1   **Short running title**

2   Far-red light regulates floral induction network

3   # Gene networks underlying faster flowering induction in response to far-red

4   # light

5   **Maria Pazos-Navarro[1], Federico M Ribalta[1], Bhavna Hurgobin[2], Janine S Croser[1] and**

6   **Parwinder Kaur[1,2]**

7   [1]Centre for Plant Genetics and Breeding, UWA School of Agriculture and Environment, The

8   University of Western Australia, 35 Stirling Hwy, Crawley, WA 6009, Australia

9   [2]Telethon Kids Institute, 100 Roberts Rd, Subiaco, WA 6008, Australia

10   Corresponding author details:

11   Maria Pazos-Navarro

12   Tel: +61 8 6488 1973

13   Email: maria.pazosnavarro@uwa.edu.au

14

ABSTRACT

Light is one of the main signals that regulates flowering. Low red to far-red ratios accelerate flowering in a wide range of species. The central gene pathways controlling flowering time in Arabidopsis, appear to be largely conserved in legumes. However, numerous examples exist of gene duplication and loss. The role of *CONSTANS-LIKE* genes as integrators of the photoperiod response has been questioned in several dicot species, including legumes. In this study on subterranean clover, using RNA-seq and controlled light spectra, we identified 13 differentially expressed genes related to light signalling, meristem identity and flowering promotion. Of these, we pinpointed genes which seem to link photoperiod and far-red light signalling coding for a With no lysine kinase, a CCT motif related to *CONSTANS*, a *FLOWERING LOCUS T b2* like protein, and their active downstream cascade. The earlier down-regulation of these genes under blue compared to far-red -enriched light may explain their role in floral induction. A second independent approach (qPCR analysis) confirmed our findings. Contrasting responses to light quality related to reproduction and defence mechanisms were also found. These results will contribute to a better understanding of the molecular basis of flowering in response to light quality in long-day plants.

KEYWORD INDEX

Flowering, gene regulation, LED light, legumes, light quality, photoperiod, red to far-red ratio, RNA sequencing.

34   **INTRODUCTION**

35   Flowering behaviour is modified by environmental cues, with light being one of the

36   most important signals that regulates flowering through quality, quantity and duration

37   (Thomas 2006). Plants perceive subtle changes in light composition, duration and direction

38   through photoreceptor pigments, which result in physiological and morphological

39   modifications necessary for adaptation to environmental changes (Rajapakse & Shakak

40   2007). In higher plants, the 'shade avoidance syndrome' is an adaptive developmental

41   strategy mediated by the perception of light spectral quality, in particular the red to far-red

42   ratio, R: FR, which acts as an early warning of potential shading (Salter *et al.* 2003; Ballaré &

43   Pierik 2017). In Arabidopsis (*Arabidopsis thaliana*), the most noticeable shade avoidance

44   responses include rapid internode elongation and accelerated floral initiation at the expense of

45   leaf expansion and chlorophyll synthesis reduction (Devlin *et al.* 1999). Similarly, research in

46   the model long-day species pea (*Pisum sativum* L.) demonstrates FR-enriched light, with a R:

47   FR ratio below 3.5, is most effective for early floral induction (Runkle & Heins 2001;

48   Cummings *et al.* 2007; Croser *et al.* 2016; Ribalta *et al.* 2017).

49   Changes in light quality are detected in the leaf by the action of a family of plant

50   photoreceptors, including phytochromes (R and FR light receptors) and cryptochromes (blue

51   light receptors) and involve complex gene regulatory networks (Andrés & Coupland 2012;

52   Viczián *et al.* 2017). In Arabidopsis significant progress has been made toward understanding

53   the role light quality plays on floral initiation pathways. The genes *GIGANTEA* (*GI*),

54   *FLAVON KELCH F BOX* (*FKF1*), *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) have

55   major regulatory roles in the promotion of flowering in response to photoperiod (Thomas

56   2006; Jiao *et al.* 2007). In particular, *CO* has been described as a network hub for the

57   integration of internal and external signals into the photoperiodic flowering pathway to

58   induce *FT* expression (Wong *et al.* 2014; Shim *et al.* 2017). *FT* acts as a mobile floral

59   promoting signal that integrates day length, light quality, circadian clock, temperature and

60   vernalisation inputs (Turck *et al.* 2008). In Arabidopsis, the role of the circadian clock and

61   light on the regulation of *CO* gene expression is well understood (Imaizumi & Kay 2006;

62   Cerdan & Chory 2003). The circadian clock sets *CO* expression in the afternoon in long-day

63   plants and light activates CO protein to induce *FT* (Imaizumi & Kay 2006).

64   In legumes, the genes and gene families central to pathways controlling flowering

65   time in Arabidopsis appear to be largely conserved. However, there are numerous examples

66   of gene duplication and loss (Weller & Ortega 2015). In pea, the conserved role of the

67   Arabidopsis genes *GI*, *EARLY FLOWERING 3* and *EARLY FLOWERING 4* in the regulation

68   of *FT* genes has been demonstrated (Hecht *et al.* 2007; Liew *et al.* 2009; Weller *et al.* 2012).

