared

to the white light control. Also, no significant difference was observed between the above mentioned three treatments for DPPH and FRAP values. However, the ABTS value under green light was significantly lower, as compared to that under far-red and red light.

Transcript levels of genes related to phenolic compounds biosynthesis

The expression of structural genes related to phenolic compounds biosynthesis was determined. The schematic showed the main phenylpropanoid pathway branches through which phenolic compounds are synthesized, and the key enzymes were marked in red (**Fig. 7A**). Under light illumination conditions, the seven analyzed genes showed higher transcript levels in both cotyledon and hypocotyl (**Fig. 7B-H**). Compared with the white light control, transcript levels of *GmCHS*, *GmF3H*, *GmF3'H*, *GmIFS,* and *GmIOMT* in both cotyledon and hypocotyl tissues were significantly increased under both blue light and UV-A ($p < 0.05$). As observed in the hypocotyls, far-red light up-regulated the transcript levels of *GmCHI*, *GmFLS,* and *GmIOMT*, red light also up-regulated gene expression of *GmFLS*, while no significant difference was made by other monochromatic lights ($p > 0.05$). Besides, the gene expression of *GmIOMT* in the cotyledon tissue was down-regulated by green light (Fig. 7H).

Discussion

It has long been established that phenolic compounds are ubiquitous in plants and are an important part of the human diet. Therefore, there is a growing interest in the studies

418 focused on increasing the phenolic compounds content in vegetables and fruits. In recent
419 years, many studies have reported that light altered the accumulation of the secondary
420 metabolites in plant seedlings.[5, 8-9, 27] However, the light spectral conditions are not
421 uniform in those studies, thus the research results are inconsistent and are less
422 comparable. In this study, the light spectra were precisely regulated using the LED lamps
423 and the effects of light spectra on the accumulation of the phenolic compounds in
424 soybean microgreens were systematically investigated. Our results further provided
425 evidence to support the beneficial role of LED light in the improvement of nutrient
426 values of microgreen vegetables.
427
428 As shown in **Fig. 1**, light exposure significantly decreased the fresh weight and the yield
429 of soybean microgreens, when compared with the dark. This could be attributed to the
430 inhibited elongation of the hypocotyl under the light during photomorphogenesis, which
431 resulted in lower water absorption ability and lower fresh weight.[39] Moreover, more
432 macro-molecules (e.g. protein, lipid and carbohydrate) were consumed to release energy
433 for the growth and to be re-used for new compounds biosynthesis in the seedlings grown
434 under light.[27] Therefore, the significantly lower yield of light-grown soybean
435 microgreens may also be attributed to the high macro-molecules metabolization level,
436 which causes the loss in dry mass. The inhibition effect of far-red light on hypocotyl
437 elongation was well documented.[40-41] Indeed, far-red light was found to significantly
438 reduce the fresh weight and hypocotyl length (data not shown) in this study, exactly

agree with the previously reported results (**Fig. 1A**). Under light conditions, there is no significant difference in yield between different light spectra treatments, even though the yield was slightly decreased under far-red light and UV-A (**Fig. 1B**). Therefore, the phenotype of the soybean microgreens could be changed to some extent by light spectra, while the yield was unaffected, indicating that light spectra treatment with low light intensity is suitable for the culture of microgreens and has the potential energy savings for commercial-scale in horticulture.

Light was reported to induce the accumulation of phenolic compounds in plants by promoting the production of malonyl CoA.[42-45] The present study confirmed these findings, showing that the TPC in soybean microgreens grown under light was higher than that grown in the dark (**Fig. 2A**). It was found that blue light and UV-A were more conductive that resulted in increased TPC, and similar results were reported on Chinese kale sprouts and basil microgreens.[45-46] The effect of green light on plant growth and development was once underestimated. Recently, green light was shown to positively regulate plant growth, photosynthesis, drought tolerance, as well as phytochemical accumulation.[47-48] For example, supplemental green light for high pressure sodium lamp (HPS) lighting or natural illumination was reported to increase ascorbic acid, anthocyanin and total phenol content in lettuce.[49] However, in this study, TFC under green light was significantly lower than that of the white light control and other light spectra treatments (**Fig. 2B**). This might be attributed to the reason that green light

functions as a signal to inform seedlings of photosynthetically unfavorable conditions, and slow down the production of precursors of phenolic compounds.[50] Given TFC, blue light and UV-A also showed promoting effects on the accumulation of TFC, indicating that blue light and UV-A are optimal monochromatic lights to produce soybean microgreens rich in phenolic compounds. It was observed that TPC and TFC were not sensitive to red and far-red light, which was inconsistent with the previous studies on pea sprouts and apple, showing that red and far-red light inhibits the phenolic accumulation.[51-52] Therefore, the effect of light spectra on phenolic metabolite might vary among different plant species.

