[Title]

Blue LED light promotes indican accumulation and flowering in indigo plant, *Polygonum tinctorium*

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Abstract

The photoperiod is a day-length-dependent seasonal change of physiological or developmental activities, such as flowering, in many plant species. *Polygonum tinctorium* (*P. tinctorium*) is an important industrial crop producing indigo blue dyes, and indican is an important substance as a precursor of indigo. Here, we report the day-length-dependent responses of growth, flowering, and indican synthesis in *P. tinctorium*. Indigo plants were grown in a hydroponic system under artificial light conditions in a completely-controlled plant factory. The growth parameters and indican content of leaves were measured and compared. Certain growth parameters (such as fresh weight and number of leaves) under 24-h continuous irradiation were significantly higher than those under other day-length conditions. Under 12-h photoperiod conditions, the flowering rate of plants with blue LED treatment increased six-fold compared with fluorescent white light treatment, while none of the plants flowered with red LED treatment. In the leaves, the relative expression levels of *Pt'IGS* and *Pt'BGL* were significantly higher in newer leaves compared to older ones. Indican content was greatly enhanced by blue light under 24-h continuous irradiation, which was reflected by increased expression levels of *Pt'IGS*. These findings demonstrate that there is a new regulatory mechanism for the indican synthesis pathway through blue light signalling. Blue light provides feasible strategy for artificially regulating indican synthesis and flowering in *P. tinctorium*.

Keywords: *Polygonum tinctorium,* Plant factory, Blue LED, Photoperiod, Flowering, Indican

1. Introduction

Light is a critical environmental factor that influences certain developmental processes in plants, all of which eventually affect plant productivity. These processes include seed germination, photomorphogenesis, chlorophyll synthesis, plant and leaf architecture, flowering, and fruit quality (Mawphlang and Kharshiing, 2017). Many flowering plants detect seasonal changes in night length or photoperiod. For instance, long-day (LD) plants, such as wheat (*Triticum aestivum*) and lettuce (*Lactuca sativa*), flower when night length falls below their critical photoperiod. In comparison, short-day (SD) plants, such as rice (*Oryza sativa*) and soybeans (*Glycine max*), flower when night length exceeds their critical photoperiod. Because the timing of flowering is determined by the photoperiod, the flowering of LD and SD plants is accelerated under LD and SD conditions, respectively. However, several studies have examined the relationship between flowering and light quality in an LD model plant *Arabidopsis*. Specifically, continuous red light is considered to inhibit flowering, while continuous blue light promotes flowering (Mockler et al., 2003; Guo et al., 1998). Rice is an SD model plant, with blue light accelerating flowering and red light delaying it (Hirose et al., 2006; Itoh et al., 2010; Izawa et al., 2000; Takano et al., 2005). Thus, light quality under LD and SD conditions represents an important environmental factor that affects the timing of flowering and gene expression through initiating the signalling cascade of photoreceptors, including red/far-red absorbing phytochromes (PHYs) (Chen and Chory, 2011), blue/UV-A absorbing cryptochromes (CRYs) and phototropins (PHOTs) (Christie et al., 2015). CRY1 has a major role in regulating seedling de-etiolation under blue light, whereas CRY2 is involved regulating flowering in response to day-length (Liu et al. 2011). PHOT1 and PHOT2 overlap in function to regulate

hypocotyl and root phototropism, chloroplast accumulation movement, stomatal opening, leaf positioning and leaf flattening (Christie et al., 2015). Among these, PHYA, PHYB, and CRY2 in *Arabidopsis* and their homologs in rice, have been identified to be related to flowering (Su et al., 2016). Molecular studies of the timing of flowering and light quality have been primarily conducted on *Arabidopsis* and rice. However, several photoreceptors are always simultaneously activated by sunlight, the signal conduction performed by different photoreceptors is not independent but interferes with or depends on each other, and the relationship among photoreceptors may be also related to the light environment and specific plant physiological activities (Chen et al., 2019). In *Arabidopsis,* antagonism or synergism between PHYs and CRYs in de-etiolation, shade avoidance responses and flowering were reported (Casal, 2000). Thus, studies on other species are needed to understand the genetic diversity of the control of flowering in relation to light quality.

Over the last 10-years, light emitting diodes (LED) have been used as source of light in plant factory systems where completely-controlled environmental conditions are needed. It is generally assumed that, compared with other light sources, LEDs irradiate more photosynthetically or photomorphogenetically effective wavelengths of light. Initially, red LEDs were used as a light source to promote photosynthesis (Morrow, 2008). Blue light has been documented to influence vegetative growth, photomorphogenesis, stomatal opening, chlorophyll synthesis, and the production of secondary metabolites (Islam et al., 2012; Nascimento et al., 2013). In *Arabidopsis,* a low intensity of blue LED light superimposed on red LED light induced increased growth in green tissue and that the growth enhancement was particularly prominent when the plants were cultivated under low red LED light (Takemiya et

al., 2005). In rice, blue LED light supplementation increased the proper growth and total nitrogen content of leaves, compared with red LED light alone (Ohashi-Kaneko et al., 2006). Interestingly, leaf lettuce grown under alternating irradiation of red and blue LEDs exhibits a greater growth-promoting effect compared to the simultaneous irradiation of red and blue LEDs (Shimokawa et al., 2014). Similarly, alternating red and blue light with an interval of 1 h enhances the accumulation of biomass, sucrose and starch in lettuce (Chen et al., 2019). In addition, monochromatic blue LED light influences the production of secondary metabolites; Blue LED light induces levels of anthocyanin accumulation in strawberry, *Fragaria* × *ananassa* cv. Sachinoka and Fengguang (Kadomura-Ishikawa et al., 2013; Xu et al., 2014). Furthermore, blue LED light promotes the accumulation of soluble sugars in non-heading Chinese cabbage (Fan et al., 2013). Thus, the effects of single lights versus combinations of lights (simultaneous or alternating) on several developmental processes and secondary metabolite production have been investigated in many plant species.

Polygonum tinctorium is an important industrial crop producing indigo blue dyes. Indigo plant has been a useful material for traditional herbal medicine in Japan to exhibit anti-oxidant, anti-allergic effect, anti-cancer and anti-inflammatory activities (Kunikata et al., 2000; Lin et al., 2009; Jang et al., 2012; Heo et al., 2014; Tokuyama-Nakai et al., 2019). It has many varieties, including “senbon” with red flowers and rounded leaves, and “kojoko” with white or red flowers and pointed leaves. The healthy leaves of indigo plants produce reactive indoxyl and efficiently glycosylate it with UDP-glucosyltransferase / indoxyl-b-D-glucoside synthase (UGT / IGS) to generate indican. Indican is a major metabolite of colourless glucoside and is a precursor of indigo (Minami et al., 2000). Thus, healthy leaves do not have any blue

colouration. However, when the tissue of leaves is damaged, indican is degraded by beta-glucosidase (BGL) to indoxyl and glucose, followed by the dimerization of indoxyl by spontaneous oxidation to form indigo via a leucoindigo intermediate (Epstein et al., 1967; Hue et al., 2018). The intracellular localization of BGL and IGS proteins has been detected in chloroplasts and the endoplasmic reticulum (ER), respectively (Minami et al., 1999; Inoue et al., 2018). In mature plants of *P. tinctorium*, the first and second new leaves contain larger amounts of indican per gram of tissue than older leaves and the other tissues (Minami et al., 2000; Inoue et al., 2018). Indican occurs in concentrations as high as a few percentage of the wet weight of indigo plant leaves during plant growth; however, knowledge remains limits about the regulatory mechanisms of synthesis and accumulation of indican.

Light control is a key component of controlled-environment agriculture, especially for closed vertical farming systems where artificial lamps are the only light source for plant growth. Optimizing LED light conditions could contribute towards developing novel agricultural technologies, such as plant factories. Here, we used *P. tinctorium* “senbon” to investigate how the quality of LED lights affects the growth and timing of flowering under various day-length conditions. We also analysed indican content to determine the correlation between indican synthesis and light quality. Our findings are expected to provide feasible strategy for artificially regulating indican synthesis and flowering in *P. tinctorium*.