69   Orthologous Arabidopsis genes play a part in the photoperiodic flowering pathway in

70   legumes. However, the role of *CONSTANS-LIKE* (*COL*) genes as integrators of the

71   photoperiod response has been questioned in several dicot species including legumes

72   (Blackman 2017). Recent studies in *Medicago truncatula* Gaertn. (Medicago) revealed that

73   none of the *COL* genes identified are functionally equivalent to *CO*, with respect to inducing

74   *FT* expression (Wong *et al.* 2014). These findings support the idea of *CO*-independent

75   pathways involved in flowering induction in legumes.

76   An improved understanding of the gene networks underlying flowering induction in

77   response to light quality will require better characterisation of the transcriptome. The recent

78   development of deep-sequencing technologies, such as RNA-Seq, has enabled the generation

79   of a high-resolution global view of the transcriptome and its organisation for some species

80   and cell types (Wang *et al.* 2009). Whole-transcriptome sequencing using RNA-Seq is a

81   convenient and rapid means to study gene expression at the whole-genome level and define

82   putative gene function (Wang *et al.* 2009; Ozsolak & Milos 2011; Singh *et al.* 2013;

83   Hirakawa *et al.* 2016; Kaur *et al.* 2017). Rapid advances have been made toward

84   understanding the transcriptional regulation of specific developmental processes in legumes

85   (Benedito *et al.* 2008; Libault *et al.* 2008; Severin *et al.* 2010). Within this framework, we

86   now seek to apply whole-transcriptome sequencing to characterise the genetic regulatory

87   mechanisms underlying the induction of flowering in legumes in response to changes in the

88   R: FR ratio using light emitting diodes (LED).

89   Despite the substantial number of grain and pasture legumes with their genome

90   sequenced and/ or significant genomic resources such as chickpea (*Cicer arietinum* L.), pea,

91   soybean (*Glycine max* (L). Merr.), Medicago and *Lotus japonicus*, no legume species has yet

92   emerged as a predominant model in the study of flowering time (Weller & Ortega 2015).

93   Recently, we established subterranean clover (*Trifolium subterraneum* L.) as a reference

94   species for genetic and genomic studies within the genus *Trifolium*. Subterranean clover is

95   diploid (2n = 16), predominantly inbreeding, and has a well-assembled and annotated

96   genome with a tissue type transcriptome atlas available (Hirakawa *et al.* 2016; Kaur *et al.*

97   2017). The improved understanding of the genetics of flowering in this model species can

98   assist to identify floral promotion pathways in other genetically complex species.

99   Within this research, we used deep-sequencing technologies to investigate

100   transcriptional activity under different light spectra at three time-points in the long-day plant,

101   subterranean clover. We have demonstrated accelerated onset of flowering across a range of

102   leguminous species in response to FR-enriched LED light spectra (Croser *et al.* 2016; Pazos-

103   Navarro *et al.* 2017; Ribalta *et al.* 2017). To elucidate the role of FR enriched spectra on

104   acceleration of time to flowering, we have compared plants grown under a FR enriched

105   regime with plants grown under a blue-enriched LED spectra. We expect that the FR-

106   enriched spectra will accelerate the up-regulation of genes related to floral initiation.

107   **MATERIALS AND METHODS**

Experimental design

Seeds of *Trifolium subterraneum* L. cv. Daliak were nicked with a scalpel prior to sowing in 70 mm plastic pots filled with steam-pasteurised potting mix (Plant Bio Mix – Richgro Garden Products Australia Pty Ltd). Plants were grown simultaneously within two walk-in phytotron rooms under tightly controlled temperatures of 24 °C day, 20 °C night and a photoperiod of 20 h, as per Ribalta *et al.* (2017). The two growth environments differed only in the spectral composition of illumination provided to the plants (Fig. 1, A and B). Environment 1 (E1) illumination was provided by LED arrays enriched in the far-red part of the spectrum with a red to far-red (R: FR) ratio of 2.9 ('B series' AP67 spectrum Valoya Helsinki, Finland). Environment 2 (E2) illumination was provided by LED tubes enriched in the blue part of the spectrum with an R: FR ratio of 5.9 (108D18-V12 tubes from S-Tech Lighting, Australia). Spectral measurements were undertaken using a Sekonic C7000 SpectroMaster spectrometer (Sekonic Corp., Tokyo, Japan) and values were averaged over three scans in the range of 380–780 nm. Ratio calculations followed the method of Runkle & Heins (2001) where the R: FR ratio was measured as a narrow band (655–665: 725–735 nm). Plants were watered daily and fertilised weekly with a water-soluble NPK fertiliser (15:2.2:12.4) with micronutrients (Peters Excel, Scotts Australia, Bella Vista, New South Wales) at a rate of 2 g per pot, as per Pazos-Navarro *et al*. (2017). Time to flowering was defined as the number of days from sowing to the open floral stage of the first flower and was recorded under the different growth conditions.