Previous studies have demonstrated that isoflavone is the main phenolic compounds in soybean.[53] For this reason, it is not surprising that isoflavone accounted for the largest proportion in the phenolic component of soybean microgreens (**Fig. 3, Fig. 4**). The decrease in isoflavones content in germinated soybean cotyledons was considered associated with the translocation of isoflavones from cotyledons to radicles.[54] It hasn't been clearly explained whether the phenolic compounds are synthesized *de novo* in the soybean microgreens, or if they are originated from the cotyledons (soybean seeds). Here, the monochromatic LED lights did not elicit a strong variation in the proportion of phenolic subfamilies. The possible explanation is that the newly biosynthesis and the transport of phenolic compounds both occurred, and more exploration is needed about metabolism and distribution of phenolic compounds. The elicit effects of light on the

481 secondary metabolites are primarily associated with their photon energy. It was
482 documented that the short-wavelength light, with high photon energy, was more
483 sufficient to excite photochemical responses than long-wavelength light, with low
484 photon energy.[55-56] On the basis of OPLS-DA, UV-A, which has the shortest light
485 wavelength, was separated from other monochromatic lights. Whereas, other
486 monochromatic lights could not be separated satisfactorily (**Fig.5 A, B**). Such a result
487 could at least partially be explained by the resource availability hypothesis, showing that
488 the secondary metabolites could be declined under low light intensity.[57] In this study, 10
489 out of 66 phenolic compounds were found to have both optimal loading score and
490 variable importance (**Fig. 5**). UV-A showed a significant effect in promoting the
491 accumulation of the selected 10 phenolic compounds, implying that those compounds
492 might play important roles in screening UV-A irradiation. Similar results were obtained
493 from the previous studies on soybean and radish sprouts.[32] Among the light spectra, red
494 and blue light have attracted much attention, not only because they fit perfectly with the
495 absorbance of chlorophylls and allowed a higher photosynthetic activity, but affect plant
496 morphogenesis through phytochromes, phytotropins, and cryptochromes.[58] Besides, red
497 and blue light were also reported to modify metabolism.[59] In this study, red and blue
498 light was found to significantly increase the content of C19 (Apigenin 7-O-glucoside)
499 and C37 (Galangin), while decreasing the content of C12 (Dihydrodaidzein 7-O-
500 glucuronide) (**Fig. S2**). It was reported that the content of phenolics was increased by
501 supplemental red light, while decreased by supplemental far-red light.[60] Similar results

were obtained in this study, for example, Apigenin 6-C-glucoside content was decreased by far-red light, while Apigenin 7-O-glucoside content was increased by red light (Fig. 4). Green light decreased the accumulation of phenolic compounds, the results are consistent with the study on lettuce which also accumulated fewer phenolics when blue light was replaced by green light.[61] Based on the points mentioned above, the effect of each monochromatic light on the monomer content of phenolic compounds is different, and it is difficult to maximize the monomer content of each compound under one light condition. Nevertheless, monochromatic light can be used to increase the content of targeted metabolites. The content of phenolics was reported to be increased or decreased under blue light and red light, respectively, as compared with white light.[45] However, it was noticed here that white light was more conducive to the enrichment of certain phenolic compounds (such as C11, C12, C29) than the monochromatic lights (**Fig. 4, Fig. 5**). Such a discrepancy could be, at least partially, explained by the different plant species used between the results of ours and Qian et al.'s[45]. As previously reported, TPC of green basil microgreens and red basil microgreens was improved under high red: blue ratios (2R:1B) and low red: blue ratio (1R:1B), respectively, as compared with the white light.[62] Thus, the combination of monochromatic light might be more conducive to the accumulation of phenolic metabolites. Therefore, further endeavors should be focused on studying the suitable light "recipes", including light spectra, light intensities, and photoperiod, to optimize the accumulation of the phenolic metabolites.