2. Materials and Methods

2.1. Growth conditions

Polygonum tinctorium cv. senbon (Tokushima Agriculture, Forestry, and Fisheries Technology Support Center, Tokushima Pref., Japan) was used and cultivated in a plant growth facility (plant factory) using circulation hydroponics and artificial LED lighting (a SHIGYO unit, Showa Denko, Japan) (Fig. 1A). For each experimental condition, *P. tinctorium* seeds were germinated in commercially available vermiculite, and grown at 22 ºC under fluorescent light (Panasonic, Japan). Light intensity was 100–160 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density (PPFD). The light cycle was 14-h light/10-h dark. When root length reached about 3 cm, seedlings were transferred to the hydroponic bed in the plant factory. Seedlings with five leaves were selected for the hydroponic experiments (Fig. 1B).

To examine the effect of LED light on the growth of *P. tinctorium*, we used 660-nm monochromatic red LED (PT R120V24, Showa Denko K.K., Japan), 450-nm monochromatic blue LED (PT B120V24, Showa Denko K.K., Japan), red/blue LED at a 2:1 ratio of 660-nm/450-nm (DPT 2RB120T33-M2, Showa Denko K.K., Japan) and fluorescent white light (Panasonic, Japan). The examined light/dark photo-cycles were 8-h/16-h, 12-h/12-h, 14-h/10-h, 16-h/ 8-h and 24-h/0-h. Fluorescent white light (FL) was used as a control. The day-length conditions for the control were 14-h/10-h light/dark photo-cycle (14-h photoperiod). Reflectors were used to prevent the contamination of lights from another shelf. The light intensity PPFD was measured by an RGB photon measuring instrument (Model-101EG, Nippon Medical & Chemical Instruments Co., Ltd, Japan). Nutrient solution Ootsuka-A formula (OAT-A formula, OAT Agrio Co., Ltd., Japan) was diluted twice, and used for hydroponic culture in the plant

151 factory. The diluted solution of Ootsuka-A formula was containing N (258 ppm), P_2O_5 (60 ppm),
152 K_2O (202.5 ppm), CaO (115 ppm), MgO (30 ppm), MnO (0.75 ppm), B_2O_3 (0.75 ppm), Fe
153 (1.35 ppm), Cu (0.015 ppm), Zn (0.045 ppm), and Mo (0.015 ppm). The pH and EC of the
154 hydroponic nutrient solution were kept at 5.8–6.9 and 0.8–1.2 $dS \cdot m^{-1}$, respectively. After 40
155 days of hydroponic culture, the plants were collected. The fresh weight of plants, number of
156 leaves, fresh weight of total leaves, length of main stem, number of branches and indican
157 content of leaves were measured.
158
159 ***2.2. Indican extraction and HPLC analysis***
160 Leaves were harvested, and the fresh weight of each sample was recorded. Leaves
161 were then frozen in liquid nitrogen. Indican was extracted by slightly modifying a previously
162 reported protocol (Minami et al., 2000). For HPLC analysis, a frozen leaf was broken in pieces
163 with a BioMasher II (Nippi, Japan) or a mortar in liquid nitrogen. It was then suspended in 0.5–
164 5.0 mL of a methanol-chloroform solution (CH_3OH:$CHCl_3$:H_2O = 12:5:3, v/v). The suspension
165 was centrifuged at 17,000 g for 15 min. The supernatant was saved, and the pellet was extracted
166 twice more in the same manner. The combined supernatants were mixed with 0.35~3.50 mL
167 chloroform and 0.5~5.0 mL H_2O. The mixture was then centrifuged. The aqueous layer was
168 used for the quantitative analysis of indican.
169
170 ***2.3. RNA isolation and quantitative RT-PCR analysis***
171 Leaves were homogenized in TRI Reagent with a BioMasher II (Nippi, Japan). Total
172 RNA extraction was performed by following the manufacturer's protocol. cDNA was

173 synthesised from 500 ng total RNA using the PrimeScript™ RT Master Mix (Perfect Real
174 Time) (TaKaRa, Japan). Quantitative RT-PCR (qPCR) was performed on a Thermal Cycler
175 Dice Real Time System (TaKaRa, Japan). Primer sequences for qPCR were based on cDNA
176 sequence of *P. tinctorium* 'senbon' *actin* (*Pt'act*) and previously reported *beta-glucosidase*
177 (*Pt'BGL*, AB003089). The following primer sets were designed using the Primer 3 software:
178 5'-GCT GGA AAC TGC CAA GAG CA-3' and 5'-GGG CAT CTG AAC CTC TCA GCA-3'
179 for *Pt'act*; 5'-CAA CCT CTT GGC AGC GTC AC-3' and 5'-CAA CCG CTC CCT CAT CGT
180 CT-3' for *Pt'BGL*. In *indoxyl-b-D-glucoside synthase* (*Pt'IGS*), 5'-AGA CGG TGT CAT GTG
181 AGT TTG C-3' and 5'-CGT CGT TCT TCC TAT CCT GAA-3' were used (Inoue et al., 2018).
182 PCR reactions were performed in a mixture containing diluted cDNA, 200 nM of each primer,
183 and 1x TB Green™ Premix Ex Taq™ II (TaKaRa, Japan). The following amplification
184 parameters were applied: 95 ºC for 30 s, followed by 40 cycles at 95 ºC for 5 s and 60 ºC for 30
185 s. The target quantity in each sample was normalised to the reference control (*Pt'act*) using a
186 comparative ($2^{-\Delta\Delta Ct}$) method following the manufacturer's instructions.
187
188 ***2.4. Transcriptome sequencing***
189 The seedlings of *P. tinctorium* were grown with monochromatic red or
190 monochromatic blue LED in a plant factory. Total RNA extraction from leaves was performed
191 using TRI reagent, as described in the previous section 2.3. Total RNA samples were used for
192 mRNA enrichment, fragmentation and cDNA library construction. cDNA library construction
193 and sequencing with NovaSeq 6000 System was performed at Macrogen Japan. Transcriptome
194 de novo assembly was performed to reconstruct the transcript sequences without the reference

genome sequence. To annotate the clustered unigenes, they were blasted against public databases with a blastx and BLASTN and BLASTX, including Kyoto Encyclopedia of Genes and Genomes (KEGG), NCBI Nucleotide (NT), Pfam, Gene ontology (GO), NCBI non-redundant Protein (NR), UniProt and EggNOG.

2.5. Statistical analysis

Data are presented as mean ± SD. Statistical analyses were performed using a two-tailed Student's *t*-test.

3. Results

3.1. Effects of various light conditions on growth and flowering during the hydroponic culture of P. tinctorium

As the control, we examined the hydroponic culture of *P. tinctorium* cv. senbon under fluorescent light (FL) under a stable 12-h light/12-h dark photo-cycle (12-h photoperiod), a stable 14-h light/10-h dark photo-cycle (14-h/10-h photoperiod) and 24-h continuous irradiation (24-h photoperiod) in the plant factory. New leaves were formed one at a time from the top of the stem, and the leaves had a rounded blade shape (Fig. 1C). Phyllotaxis was an alternate type of leaf that formed at each knot on the stem, and then new branches began to form sequentially at each petiole from the bottom of the main axis (Fig. 1D, E). The fresh weight (FW) of shoots was greatest under 24-h photoperiod at 40 days after planting, reaching significant level compared with other treatment in the present study (Fig. 2). In brief, the 24-h photoperiod produced the highest growth parameters, including number of the leaves (NL) and FW, the length of main stem and number of branched shoots (Table 1). No significant differences were detected for certain growth parameters, including FW and NL between the 14-h and 16-h photoperiods (data not shown). In comparison, the 12-h photoperiod resulted in flowering. Flower bud formation and blooming flowers were observed at the apical meristem of main stem within 66 days after planting during hydroponic culture under the 12-h photoperiod (Fig. 1F-H). However, flowering was not observed under a 14-h photoperiod nor other LD conditions.