Leaf samples were collected simultaneously 6 h after lights were switched on from E1 and E2 grown plants at three time-points (TPs) when plants in the E1 environment reached the following developmental milestones: third-leaf stage (TP1), appearance of the first flower bud (TP2) and open flower (TP3; Fig. 2A). This correlated to 14 growing days for TP1 (at this time-point, plants in both environments were at the third-leaf stage), 42 growing days for

6

133   TP2, and 47 growing days for TP3. Leaf tissue (75–100 mg FW) was harvested and snap-

134   frozen in liquid nitrogen and stored at –80 °C until RNA extraction.

RNA isolation and library preparation

136   Total RNA from all tissue samples was extracted using the Spectrum™ Plant Total RNA Kit

137   (Sigma-Aldrich, St Louis, USA) following the manufacturer's instructions. Aliquots of

138   purified RNA were stored at −80 °C. The concentration of RNA was confirmed using a Qubit

139   fluorometer with the Qubit RNA assay kit (Life Technologies, Carlsbad, USA). The integrity

140   of total RNA was determined by electrophoretic separation on 1.2% (w/v) denaturing agarose

141   gels. Sequencing libraries were constructed using 500 ng of total RNA with a TruSeq®

142   Stranded Total RNA Sample Prep Kit with Ribo-Zero (Illumina Inc., San Diego, USA)

143   following the manufacturer's instructions. Concentrations of libraries were measured using

144   the Qubit fluorometer with the Qubit dsDNA HS assay kit (Life Technologies, Carlsbad,

145   USA) and Agilent high-sensitivity DNA chips (Agilent Technologies, Santa Clara, USA).

146   The amplified libraries were pooled in equimolar amounts, and quality was assessed with

147   Agilent high-sensitivity DNA chips (Agilent Technologies, Santa Clara, USA). All reads

148   were pair-end sequenced using the HiSeq 2000 platform (Illumina Inc., San Diego, USA).

Differential gene expression analysis

150   The sequencing quality of the Illumina reads was assessed using FastQC (Andrews 2010).

151   The reads were quality filtered using Trimmomatic (Bolger *et al.* 2014) to remove adapter

152   sequences. Filtered reads were mapped to the advanced genome assembly (Tsub_Refv2.0)

153   (Kaur *et al.* 2017) using TopHat v2.1.1 (Trapnell *et al.* 2009) with default parameters and

154   passing the reference annotation with the-G option. Paired-end and single-end reads were

155   mapped separately, and the BAM files were used to calculate the read counts for each gene

156   using featureCounts (Liao *et al.* 2014) (Table S1.1 and Table S1.2). For each sample, the sum

157   of read counts at each gene locus was taken by adding the read counts from the paired-end

158   BAM file and single-end BAM files. Differential expression analysis was carried out on the

159   matrix of read counts for each gene (rows) and sample (columns) using the perl script

160   run_DE_analysis.pl from the Trinity v2.2.0 (Haas *et al.* 2013) suite of programs based on the

161   edgeR method (Robinson *et al.* 2010). Volcano plots were generated for each time-point and

162   differentially expressed genes with false discovery rate (FDR) less than 5% were output for

163   each time-point.

164   **Functional characterisation and GO enrichment analyses**

165   Gene ontology (GO) enrichment was performed using the Fisher exact test as implemented in

166   R using the topGO package (Alexa & Rahnenfuhrer 2010) with method 'weight01' used to

167   adjust for multiple comparisons. Up- and down-regulated DEGs (logFC>2 and FDR<0.05)

168   were considered for the analysis.

169   **Quantitative reverse-transcriptase PCR (qPCR) analysis**

170   Quantitative reverse transcriptase (qPCR) analysis were performed to study the relative gene

171   expression of *TSub_g7401* (CCT motif protein), *TSub_g3280* (*AGL6: AGAMOUS*-like 6),

172   *TSub_g25875* (*AGL20: AGAMOUS*-like 20), *TSub_g20101* (*AGL12: AGAMOUS*-like 12),

173   *TSub_g15483* (*AGL8: AGAMOUS*-like 8), *TSub_g12538* (*FTb2: FLOWERING LOCUS T*

174   *b2), TSub_g11956* (*WNK5:* With no lysine (K) kinase.) and *TSub_g21607* (*AGL4:*

175   *AGAMOUS*-like 4) in response to FR-enriched and B-enriched lights. The *Pisum sativum* L.

176   (Alaska) ubiquitin (PUB3) gene L81141.1 was used as reference gene. Leaf tissue was

177   collected at the same time of the day (6 h after lights were switched on) from plants at third-

178   leaf stage grown in both environments (E1 and E2) and used for isolation of total RNA using

179   Spectrum^{TM} Plant Total RNA kit (Sigma). The High-Capacity cDNA reverse transcription kit

180   with RNase inhibitor kit (Appliedbiosystems) was used to synthesise cDNA from 500 ng

181   RNA. Primers for qPCR were designed using NCBI Primer-BLAST (Ye *et al.* 2012) (Table

182   S7). Primer efficiency was determined using LinRegPCR 2014.x software (Ruijter et al.

183   2009) and was close to the ideal value of 2 for all primer pairs (between 1.8 and 1.9).