523 Phenylpropanoid pathway genes could be activated by light.[63] The results of the present
524 work also showed that the accumulation of the phenolic compounds is correlated with
525 the transcriptional levels of the key structural genes in soybean microgreens phenolic
526 compounds biosynthesis (**Fig. 6**). After establishing the expression levels of those key
527 genes, we identified two sets of gene expression patterns that were light-dependent and
528 tissue-dependent. One clear expression pattern showed that UV-A and blue light induced
529 greater expression of seven major structural genes and significantly changed the profile
530 of total phenolic contents, especially the antioxidant capacity (DPPH, FRAP, ABTS)
531 (**Fig. 6**, **Fig. 7**). Another pattern was found for the genes regulated by other light spectra
532 (white, far-red and red), which were able to activate expression of all seven genes, but
533 the expression level of these genes was much lower than that under UV-A and blue light.
534 This result suggests that the longer wavelength lights with lower light intensity may be
535 not sufficient to trigger the transcription of these genes, or that other genes of the
536 phenylpropanoid pathway we have not identified may be involved in the biosynthetic
537 regulation. On the other hand, the above-mentioned difference might also be attributed
538 to the fact that UV-A and blue light are more energetic than that of far-red, red and white
539 light, which give fewer photons within the longer wavelength. These differences could
540 affect the accumulation of the phenolic metabolites. Furthermore, among these seven
541 genes we studied, the expression of *GmIFS* was most upregulated in both hypocotyl and
542 cotyledon under UV-A. This indicates that the effect of UV-A on the phenolic
543 accumulation and antioxidant capacity might be related mostly to the activation of

544 *GmIFS*, whose expression was much higher than that of the *GmCHI*, *GmF3'H*, *GmF3H*
545 and *GmIOMT* genes. However, no increase in the expression of *GmCHS*, *GmIFS* under
546 green light treatment was observed except *GmIOMT* and *GmF3'H* that showed a slightly
547 higher expression in the hypocotyl. The results agree with the previous report showing
548 green light was less effective than other LED light spectra in triggering genes involved
549 in flavonoid compounds biosynthetic pathways.[64] It was also noticed that the expression
550 of *GmCHI*, *GmFLS* and *GmIOMT* far-red light was much higher than that under white
551 light control, which was consistent with the study on *Arabidopsis*, showing that far-red
552 light promotes the accumulation of anthocyanin.[65] Hence, our results indicated that the
553 biosynthesis of these phenolic compounds in soybean microgreens under different light
554 regimes might be precisely controlled by the cooperation of multiple phenylpropanoid
555 biosynthesis genes in response to different light illumination.[66] The gene activation is
556 more likely coordinately regulated with structural genes by stress stimuli such as UV
557 light or other monochromatic light. This coordinate control is due to key regulators that
558 are activated by stress signals in the metabolic pathways. However, the amount of
559 metabolites cannot be completely explained by the transcript levels of the structural
560 genes. Further study on the identification of the transcription factors and additional
561 potential transcriptional regulators involved in phenylpropanoid pathway and phenolic
562 biosynthesis may provide deep insight into the results obtained in this study.[67] Besides,
563 the role of the photoreceptors in light-regulated phenolic biosynthesis remained to be
564 clarified. It is clear that extensive transcriptomic and reverse genetic studies are required

565 to understand transcriptional regulation of metabolic pathways.