The effects of light quality on plant growth and flowering time were examined in hydroponic system with simultaneous red and blue (R+B), monochromatic red (R) and monochromatic blue (B). The means of these light intensities were 197.8 ± 21.0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for

R+B, 151.5 ± 16.6 μmol·m^{-2}·s^{-1} for R, and 55.3 ± 6.5 μmol·m^{-2}·s^{-1} for B, respectively. Compared with the 14-h and 24-h photoperiods under simultaneous R+B treatments, there was no significant difference in the fresh weight of shoots (Fig. 2). The only difference occurred for the length of the main stem between the growth parameters of 14-h and 24-h photoperiod treatments (Table 1). Under the 12-h photoperiod, growth parameters were lower, with flowering being observed at a rate of 15.7% in the total shoot apical (Table 2). In the monochromatic R treatment, the fresh weight of shoots and their growth parameters were the highest under the 24-h photoperiod (Table 1). Of note, even though the growth parameters under the 12-h photoperiod with monochromatic R were similar to those under 12-h photoperiod with FL, flower bud formation was not observed in any shoot apicals under the 12-h photoperiod after 66 days of hydroponic culture (0.0%, Table 2). Compared with FL and simultaneous R+B, no morphological changes were observed in leaf shape and the phyllotaxis of plants under monochromatic R. In comparison, under monochromatic B resulted in poor growth based on the fresh weight of shoots and their growth parameters (Fig. 2 and Table 1). The length of the main stem under the 24-h photoperiod with monochromatic B was statistically indistinguishable from that under the 24-h photoperiod with FL (Table 1); however, there were significant differences in the number of nodes (10.8 ± 0.9 in monochromatic B vs 14.5 ± 1.3 in FL) and internode length (3.0 ± 0.2 cm in monochromatic B vs 2.5 ± 0.1 cm in FL). Thus, this phenomenon might be the increased internode elongation (spindly growth). In contrast to the monochromatic R treatment, the rate of flowering was highest under the 12-h photoperiod with monochromatic B treatment (87.6%, Table 2). Thus, flowering was noticeably enhanced by blue light in hydroponic *P. tinctorium*.

3.2. Effects of light quality on indican synthesis

To examine the effects of light conditions on indican synthesis, the indican content of leaves at three positions (1st, 4th and 7th) was compared under various light conditions. Under the 14-h photoperiod with FL, indican was present in 1.01 ± 0.18, 0.19 ± 0.04 and 0.14 ± 0.02 % at the 1st, 4th and 7th leaf position, respectively (Fig. 3). Under FL or simultaneous R+B treatment, indican content at the 4th and 7th positions of leaves was significantly higher for the 24-h photoperiod compared to the 14-h photoperiod. Similarly, under monochromatic R or B treatment, indican content at the 4th and 7th positions of leaves was higher for the 24-h photoperiod compared to the 14-h photoperiod. Under 12-h photoperiod with monochromatic B treatment, the 1st position of leaves had the lowest levels of indican content, but the highest flowering rate. *Pt'IGS* was expressed at relatively high levels in the 1st position of leaves compared with the other leaf positions (Fig. 4). The relative expression levels of *Pt'BGL* in the 1st position of leaves were also high dramatically, because the expression levels of *Pt'BGL* in the 4th and 7th leaves were not detectable with qPCR (Fig. 4).

3.3. Time-dependent change to the indican content of leaves

There was a significant difference in plant growth between blue light and other light conditions. The size of the 4th and 7th leaves also differed under each light condition. Of note, because the leaves at the 1st position where indican content was measured were approximately the same size, we expected that the amount of indican contents in the 1st leaves treated with blue light to be relatively higher. Under fluorescent lamps, the indican content of new leaves

gradually increased. After reaching 1.1%, no increase was observed. The results were the same for both the 14-h photoperiod and 24-h photoperiod (Fig. 5A). However, under the blue LED, the 14-h photoperiod and 24-h photoperiod differed. For the 24-h photoperiod, no further increase was observed in new leaves once 1.7% indican content was reached (Fig. 5A). In additional experiments, we found that the indican contents of leaves significantly increased (0.62 ± 0.05 % to 0.89 ± 0.13 %) when monochromatic blue LED light was changed from 60 to 120 μmol m^{-2} s^{-1} under the 12-h photoperiod, while no other significant changes were detected under LD conditions. Furthermore, when the light intensity of the red and blue LEDs was the same under 24-h photoperiod for 23 days, indican content was higher under blue light conditions (1.11 ± 0.14 % in red vs 1.45 ± 0.16 % in blue).

Time dependent changes to *Pt'IGS* and *Pt'BGL* expression differed under the 24-h photoperiod with blue LED, which promoted indican synthesis (Fig. 5B). For plants grown under a 24-h photoperiod, *Pt'IGS* and *Pt'BGL* expression increased, whereas *Pt'IGS* expression decreased at 38 days of growth. *Pt'IGS* expression under the 24-h photoperiod with blue LED was significantly higher than that under the 24-h photoperiod with FL during the early stage of growth. *Pt'BGL* expression under the 24-h photoperiod with blue LED was significantly higher than that under the 24-h photoperiod with FL at all times of day.

4. Discussion

Light controls many of the physiological processes of plants as signals. Plant responses are triggered by changes to light intensity, light quality and photoperiod. Thus, here, we investigated the growth, flowering and indican synthesis of indigo plant *P. tinctorium* under various photoperiod conditions with red and blue LED lights. In examination of the effect of photoperiods, the 24-h photoperiod promoted the greatest shoot biomass in *P. tinctorium*. Monochromatic red light increased the growth parameters more than monochromatic blue light; however, simultaneous red and blue conditions had higher growth parameters than monochromatic red conditions. Thus, simultaneous blue and red lights appear to be synergistic for *P. tinctorium* growth. In lettuce, alternating irradiation of red and blue LEDs exhibits a greater growth-promoting effect compared to the simultaneous irradiation of red and blue LEDs (Shimokawa et al., 2014; Chen et al., 2019). In *P. tinctorium*, compared to the simultaneous irradiation of red and blue light in the 12-h photoperiod, alternating red and blue light at 4-h and 12-h intervals produced low numbers of branches but increased stem length significantly (data not shown). Thus, alternating irradiation may negatively affect growth in *P. tinctorium*, likely repressing photomorphogenesis. In addition, we demonstrated that blue light intensity affects the stem growth: When the amount of blue and red light was reduced to 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$, the length of the main stem and number of nodes were lower in both cases. When the same intensity of red and blue LEDs was compared (60 $\mu mol \cdot m^{-2} \cdot s^{-1}$) under the 24-h photoperiod for 40 days, growth parameters (number of leaves, fresh weight, number of branches, etc.) were significantly lower in blue light condition. In the latter case, the length of the main stems reached 25 cm under monochromatic blue light faster than under monochromatic red light. There was no

310 difference in the number of nodes (11.8 ± 0.5 in blue vs 12.0 ± 0.8 in red), but the internodes
311 were increased under blue light (2.0 ± 0.1 cm in blue vs 1.6 ± 0.1 cm in red), implying that
312 promoted internode elongation (spindly growth). Thus, spindly stem growth may depend on
313 blue light intensity.