184   Reactions were set up with 20 ng cDNA and 3 pmol (each) forward and reverse primers using

185   KiCqStart SYBR Green qPCR Low ROX Ready Mix (Sigma-Aldrich, St Louis, USA), and

186   run on an Applied Biosystems 7500 Fast Real-Time PCR System under the following

187   conditions: 95°C initial denaturation for 30 sec, followed by 40 cycles of 95°C denaturation

188   for 3 sec plus primer annealing/extension at 60°C for 30 sec. Finally, melt-curve analyses

189   were performed (one cycle of 95°C for 15 min, 60°C for 1 min, increasing by $0.1°C\ min^{-1}$ to

190   95°C for 15 sec, 60°C for 15 sec). Data were analysed with the Applied Biosystems 7500

191   software (version 2.0.6), and values for ΔC_T and $\Delta\Delta C_T$ calculated according to Schmittgen &

192   Livak (2008). Each sample was run in three technical replicates for each gene.

193   **Statistical analysis**

194   Differences on gene expression were analysed by one-factor analysis of variance (ANOVA)

195   and the least significant difference (LSD) test at the 5% level of significance.

196   **RESULTS**

197   **Far-red enriched light accelerates floral onset**

198   To study the effect of light quality on time to flowering, plants were grown

199   simultaneously in controlled environments under the same temperature (24/20 °C day/night)

200   and photoperiod (20 h) regime, but different light spectra. Environment 1 (E1) consisted of a

201   R: FR ratio of 2.89 (FR-enriched light) and Environment 2 (E2) consisted of a R: FR ratio of

202   5.86 (blue-(B) enriched light). We observed a clear effect of light spectra on floral onset in

203   subterranean clover. Time to flowering was significantly reduced ($P < 0.001$) under FR-

204   enriched light by 14 days compared to B-enriched light (Fig. 1, A and B).

205   **Gene response to different light spectra**

206   Given the clear effect of the FR-enriched spectrum on the acceleration of floral onset,

207   we aimed to identify the influence of light on gene expression related to flowering induction.

208   *Transcriptome dynamics in response to light spectra*

209   RNA-Seq technology was used to analyse variations in gene expression related to

210   changes in the R: FR ratio across three time-points (TP) based on the attainment of precise

211   developmental milestones under E1 growing conditions (low R: FR ratio). This correlated to

212   14 growing days for TP1 (third-leaf stage), 42 growing days for TP2 (pre-flowering stage

213   under FR-enriched light) and 47 growing days for TP3 (flowering stage under FR-enriched

214   light). From a total of 31,272 protein-coding genes identified in the subterranean clover

215   advanced assembly Tsub_Refv2.0 (Kaur *et al.* 2017), 644 genes were differentially expressed

216   in response to different light spectra in at least one of the three time-points analysed (Table

217   S2). Clear differences in the number of differentially expressed genes (DEGs) were observed

218   in response to the light treatments (Fig. 2B; Fig. 3, A and B; Table S2). The highest number

219   of DEGs was found at TP1 with a total of 448 genes, of which 418 were exclusively

220   differentially expressed at this stage (93%; Table S3). Of the 448 DEGs at TP1, 241 were up-

221   regulated and 207 were down-regulated in B-enriched light compared to FR-enriched light.

222   At TP2, we found 156 DEGs (135 up- and 21 down-regulated) with 122 of them exclusively

223   differentially expressed at this point (78%; Table S4). At TP3, we identified 80 DEGs (16 up-

224   and 64 down-regulated), with 65 of them exclusively differentially expressed at this point

225   (81%; Table S5).

226   **Far-red and blue-enriched lights affect different biological processes**

227   To identify the major functional biological categories represented by DEGs in

228   response to light spectra, we performed gene ontology (GO) enrichment analysis. Changes in

229   gene expression were seen in the three different time-points in GO terms, suggesting

230   biological processes are differentially affected by light quality. A total of 459 (71.3%) of all

231   DEGs were assigned GO categories corresponding to several aspects of metabolic and

232   cellular processes, response to stress, plant defence, ion localization and transport, and

233   flowering-related processes among others (Table S6a–c).

234   When analysing DEGs in both environments at TP1, the most significantly enriched

235   GO categories with respect to up-regulated genes (p-value < 0.05) were related to defence

236   response, mainly to biotic stimuli such as bacteria or fungus (Table S6a). Some of those

237   genes were related to flavonoid pathways (Tsub_g874 and Tsub_g13273) and LRR disease

238   resistance related proteins (Tsub_g14165, Tsub_g23725 and Tsub_g6245; Table S3). On the

239   other hand, the GO terms related to 'ion transport' and floral development were

240   predominantly associated with down-regulated genes in TP1 (Table S6a). Related to the red

241   light signalling category, a CCT motif protein (TSub_g7401) was down-regulated under B-

242   enriched light. Most of the genes identified in the floral development categories were MAD-

243   box proteins (TSub_g15483, TSub_g25875, TSub_g21607 and TSub_g3280; Table S3).