566

567 The antioxidants in our dietary have become a topic of increasing interest in recent years.
568 Dietary antioxidants, including phenolic compounds, are of great help to increase the
569 nutrient value and maintain human health.[68] To date, no single test can explain all of the
570 antioxidant capacity *in vitro*, thus three assays were used to give comparable results of
571 the antioxidant capacity in soybean microgreens. In this study, the maximum DPPH,
572 FRAP and ABTS values were found in soybean microgreens grown under UV-A,
573 followed by blue light and white light (**Fig. 6**), agreeing with the results on pea sprouts
574 and Chinese kale sprouts.[45, 52] The higher antioxidant capacity under white light, blue
575 light, and UV-A might be related to the higher phenolic compounds content, more
576 precisely to the higher transcript level of phenylpropanoid pathway genes. The above-
577 mentioned result might be explained by the structure-activity relationship, for example,
578 the presence and number of hydroxyl groups and resonance effects.[69] Also, in our
579 experiment condition, the difference in antioxidant capacity between far-red, red and
580 green light was only observed when using the ABTS assay. Therefore, the ABTS assay
581 might be more effective for further researches on the antioxidant capacity analysis in
582 soybean microgreens.

583

584 In summary, our results showed that LED light spectra treatment, especially blue light
585 and UV-A, could alter the profile of phenolic compounds and the antioxidant capacity

586 of soybean microgreens. The antioxidant capacity is positively affected by the light
587 spectra treatment (**Fig. 8**). Therefore, the application of LED light can be beneficial for
588 the production of phenolic compounds-rich functional vegetable with higher antioxidant
589 capacity. Future experiments are needed to optimize the effect of blue and UV-A light,
590 and LED "recipes" (a combination of different light spectra) can be customized to
591 maximize the content of phenolic compounds. The profile of the phenolic compounds
592 could be partially explained by the expression of genes related to phenylpropanoid and
593 flavonoid biosynthesis. Consequently, further studies on the transcriptomic and
594 metabolomics will be able to provide a better understanding of light-regulated phenolic
595 compounds biosynthesis.
596

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603 Notes

604 The authors declare no competing financial interest.

Supporting information

Supplementary Fig. S1 Relative spectral distribution of different LED light spectra from 300nm to 800nm.

Supplementary Fig. S2 Effect of light spectra on the morphology of soybean microgreens. Photo were taken at 6 days after sowing. D, dark; W, white light; FR, far-red light; R, red light; G, green light; B, blue light; UV-A, ultraviolet A. Bar = 1 cm.

Supplementary Fig. S3 Effect of light spectra on the relative abundance of ten selected phenolic compounds. SS, soaked seeds; D, dark; W, white light; FR, far-red light; R, red light; G, green light; B, blue light; UV-A, ultraviolet A. A, C5: 6''-O-Malonyldaidzin; B, C6: 6''-O-Malonylgenistin; C, C11: Daidzin; D, C12: Dihydrodaidzein 7-O-glucuronide; E, C17: Apigenin-6-C-glucoside; F, C18: Apigenin 7-O-glucoside; G, C19: Apigenin 7-O-glucoside; H, C20: Chrysin; I, C24: Luteolin 7-O-malonyl-glucoside; J, C29: Naringenin 4'-O-glucuronide; K, C37: Galangin. ND means not detected. Values are the mean ± SE of triplicate (n = 3). The different letters represent significant differences among various treatments ($p < 0.05$).

Supplementary Table S1 Sequence-specific primers used in this study

Supplementary Table S2 Identification of phenolics compounds by HPLC-ESI-TOF/MS in the extracts of soybean microgreens.

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Figure Legends

Fig. 1 Effects of light spectra on the fresh weight (A) and yield (B) of soybean microgreens at 6 days after sowing. D, dark; W, white light; FR, far-red light; R, red light; G, green light; B, blue light; UV-A, ultraviolet A. Values are the mean ± SE of triplicate (n = 3). The different letters represent significant differences among various treatments ($p < 0.05$).

Fig. 2 Effects of light spectra on the total phenolic content (A) and total flavonoid content (B) of soaked seeds (SS) and soybean microgreens. For the microgreens, plants were grown in the dark for 2 days, and then exposed to different LED lamps (W, white light; FR, far-red light; R, red light; G, green light; B, blue light; UV-A, ultraviolet A). The microgreens constantly grown in the dark was referred as dark treatment (D). The microgreens were harvested at 6 days after sowing. Values are the mean ± SE of triplicate (n = 3). The different letters represent significant differences among various treatments ($p < 0.05$).