314 Light quality affects the flowering of *P. tinctorium*. A stable 12-h photoperiod under
315 FL induced flowering in hydroponic *P. tinctorium* (14.2%, Table 2), we used a stable 12-h
316 photoperiod as the SD condition for flowering. Monochromatic blue light had the highest
317 flowering rate. In comparison, blue and red light combined (e.g., blue + red) decreased the
318 flowering rate, while monochromatic red light entirely inhibited flowering. Blue light strongly
319 promoted flowering in *P. tinctorium*, whereas red light was suitable for growth without
320 flowering. Changing light at appropriate intervals (e.g., from red to blue) could induce the
321 formation of buds for flowering within 30 days (data not shown). Previous studies reported that
322 PHYA, PHYB and CRY1 regulate the timing of flowering in *Arabidopsis* and rice (*Oryza*
323 *sativa*). *PHYA* mutations in rice coupled with mutations in either *PHYB* or *PHYC* cause
324 dramatic early flowering (Takano et al., 2005). Thus, light receptors play an important role in
325 the photoperiodic control of flowering and red light might inhibit flowering in SD plants. *CRY2*
326 antisense transgenic rice flowers later than WT under both LD and SD conditions (Hirose et al.,
327 2006). Thus, blue light signalling can promote flowering in SD plants. Blue light accelerated
328 flowering, while additional red light delayed it, in the current study; thus, negative effects, such
329 as later flowering, was likely caused by red light under SD conditions in *P. tinctorium*.
330 Monochromatic blue light produced the highest indican content in leaves under LD conditions,
331 but the lowest under SD conditions (Fig. 3). For *P. tinctorium* cultivated throughout the whole

332 growth period, red and blue light had synergistic effects on growth under LD conditions;
333 however, blue light was the most efficient lighting strategy at producing high indican content in
334 leaves and greater flowering. The blue-light receptor gene *CRY2* and the far-red-light receptor
335 gene *PHYA* promote flowering under LD conditions in *Arabidopsis*, by contrast, red-light
336 receptor gene *PHYB* inhibits flowering under both LD and SD conditions in *Arabidopsis*
337 (Johnson et al., 1994; Guo et al., 1998; Mockler et al., 1999). These findings indicate that light
338 receptors play an important role in the photoperiodic control of flowering.

339 Under the 24-h photoperiod with monochromatic blue LED light, *Pt'IGS* transcripts
340 increased with indican content until 31 days of growth (Fig. 5). Thus, the accumulation of
341 indican was likely related to an increase in *Pt'IGS* transcripts. Subsequently, indican did not
342 increase any further under FL and monochromatic blue LED. Thus, this equilibrium might be
343 related to a decline in *Pt'IGS* expression. Interestingly, the number of *Pt'BGL* transcripts under
344 blue light increased significantly until 38 days of growth, compared with that under control.
345 Thus, blue light might regulate to enhance the accumulation of both indican and *Pt'BGL*
346 expression in *P. tinctorium* leaves. Although indican concentration reaches a few percentage of
347 the wet weight of leaves during plant growth, knowledge remains limited about the
348 accumulation mechanisms of indican *in vivo*.

349 The major bioactive compounds of indigo plants were identified to be indigo,
350 indirubin, and tryptanthrin (Liau et al., 2007). Indirubin is an isomer of indigo, and it is also a
351 metabolite of *P. tinctorium* (Xiao et al., 2002). In general, the yield of indigo and indirubin are
352 influenced by environmental factors and the quality of growing season (Wang et al., 2019).
353 Tryptanthrin was detected exclusively in the extracts of the leaves, but not in stems

(Tokuyama-Nakai et al, 2018). However, knowledge remains limited about the mechanisms of accumulation for indirubin and tryptanthrin. Indirubin is produced from indoxyl in a process similar to indigo *in vivo*, but no reports describe the tryptanthrin synthesis pathway in plants. RNA-seq analyses provide a powerful tool for acquiring transcriptomic information and identifying the genes involved in the biosynthesis and metabolism of natural products in plants. RNA-seq analyses for indican biosynthesis pathway genes were performed in two indigo plants, *Indigofera tinctoria* and *P. tinctorium* (Sarangi et al., 2015; Minami et al., 2015; Wang et al., 2019). We performed RNA-seq analysis of *P. tinctorium* grown under blue light versus red light in a plant factory. Interestingly, the relative expression of *Pt'IGS* and *Pt'BGL* in 1st leaves grown under monochromatic blue light was higher compared to that under monochromatic red light based on RNA-seq analysis, which was confirmed by qPCR analysis (Fig. 6).

RNA-seq analysis revealed that the major enzymes involved in indican accumulation were highly expressed under blue light condition, providing a credible insight towards understanding why indican accumulated at this treatment. It has been reported that indole can be oxidized to form indoxyl in the presence of CYP450, which promotes the transformation of accumulated indole to indoxyl (Song et al., 2011). The transgenic tobacco plants expressing both human *CYP2A6* and maize *indole synthase* (*bx1*) resulted in the formation of indoxyl and lower level of indican compared with in *P. tinctorium*, but did not develop the formation of indigo (Warzecha et al., 2006), indicating that the most indoxyl was less transformed to indican by ubiquitous glucosyl transferases (GTs). Thus, repression of *Pt'IGS* function in indigo plants or overexpression of specific *Pt'*IGS/UGTs in other indigo plants may be efficiently mimicked for biosynthetic pathway in metabolic engineering of plants to accumulate indigo or indirubin or

tryptanthrin. *Pt'*IGS is associated with ER membranes and may interact with ER membrane localized CYP450s in *P. tinctorium* (Inoue et al., 2018). There are seven unigenes encoding CYP450s and six unigenes encoding UGTs including *Pt'IGS* in *P. tinctorium* by RNA-seq analysis (Wang et al., 2019). However, the function of these genes and what controls the transcription of those genes including *Pt'IGS* and *Pt'BGL* were still unknown. Our findings revealed that LD condition with blue light, i.e. a blue light signaling is involved in transcription of *Pt'IGS* and *Pt'BGL* genes. Monochromatic blue light promoted the increase of *Pt'IGS* mRNA transcription in *P. tinctorium* compared with those under fluorescent light or red light, indicating that up-regulated *Pt'*IGS may promote indican accumulation in vacuoles. In addition, *Pt'BGL* was up-regulated by monochromatic blue light, indicating that up-regulated *Pt'*BGL may be stored in chloroplasts. Therefore, the leaf tissues highly accumulated indican and BGL can be used to produce more indigo and indirubin. In summary, it was found that blue light provides feasible strategy for artificially promoting the indican synthesis pathway and also flowering in *P. tinctorium*.

5. Conclusions

Regulating light conditions is a key component of controlled-environment agriculture, especially for closed vertical farming systems where artificial lamps are the only source of light for plant growth. *Polygonum tinctorium* is an SD plant, for which flowering is drastically enhanced by monochromatic blue LED light under 12-h photoperiods. In the blue light treatment, under 12-h or 24-h photoperiod showed the lowest or highest levels of indican content of leaves. Compared with the plants treated with fluorescent white light, treatment with monochromatic blue LED light under a 24-h photoperiod increased *Pt'IGS* and *Pt'BGL* expression and indican content of leaves. These results reveal the novel mechanism by which the indican synthesis pathway is controlled by blue light signalling to promote the accumulation of indican and BGL proteins in vacuoles and chloroplasts of *P. tinctorium* leaves, respectively. Blue light treatment could be used to control the indican synthesis pathway and flowering in *P. tinctorium* with high efficiency, by strictly using day-length in plant factories.

Declaration of Competing Interest

There are no conflicts to declare.

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Figure legends

Fig. 1. Hydroponic culture of the indigo plant *Polygonum tinctorium* under fluorescent light in a plant factory.

A, Type of circulation in the hydroponic system with artificial lights. B, Seedlings with five leaves, just after planting. C, New leaves are indicated by arrows, lateral and top view. No.1–5 in B and C correspond to each leaf position, respectively. D, New branches are indicated by arrows. E, Growth of *P. tinctorium* plants 14 days after planting. F, Initial flower bud is indicated by an arrow. G, Developing flower buds. H, Blooming flowers.

Fig. 2. Comparison of the fresh weight of plant shoots grown under SD and LD conditions.

Graphs show the effect of simultaneous red and blue (R+B), monochromatic red (R) or blue (B) LEDs on the growth of *P. tinctorium* and fluorescent light (FL) for control, in three different lighting conditions. 12-h represents a stable 12-h light/12-h dark photo-cycle, 14-h represents 14-h light/10-h dark photo-cycle and 24-h represents 24-h continuous lighting. Data are the means ± SD, n = 4~15 from two to three independent experiments, $P^{\#} < 0.05$ (14-h vs 24-h).

Fig. 3. Effects of light quality and photoperiod on the indican content of leaves at various positions.

Comparison of indican content in leaves at various positions using LED lighting (R+B, R, B) and control (FL) under the three photoperiods (12-h, 14-h, 24-h) for 30 days. 1st, 4th and 7th correspond to leaf position; 1st is the youngest leaf, 7th is the oldest leaf, and 4th is the

intermediate aged leaf. Data are the means ± SD, n = 4~15 from two to three independent experiments. $P^{\#} < 0.05$, $P^{\#\#} < 0.01$ (14-h vs 24-h); $P* < 0.05$, $P** < 0.01$ (12-h vs 14-h).