244   At TP2, biological processes related to nodulation (GO: 0009877), defence response

245   (GO: 0006952), and vegetative to reproductive transition (GO: 00110228) were up-regulated

246   in B-enriched light compared to FR-enriched light. In contrast, genes involved in the

247   secondary compound biosynthesis of isoflavonoids and flavonoids (GO: 0009717 and GO:

248   0009813) and anthocyanin-containing compounds (GO: 0009718) were down-regulated

249   (Tables S4 and S6b).

250   At TP3, genes associated with biological processes related to photoperiodism,

251   flowering (GO: 2000028 and GO: 0048573) and developmental vegetative growth (GO:

252   0080186) were up-regulated under B-enriched light, and therefore their expression was lower

253   under FR-enriched light (Table S6c). We found that TSub_g17978 was the same gene

254   involved in those biological processes (Table S5). On the other hand, genes associated with

255   the response to stress (GO: 0006950) were down-regulated. Additionally, we found other

256   categories related to stress response: high light intensity (GO: 0009644), response to

257   hydrogen peroxide (GO: 0042542), L-proline biosynthetic process (GO: 0055129), and

258   detection of visible light (GO: 0009584; Table S6c).

259   **Far-red enriched light promotes the floral induction network**

260   The expression of genes related to flowering promotion across species occurred at an

261   earlier stage of plant development under FR-enriched light than B-enriched light. We

262   identified 13 DEGs related to light signalling, meristem identity and flowering promotion, of

263   which ten were exclusively expressed at TP1, one at TP2 and two at TP3. One of the DEGs

264   was shared between TP1 and TP2 (Table 1). All of the flowering-related DEGs identified at

265   TP1 were down-regulated under B-enriched light, as their expression was higher under FR-

266   enriched light. Of those, we found a gene-coding protein related to light signalling, a CCT

267   motif protein (TSub_g7401), and an uncharacterised protein identified as a probable

268   serine/threonine-protein kinase (with no lysine kinase 5-*WKN5*; TSub_g11956). Additionally,

269   we observed five different MADS-box transcription factors involved in floral promotion

270   (TSub_g15483, TSub_g21607, TSub_g3280, TSub_g25875 and TSub_g20101; Tables I and

271   Table S2). The floral promoter TSub_g12538, which is a possible pea *FLOWERING LOCUS*

272   *T b2* (*FTb2*), was among the down-regulated genes. Gene-coding proteins potentially linked

273   with floral promotion were also identified: an uncharacterised protein (TSub_g22633), a

274   possible *GIBBERELLIN 20 OXIDASE 2* (*GA20OX2*) involved in the promotion of floral

275   transition, a sugar transporter (TSub_g15042), possible *SUCROSE-PROTON SYMPORTER 2*

276   (*SUC2*), and a homeobox related protein that may be related to meristem identity

277   (TSub_g491; Table 1 and Table S2).

278   All of the DEGs related to flowering induction identified at TP2 and TP3 were up-

279   regulated under B-enriched light. At TP2, one DEG was identified, a MADS-box protein

280   (TSub_g20101). Subsequently, at TP3, we found two DEGs associated with flowering

281   promotion factors (TSub_g30315 and TSub_g30317; Fig. 4 and Table 1).

282   **Expression of flowering genes in response to far-red and blue-enriched lights**

283   The relative expression of flowering induction related genes at the third-leaf stage

284   (TP1) decreased between 1.6 – 104.28 fold changes under B compared to FR-enriched lights.

285   (Fig. 5A). *TSub_g21607,* a MADS-box transcription factor involved in floral promotion,

286   showed the highest fold change (-104.28) compared to the other genes studied. The rest of

287   MADS-box presented lower fold changes, between -1.60 and -20.79, together with

288   *TSub_g7401* (-14.16 change-fold) and *TSub_g12538* (-4.09).

289   Relative expression ($2^{-\Delta Ct}$) of the studied genes was significantly higher in response to

290   FR-enriched light (Fig.5B). Two genes showed the biggest response to light quality,

291   *TSub_g21607,* a MADS-box transcription factor involved in floral promotion and

292   *TSub_g11956*, a WNK related to the photoperiod pathway.

293   **DISCUSSION**

294   For the first time, we have demonstrated growing plants under FR light accelerates the

295   up-regulation of genes related to floral initiation pathways using the long-day plant

296   subterranean clover as a model. We identified novel genes which link photoperiod and FR-

297   light signalling in addition to the up-regulation of genes related to plant defence under B-

298   enriched light.

299   RNA-Seq time-course analysis identified 13 DEGs related to light signalling,

300   meristem identity and flowering promotion. There was clear evidence in the transcriptome of

301   up-regulation of gene networks related to flowering at a very early stage of plant

302   development (TP1, third-leaf stage) when plants were grown under FR-enriched light. Under

13

303   B-enriched light, the up-regulation of this network was not expressed until TP2 and TP3

304   when the expression of *AGL12* and floral promoting factors was identified. These results are

305   in accordance with the acceleration of time to flowering observed under FR-enriched light

306   (low R: FR ratio; 14 days faster) compared to B-enriched light (high R: FR ratio).