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Fig. 4 Heat map of identified phenolic compounds in soaked seeds (SS) and soybean microgreens cultured under different light spectra. The microgreens were grown in the

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859 metabolites are shown in blue (colour key scale can be found in bottom left corner of the
860 heat map).
861
862 **Fig. 5** Profiling of phenolic metabolites in soybean microgreens using OPLS-DA to
863 discriminate the effects of different light spectra. The microgreens were harvested at 6
864 days after sowing. (A) PCA score plot, R^2X=0.491, Q^2=0.152; (B) OPLS-DA score plot,
865 using metabolites as X variables and light treatments as predicted Y variables. R^2X =
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867 plot showing the best biomarker phenolic metabolites, using VIP along vertical axis
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869 light control (W); (F) OPLS-DA S-plot of UV-A vs white light control. The red circles
870 represent the most differential phenolic compounds.
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872 **Fig. 6** Effects of light spectra on the antioxidant capacity of soybean microgreens
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880 **Fig. 7** Effects of light spectra on the transcript levels of genes related to phenolic
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885 Schematic of the representation of phenolic compound biosynthetic pathways.
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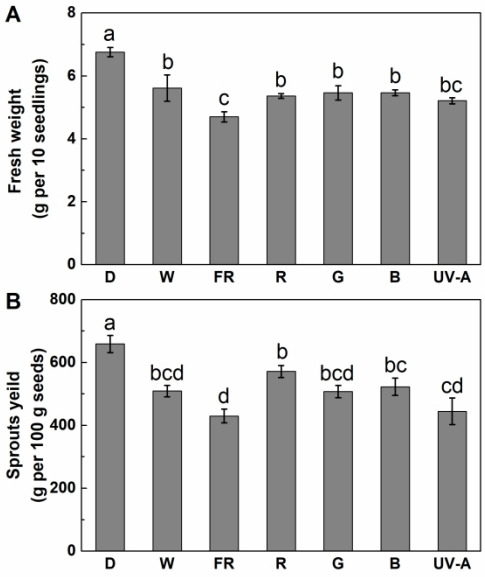

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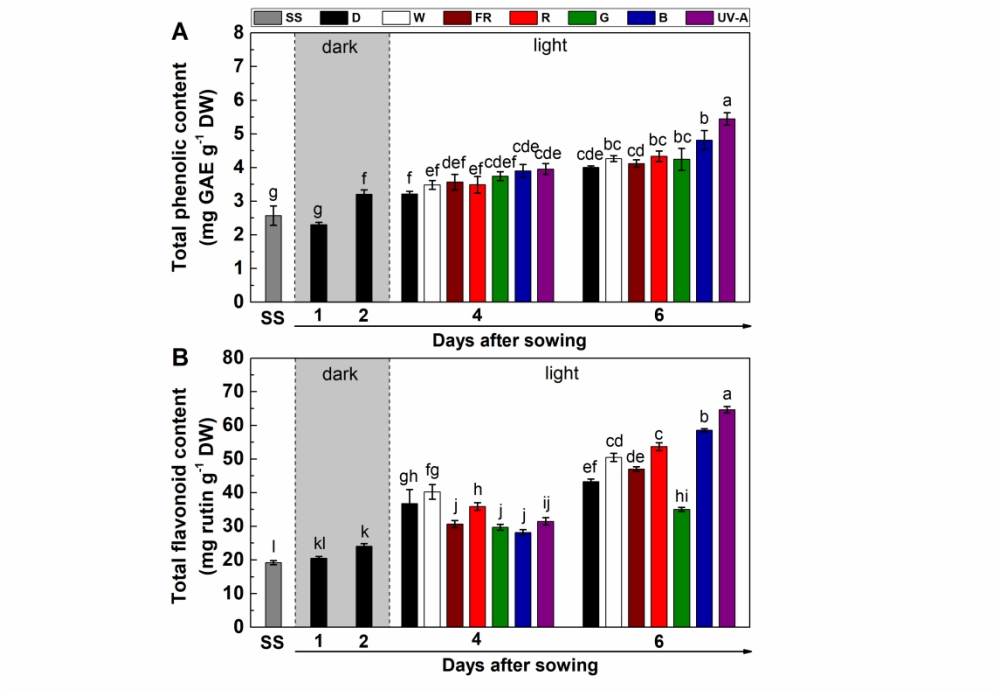