Fig. 4. Relative expression levels of genes involved in the indican synthesis pathway.

Relative expression levels of *Pt'IGS* and *Pt'BGL* at different leaf positions. 1st is the youngest leaf, 7th is the oldest leaf, and 4th is the intermediate aged leaf.

Fig. 5. Time-dependent changes to indican content and the expression of *Pt'IGS* and *Pt'BGL* during growth under LD conditions in *Polygonum tinctorium*.

A, Changes to indican contents under 14-h and 24-h photoperiods with FL or monochromatic blue LED. B, Changes to the relative expression level of *Pt'IGS* and *Pt'BGL* are shown for the 24-h photoperiod with FL or monochromatic blue LED. Data are the means ± SD, n = 4.

Fig. 6. Relative expression levels of *Pt'IGS* and *Pt'BGL* in the 1st leaves.

Plants were grown under SD conditions with monochromatic red LED (R) or monochromatic blue LED (B) treatment. A, Relative expression levels of *Pt'IGS* by quantitative RT-PCR (qPCR) and RNA-seq analyses. B, Relative expression levels of *Pt'BGL* by qPCR and RNA-seq analyses.

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Table 1 Results of the hydroponic culture of the indigo plant *Polygonum tinctorium* in a plant factory.

Light conditions		Number of leaves	Fresh weight of leaves (g)	Length of main stem (cm)	Number of branched shoots
FL(white)	12-h	211.0 ± 20.6	27.7 ± 5.4	15.8 ± 1.0*	27.0 ± 5.4
	14-h	223.0 ± 55.3	33.0 ± 11.5	22.7 ± 4.6	33.8 ± 7.2
	24-h	336.5 ± 69.2#	59.3 ± 14.4##	33.9 ± 2.4##	51.1 ± 12.8#
R+B	12-h	253.0 ± 35.5*	39.8 ± 6.6	16.8 ± 1.5**	38.0 ± 9.0
	14-h	356.3 ± 59.3	60.1 ± 19.3	23.1 ± 2.0	50.3 ± 10.4
	24-h	356.9 ± 40.7	60.9 ± 16.2	31.2 ± 3.6##	56.9 ± 9.3
R	12-h	217.3 ± 20.7	28.8 ± 3.4	18.0 ± 0.9*	32.5 ± 3.0
	14-h	236.5 ± 98.8	42.1 ± 18.3	20.6 ± 1.1	40.8 ± 12.5
	24-h	317.9 ± 37.0#	61.6 ± 13.5#	29.8 ± 3.3##	50.5 ± 5.0
B	12-h	91.3 ± 7.5	8.1 ± 1.3	13.9 ± 1.2*	12.5 ± 2.4
	14-h	94.7 ± 16.8	8.8 ± 2.9	17.7 ± 2.6	12.3 ± 2.6
	24-h	117.5 ± 20.1#	21.2 ± 6.6##	31.5 ± 5.8##	17.8 ± 2.9##

Plants were cultivated in a plant factory for 40 days under three different photoperiods (12-h, 14-h, 24-h) using LED lighting; simultaneous red and blue LEDs (R+B), monochromatic red LED (R), monochromatic blue LED (B) and control (FL). Data are the means ± SD, n = 4~12 from two to three independent experiments. $P^{\#} < 0.05$, $P^{\#\#} < 0.01$ (14-h vs 24-h); $P^{*} < 0.05$, $P^{**} < 0.01$ (12-h vs 14-h).

641 **Table 2 Effects of light quality on the flowering of *Polygonum tinctorium* under SD**
642 **condition.**
643

Light treatment	Number of flowering	Number of branched shoots	Rate of flowering (%)
FL (white)	8.1 ± 5.9	57.0 ± 11.5	14.2
R+B	11.8 ± 4.9	75.1 ± 15.2**	15.7
R	0.0 ± 0.0**	64.8 ± 14.7	0.0
B	30.9 ± 12.3**	35.3 ± 8.8**	87.6

644

645 Plants were cultivated under 12-h photoperiod conditions with fluorescent white light (FL),
646 simultaneous red and blue LEDs (R+B), monochromatic red LED (R) or monochromatic blue
647 LED (B) for 66 days in a plant factory. Data are the means ± SD (n = 8 or 12). Flowering rate is
648 the percentage of branch shoots with flower buds out of total branched shoots. Days to
649 flowering after the start of a stable 12-h photoperiod treatment. $P^{**} < 0.01$ (vs FL).