307   The genetic link for the integration of photoperiod, light perception and circadian

308   clock pathways has been well characterised in Arabidopsis and involves the B-box

309   transcription factor *CO*. The floral promoter *CO* protein is up-regulated by long-day growing

310   conditions and its expression is stabilised by FR light through *PHYTOCHROME A* activity

311   (Kim *et al.* 2008; Pin & Nilsson 2012; Song *et al.* 2013). In Medicago, a temperate legume as

312   subterranean clover, it has been suggested that the integration of responses to day length and

313   light quality may not be regulated by *CO*-like (*COL*) genes (Wong *et al.* 2014; Weller &

314   Ortega 2015). In contrast, in subterranean clover, we identified the expression of a CCT motif

315   (*TSub_g7401*) at an early developmental stage (TP1) under FR-enriched light. That result

316   was supported by qPCR analysis. We found that the relative expression ($2^{-\Delta Ct}$) of

317   *TSub_g7401* gene, homologous to a conserved domain related to *CO* and *COL* proteins, was

318   higher under the FR compare to the B-enriched light at the same 20h photoperiod with a -

319   14.16 fold change. Therefore our results suggest that, in subterranean clover, at same

320   photoperiod conditions (20 h) FR enriched light induces *COL* gene expression. In addition,

321   we identified a serine/threonine-protein kinase (TSub_g11956), *WNK5* known to be involved

322   in regulating time to flowering in response to photoperiod in Arabidopsis. Wang *et al.* (2008)

323   reported that *WNK5* acts to delay flowering in Arabidopsis. In our case, the higher relative

324   expression of *TSub_g11956* was observed under FR-enriched light, it is suggested that this

325   homologous gene in subterranean clover is related to flowering induction and its expression

326   is modulated by light quality. Our results suggest floral initiation under FR-enriched light is

327   mediated by CO-like and WNK-like proteins. To our knowledge, a direct interaction between

328   WNK-like protein and *FTb2* has not previously been reported. For subterranean clover under

329   FR-enriched light, the relative expression of *TSub_g11956* (homologous of *WNK*-like gene)

330   was higher than for *TSub_g7401* (CCT motif related to *CO* and *COL*), so although a direct

331   interaction can be inferred, further work is suggested to elucidate changes in pattern

332   expression throughout the day.

333   The CO protein has a role in activating the expression of FT (Kim et al. 2008). FT-

334   like proteins from several species function similarly to FT with respect to the induction of

335   flowering, transport in phloem, and interaction with FLOWERING LOCUS D-like proteins

336   (Hecht et al. 2011). In our experiment, at the first time-point (TP1) under FR-enriched light,

337   we found a gene-coding protein (TSub_g12538) homologous of a pea FTb2 whose relative

338   expression was also higher under FR compared to B-enriched lights. This protein is expressed

339   specifically in pea and Medicago leaves under long days and meets the characteristics of the

340   classical 'florigen' (Weller & Ortega 2015). The differential expression of this homologous

341   gene may suggest its role as a floral promoter in subterranean clover.

342   Downstream in the photoperiod pathway, we identified four MADS-box transcription

343   factors exclusively expressed at TP1 under FR-enriched light (TSub_g15483, TSub_g21607,

344   TSub_g3280, and TSub_g20101). We also identified one MAD-box TF (*AGL*12) that was

345   up-regulated at TP1 under FR-enriched light and not identified until TP2 under B-enriched

346   light (TSub_g25875). These TFs have been identified as key components of the networks that

347   control the transition from vegetative to reproductive stages and flower development in

348   Arabidopsis (Tapia-López *et al.* 2008). These flowering promoters are homologous to

349   *AGAMOUS-LIKE* (*AGL*) MADS-box proteins: *AGL4, AGL6, AGL8, AGL12* and *AGL20*.

350   *AGL8* promotes floral determination in response to FR-enriched light (Hempel *et al.* 1997).

351   *AGL20* has been described as an integrator of the vernalisation, autonomous and photoperiod

352   pathways controlling flowering in Arabidopsis (Lee *et al.* 2000). It has also been associated

353   with gibberellins in the induction of flowering (Moon *et al.* 2003). *AGL12* has been described

354   as an important floral promoter through the up-regulation of A*GL20* (Tapia-López *et al.*

355   2008). The expression of *AGL4* and *AGL6* is associated with gene regulation during floral

356   meristem and floral organ development, with both found mainly in flowers (Ma *et al.* 1991;

357   Pelaz *et al.* 2000; Dreni & Zhang 2016). The fact that *AGL12* was found at TP1 under FR-

358   enriched light and at TP2 under B-enriched light provides further evidence that FR-enriched

359   spectra accelerate flowering induction.