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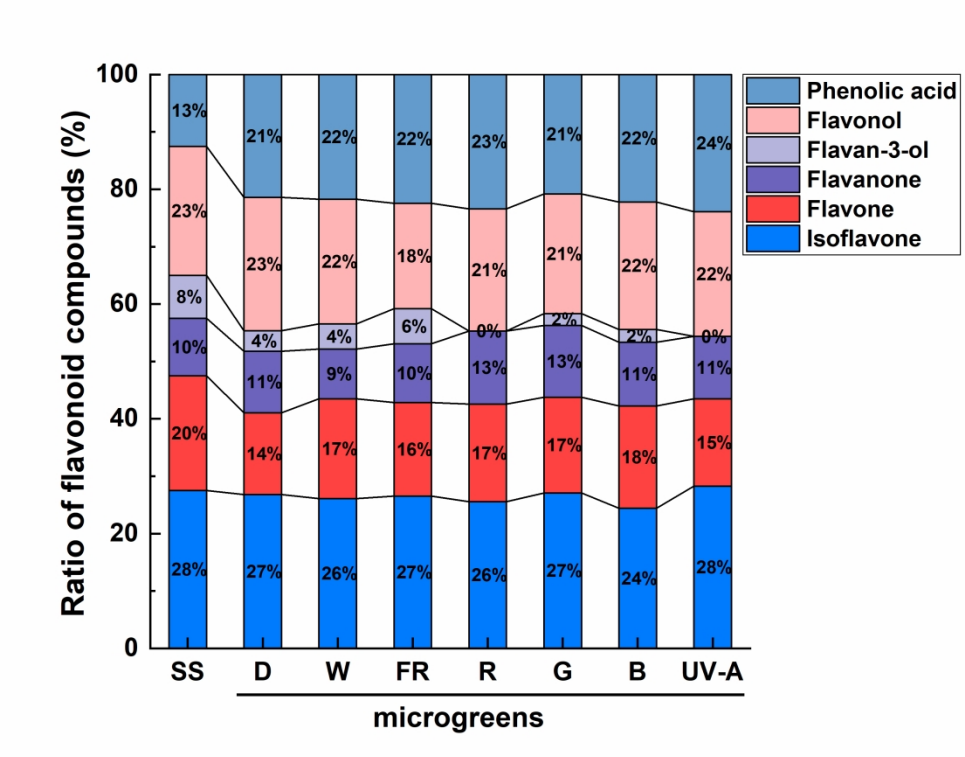

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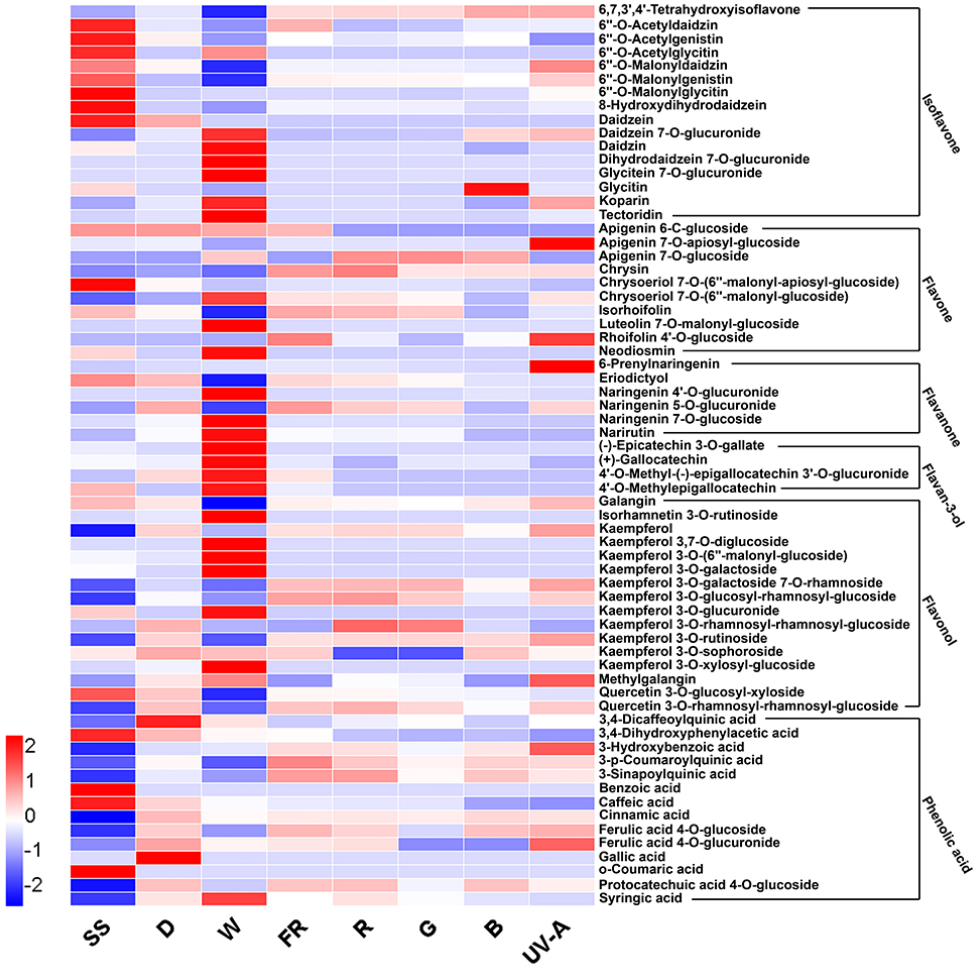