Fig 1

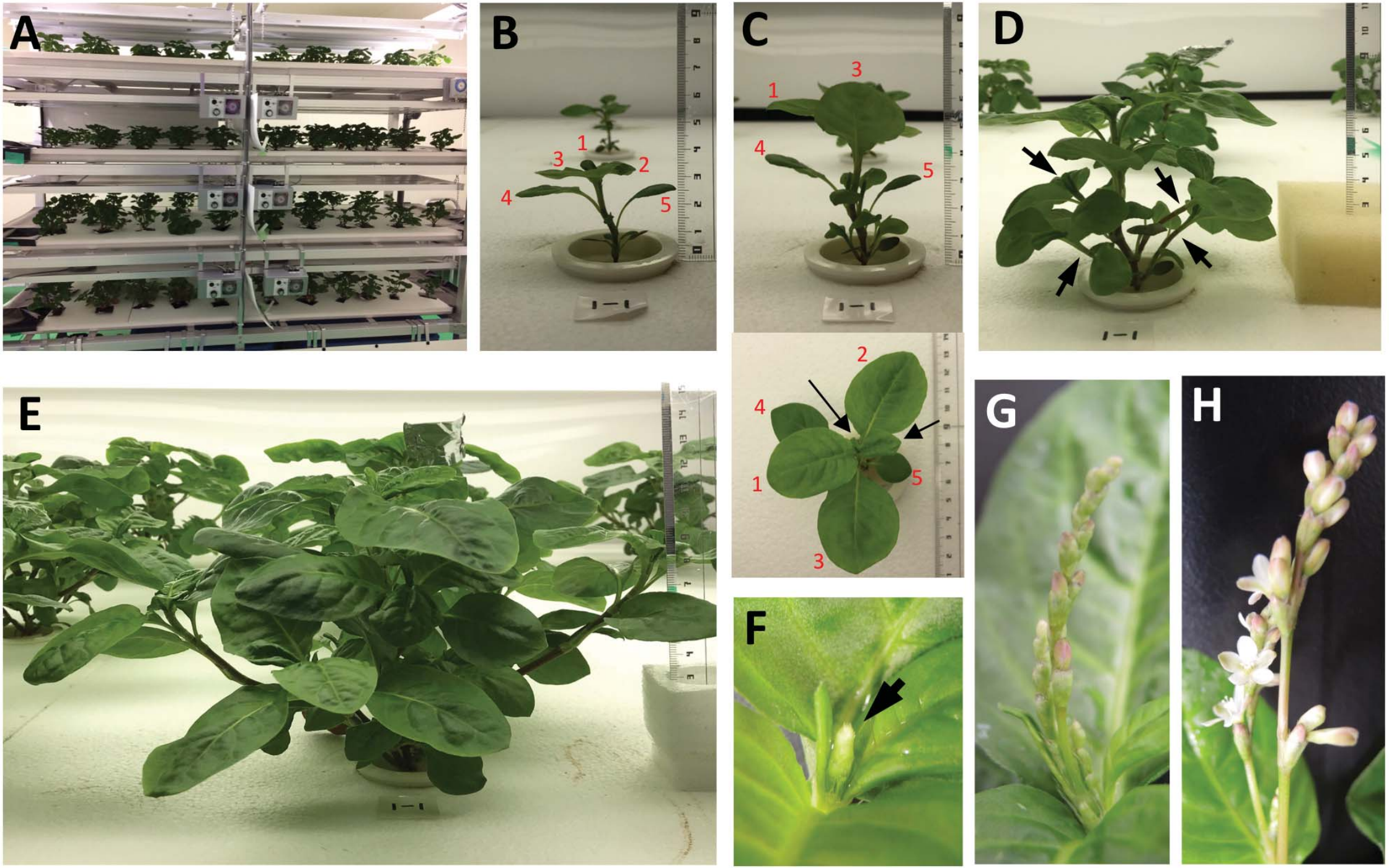


Fig 2

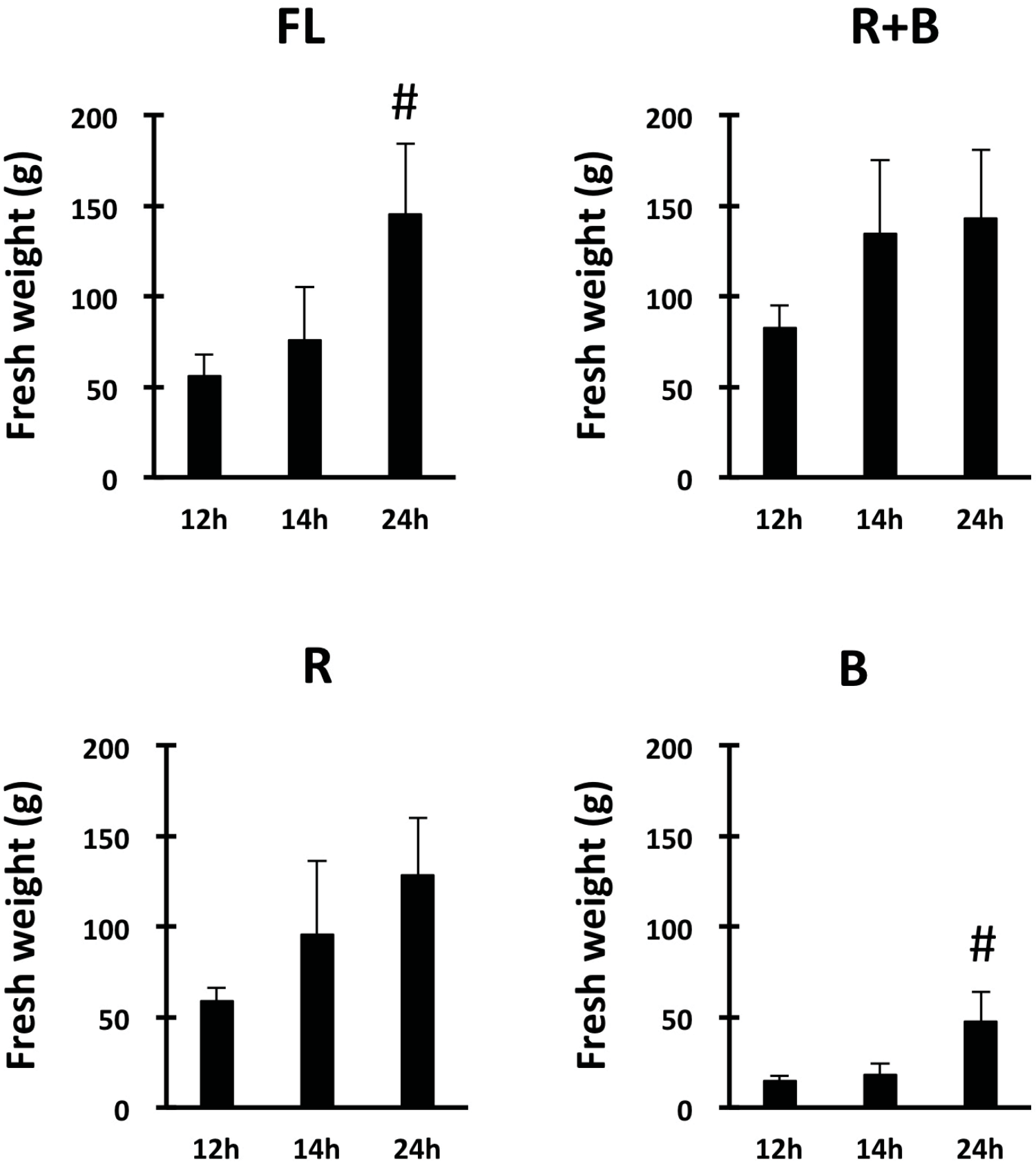