360   In addition to the DEGs related to the photoperiod flowering pathway and

361   downstream cascade, we identified a further three DEGs related to flowering at TP1 under

362   FR-enriched light: TSub_g491, TSub_g22633 and TSub_g15042. The TSub_g491 gene-

363   coding protein is a homolog of the *KNAT1* protein and involved in the development of both

364   vegetative and reproductive meristems (Scofield *et al.* 2007; Aguilar-Martinez *et al.* 2015).

365   The TSub_g22633 is homologous to *GAS20OX2*, which is involved in the promotion of floral

366   transition in Arabidopsis (Andrés *et al.* 2014). The TSub_g15042 is a sugar transporter,

367   *SUC2* involved in the transport of *FT* through phloem companion cells in leaves to the

368   meristem for the induction of floral organ formation (Corbesier *et al.* 2007). At the later

369   developmental time-points (TP2 and TP3), only three genes were exclusively differentially

370   expressed under B-enriched light. At TP2, we identified a gibberellin-related protein

371   (TSub_15579) homologous to *AT5G14920*, which is shown to be related to flowering

372   induction in Arabidopsis over-expressing the *ZEITLUPE/LOV KELCH PROTEIN 1,* a blue

373   light photoreceptor (Saitoh *et al.* 2015). At TP3, we found two genes (TSub_g30315 and

374   TSub_g30317) described as homologs of flowering-promoting factor-like proteins in

375   Medicago. The fact that genes related to reproductive processes were identified at TP1 under

376   FR-enriched light and at TP2 and TP3 under B-enriched light is in accordance with the

377   accelerated flowering observed under a low R: FR ratio.

378   Interestingly, we found contrasting responses to light quality related to reproduction

379   and defence mechanisms. In our experiment, at TP1 and TP2, growing plants under B-

380   enriched light (high R: FR ratio) enhanced the expression of genes involved in flavonoid and

381   anthocyanin pathways. At TP3, we found down-regulation of genes related to the L-proline

382   biosynthetic process, which is involved in the response to abiotic stress (Devlin 2016). This is

383   in agreement with studies indicating plant health responses are modulated by B-enriched and

384   FR-enriched light. For example, B-enriched light can enhance the production of secondary

385   metabolites such as flavonoids and anthocyanins, which are defensive compounds against

386   fungus, bacteria and environmental stresses (Johkan *et al.* 2010). Similarly, plants grown

387   under FR-enriched light (low R: FR ratio) can express a weak defence phenotype (Cerrudo *et*

388   *al.* 2012; Ballaré 2014). Our findings provide further support that the stress response is active

389   under FR-enriched light, which may be due to the preferential allocation of resources to

390   reproduction over defence.

391   The results from this study will contribute to a deeper understanding of the molecular

392   basis of flowering and the possible negative correlation between reproductive and defence

393   mechanisms in response to different light quality in long-day plants. Using RNA-Seq and

394   tightly controlled light spectra, we have identified key genes which link photoperiod and FR-

395   light signalling coding for a CCT motif, a WNK-like and an *FTb2*-like protein, and the active

396   downstream cascade. The earlier down-regulation and the lower relative expression of these

397   genes observed under B-enriched light compared to FR-enriched light may explain their role

398   in the acceleration of floral onset.

399

400

401

408   **ACCESSION CODE**

409   The RNA-Seq sequencing data has been made available (under embargoed) at

410   https://zenodo.org (DOI: 10.5281/zenodo.1184974).

411   **CONFLICT OF INTEREST**

412   The co-authors have read and agreed to the content of the manuscript and declared no conflict of

413   interest.

414

REFERENCES

Aguilar-Martinez J.A., Uchida N., Townsley B., West D.A., Yanez A., Lynn N., …, Neelima S. (2015) Transcriptional, posttranscriptional, and posttranslational regulation of SHOOT MERISTEMLESS gene expression in Arabidopsis determines gene function in the shoot apex. *Plant Physiology* **167**, 424–442.

Alexa A. & Rahnenfuhrer J. (2010) topGO: enrichment analysis for gene ontology. R package. Version 2.

Andrés F. & Coupland G. (2012) The genetic basis of flowering responses to seasonal cues. *Nature Reviews Genetics* **13**, 627–639.

Andrés F., Porri A., Torti S., Mateos J., Romera-Brachat M., Garcias-Martinez J.L., …Coupland G. (2014) SHORT VEGETATIVE PHASE reduces gibberellin biosynthesis at the Arabidopsis shoot apex to regulate the floral transition. *Proceedings of the National Academy of Sciences of the United States of America* **111**, E2760–E2769.

Andrews S. (2010) FastQC: A quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Ballaré C.L. (2014) Light regulation of plant defense. *Annual Review of Plant Biology* **65**, 335–363.

Ballaré C.L. & Pierik R. (2017) The shade-avoidance syndrome: multiple signals and ecological consequences. *Plant, Cell & Environment* doi: 10.1111/pce.12914

Benedito V.A., Torres-Jerez I., Murray J.D., Andriankaja A., Allen S., Kakar K., …, Udvardi M.K. (2008) A gene expression atlas of the model legume *Medicago truncatula*. *Plant Journal* **55**, 504–513.