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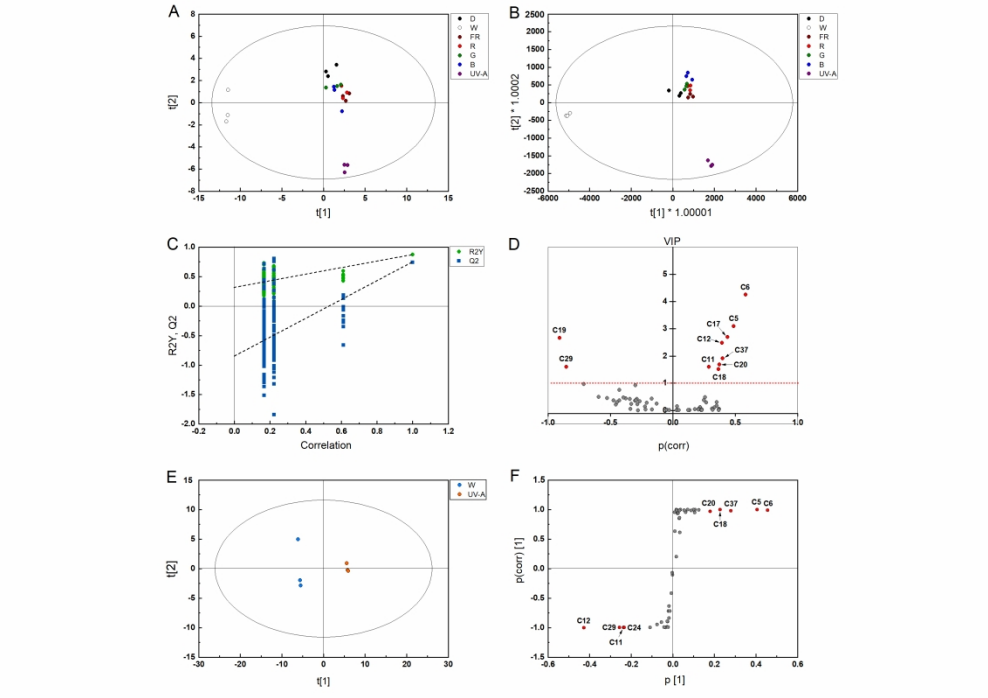

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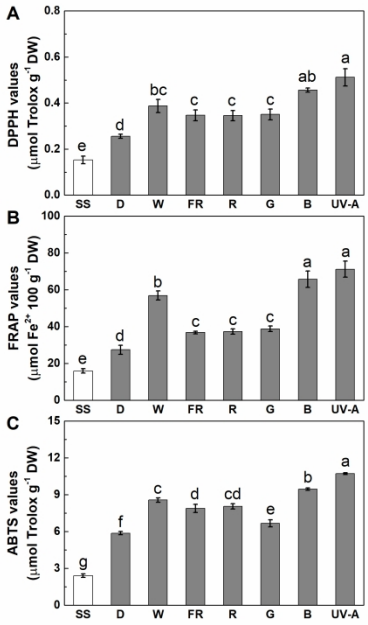