Fig 3

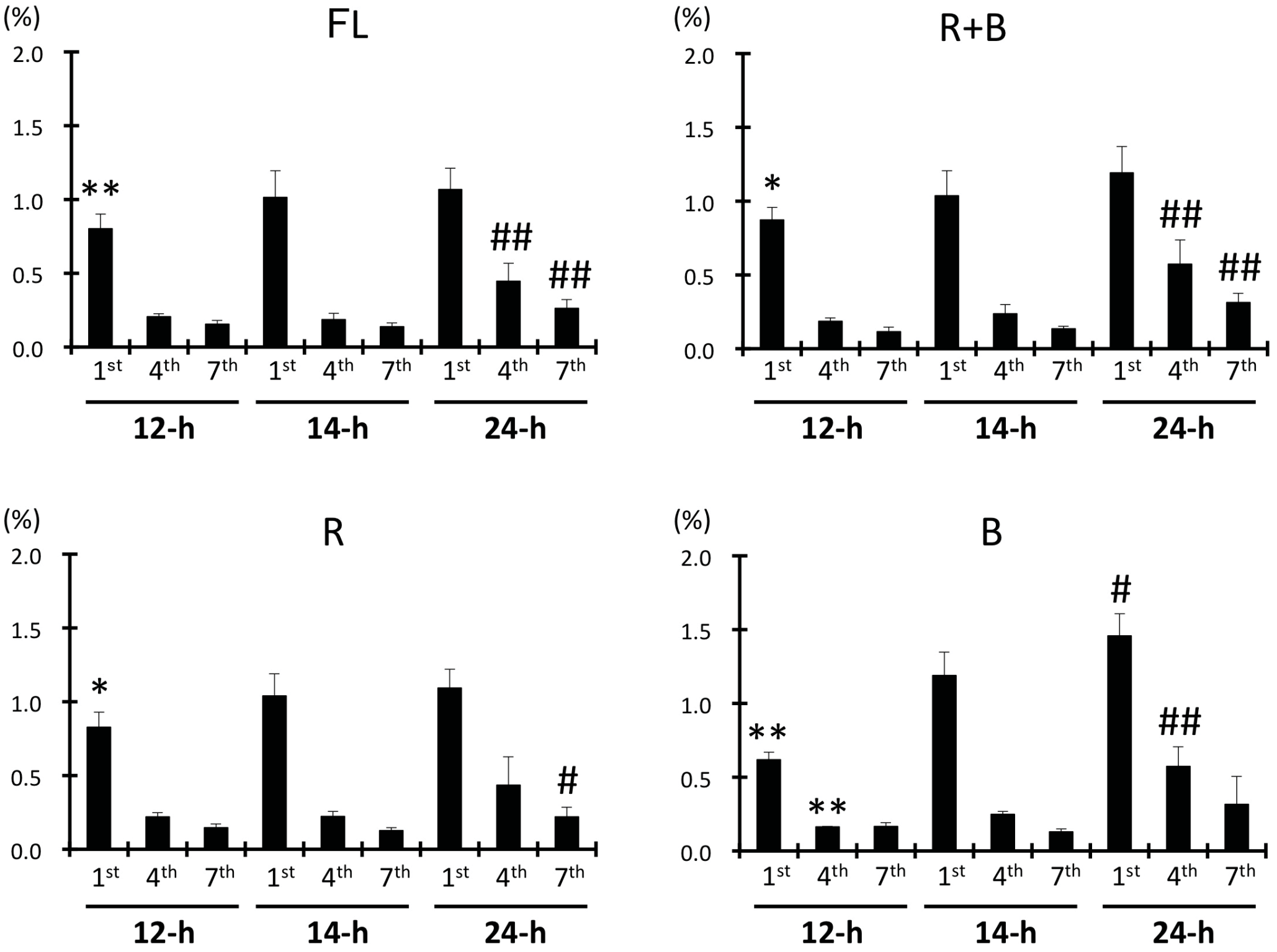
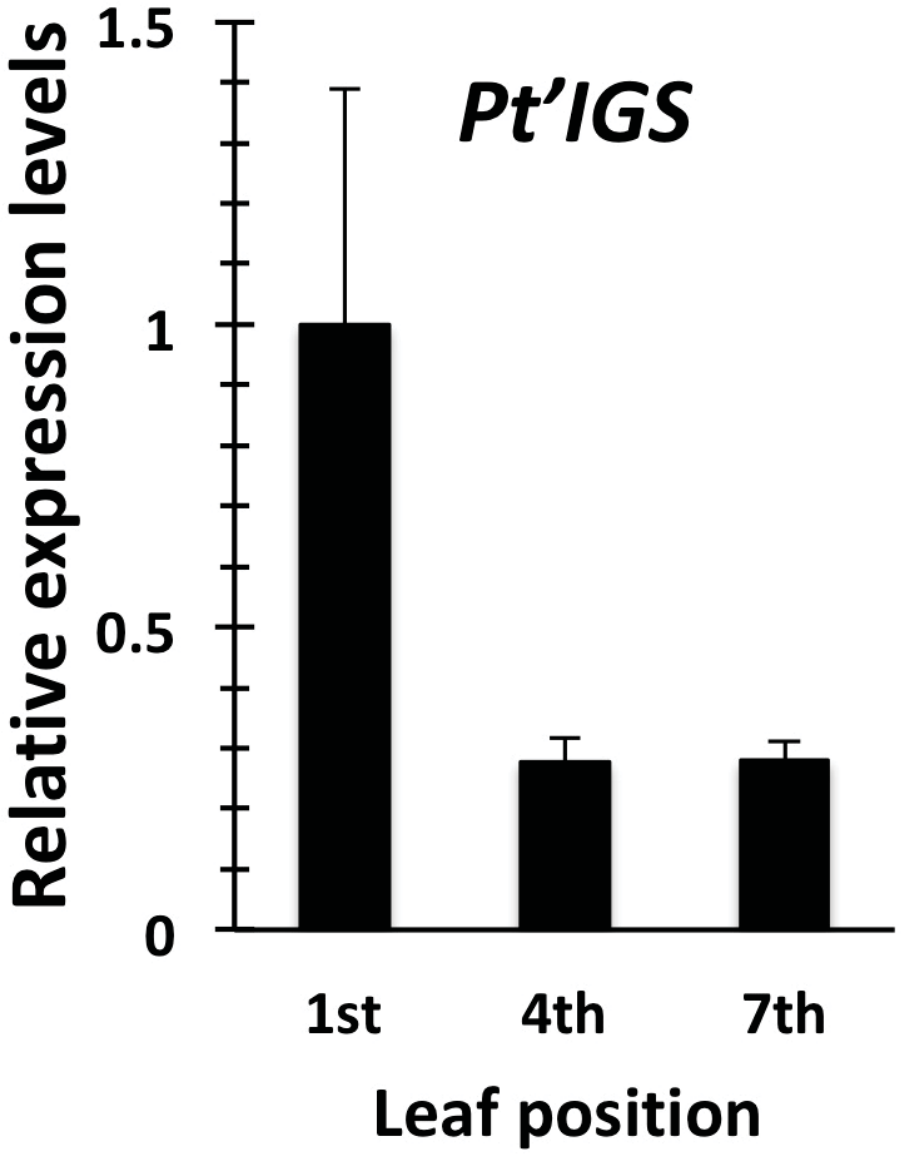

FL
(%)
2.0
1.5
1.0
0.5
0.0
**
##
##
1st 4th 7th 1st 4th 7th 1st 4th 7th
12-h
14-h
24-h
R+B
(%)
2.0
1.5
1.0
0.5
0.0
*
##
##
1st 4th 7th 1st 4th 7th 1st 4th 7th
12-h
14-h
24-h
R
(%)
2.0
1.5
1.0
0.5
0.0
*
#
1st 4th 7th 1st 4th 7th 1st 4th 7th
12-h
14-h
24-h
B
(%)
2.0
1.5
1.0
0.5
0.0
**
**
#
##
1st 4th 7th 1st 4th 7th 1st 4th 7th
12-h
14-h
24-h

Fig 4


Pt'IGS
Relative expression levels
1.5
1
0.5
0
1st
4th
7th
Leaf position

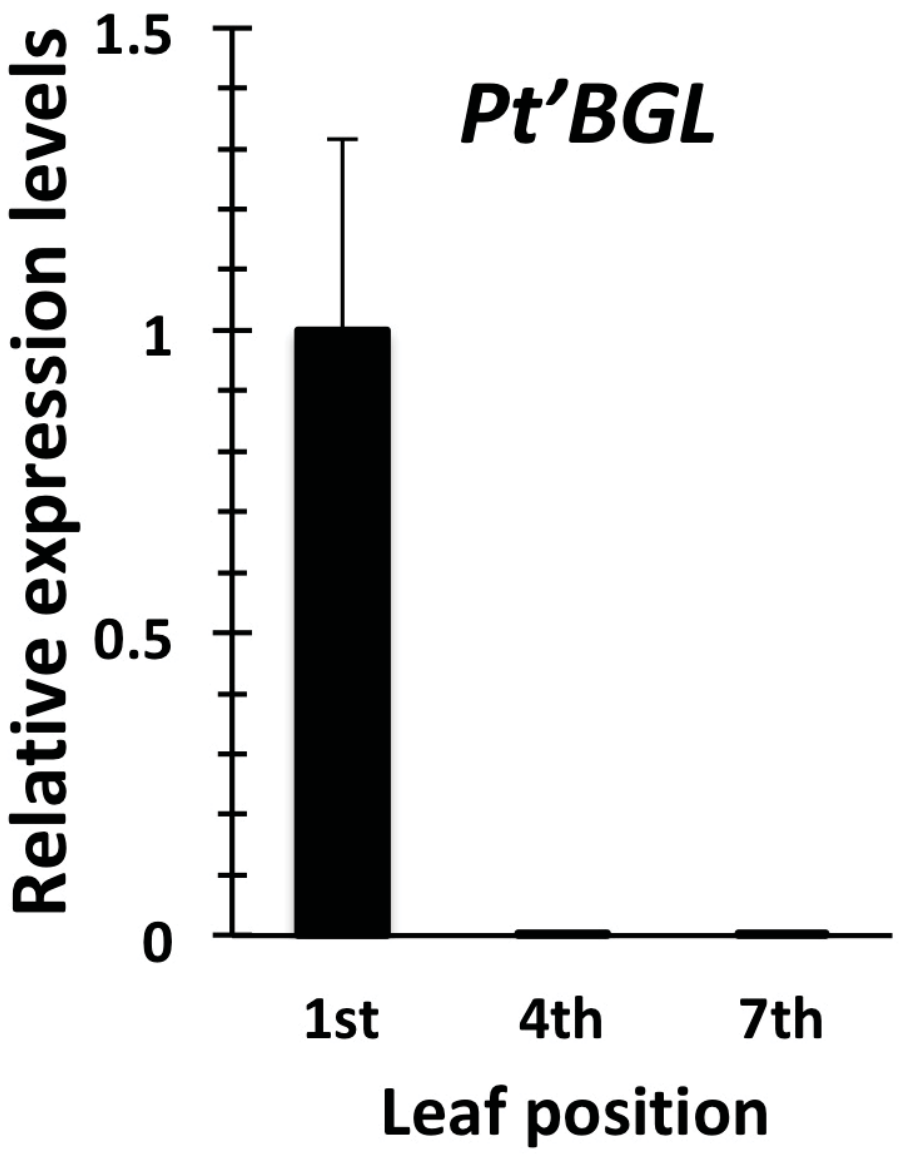

Pt'BGL
Relative expression levels
1.5
1
0.5
0
1st
4th
7th
Leaf position

Fig 5

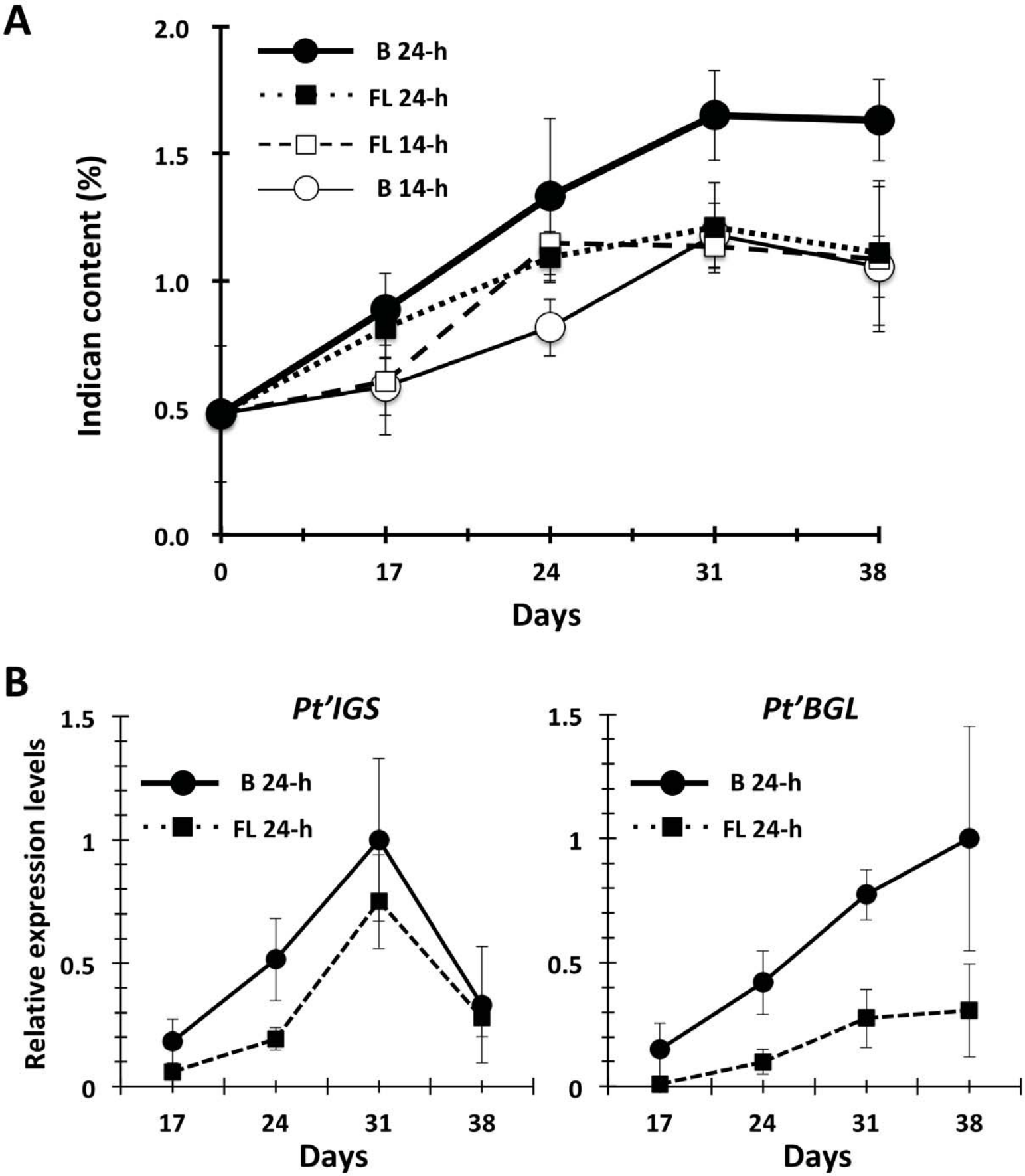
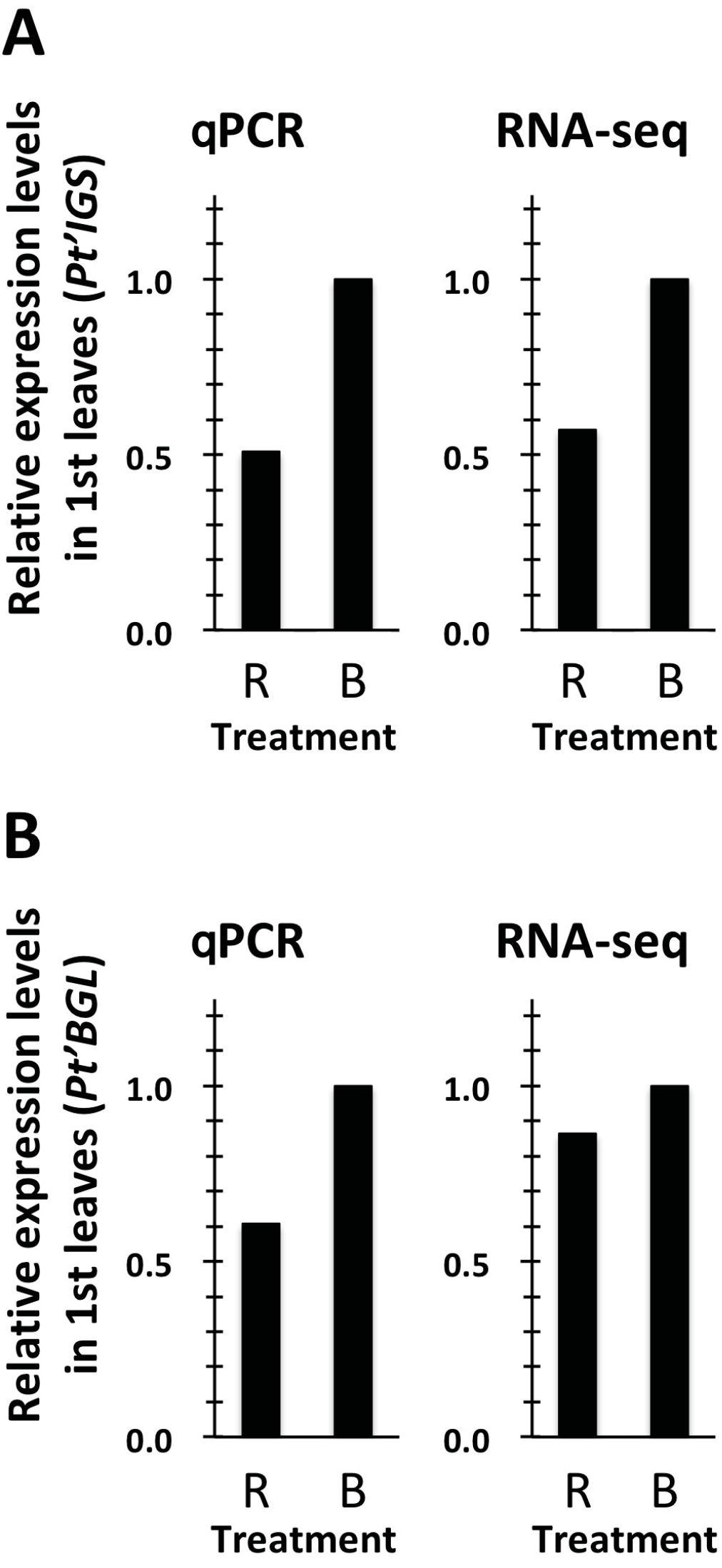

A
Indican content (%)
2.0
1.5
1.0
0.5
0.0
B 24-h
FL 24-h
FL 14-h
B 14-h
0
17
24
31
38
Days
B
Relative expression levels
Pt'IGS
1.5
1
0.5
0
B 24-h
FL 24-h
17
24
31
38
Days
Pt'BGL
1.5
1
0.5
0
B 24-h
FL 24-h
17
24
31
38
Days

Fig 6


A
qPCR
RNA-seq
Relative expression levels in 1st leaves (Pt'IGS)
1.0
0.5
0.0
R
B
Treatment
B
qPCR
RNA-seq
Relative expression levels in 1st leaves (Pt'BGL)
1.0
0.5
0.0
R
B
Treatment