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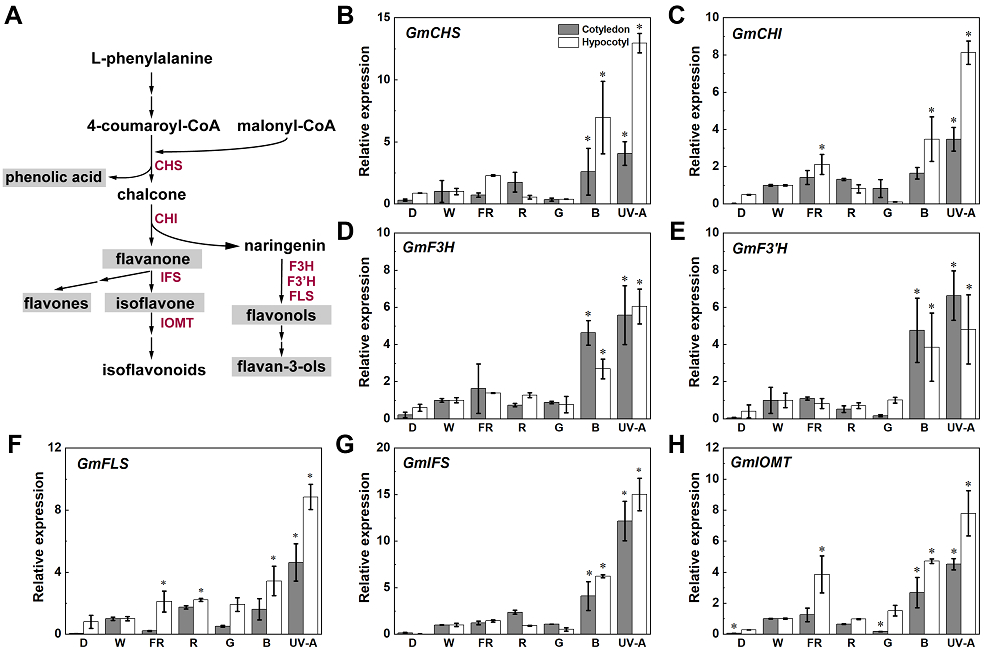

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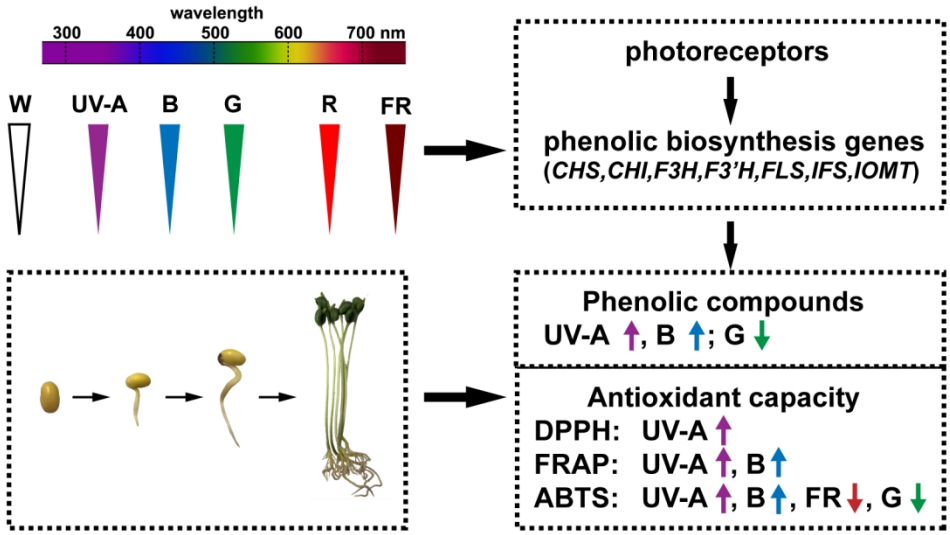

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