Blackman B.K. (2017) Changing responses to changing seasons: natural variation in the plasticity of flowering time. *Plant Physiology* **173**, 16–26.

Bolger A.M., Lohse M. & Usadel B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120.

Cerdán P. D. & Chory J. (2003) Regulation of flowering time by light quality. *Nature* **423**, 881 – 885.

Cerrudo I., Keller M.M., Cargnel M.D., Demkura P.V., de Wit M., Patitucci M.S., …, Ballaré C.L. (2012) Low red/far-red ratios reduce Arabidopsis resistance to Botrytis cinerea and jasmonate responses via a COI1-JAZ10-dependent, salicylic acid-independent mechanism. *Plant Physiology* **158**, 2042–2052.

Corbesier L., Vincent C., Jang S., Fornara F., Fan Q., Searle I., …, Coupland G. (2007) FT protein movement contributes to long-distance signalling in floral induction of Arabidopsis. *Science* **316**, 1030–1033.

Croser J.S., Pazos-Navarro M., Bennett R.G., Tschirren S., Edwards K., Erskine W., …, Ribalta FM (2016) Time to flowering of temperate pulses *in vivo* and generation turnover *in vivo–in vitro* of narrow-leaf lupin accelerated by low red to far-red ratio and high intensity in the far-red region. *Plant Cell, Tissue and Organ Culture* **127**, 591–599.

Cummings I.G., Reid J.B. & Koutoulis A. (2007) Red to far-red ratio correction in plant growth chambers—growth responses and influence of thermal load on garden pea. *Physiologia Plantarum* **131**,171–179.

Datta S., Hettiarachchi G.H.C.M., Deng X.W. & Holm M. (2006) Arabidopsis CONSTANS-LIKE3 is a positive regulator of red light signaling and root growth. *The Plant Cell* **18**, 70–84.

Devlin P.F. (2016) Plants wait for the lights to change red. *Proceedings of the National Academy of Sciences of the United States of America* **113**, 7301–7303.

Devlin P.F., Robson P.R., Patel S.R., Goosey L., Sharrock R.A. & Whitelam G.C. (1999) Phytochrome D acts in the shade-avoidance syndrome in Arabidopsis by controlling elongation growth and flowering time. *Plant Physiology* **119**, 909–916.

Dreni L. & Zhang D. (2016) Flower development: the evolutionary history and functions of the AGL6 subfamily MADS-box genes. *Journal of Experimental Botany* **67**, 1625–1638.

Haas B.J., Papanicolaou A., Yassour M., Grabherr M., Blood P.D., Bowden J., …, Lieber M. (2013) De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nature Protocols* **8**, 1494–1512.

Hecht V., Knowles C.L., Vander Schoor J.K., Liew L.C., Jones S.E., Lambert M.J. & Weller J.L. (2007) Pea LATE BLOOMER1 is a GIGANTEA ortholog with roles in photoperiodic flowering, deetiolation, and transcriptional regulation of circadian clock gene homologs. *Plant Physiology* **144**, 648–661.

Hecht V., Laurie R.E., Vander Schoor J.K., Ridge S., Knowles C.L., Liew L.C., …, Weller J.L. (2011) The Pea GIGAS gene is a FLOWERING LOCUS T homolog necessary for graft-transmissible specification of flowering but not for responsiveness to photoperiod. *The Plant Cell* **23**, 147–161.

Hempel F.D., Weigel D., Mandel M.A., Ditta G., Zambryski P.C., Feldman L.J. & Yanofsky M.F. (1997) Floral determination and expression of floral regulatory genes in Arabidopsis. *Development* **124**, 3845–3853.

Hirakawa H., Kaur P., Shirasawa K., Nichols P., Nagano S., Appels R., …, Isobe S.N. (2016) Draft genome sequence of subterranean clover, a reference for genus Trifolium. *Science Reports* **6**. doi: 10.1038/srep30358.

Imaizumi T. & Kay S. A. (2006) Photoperiodic control of flowering: not only by coincidence. *TREND in Plant Science* **11**, 550 – 558.

Jiao Y., Lau O.S. & Deng X.W. (2007) Light-regulated transcriptional networks in higher plants. *Nature Reviews Genetics* **8**, 217–230.

Johkan M., Shoji K., Goto F., Hashida S. & Yoshihara T. (2010) Blue light-emitting diode light irradiation of seedlings improves seedling quality and growth after transplanting in red leaf lettuce. *Hortscience* **12**, 1809–1814.

Kaur P., Bayer P.E., Milec Z., Vrána J., Yuan Y., Appels R., …, Doležel J. (2017) An advanced reference genome of *Trifolium subterraneum* L. reveals genes related to agronomic performance. *Plant Biotechnology Journal* **15**, 1034–1046.

Kim S.Y., Yu X. & Michaels S.D. (2008) Regulation of CONSTANS and FLOWERING LOCUS t expression in response to changing light quality. *Plant Physiology* **148**, 269–279.

Lee H., Suh S., Park E., Cho E., Ahn J.H., Kim S., …, Lee I. (2000) The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in Arabidopsis. *Genes & Development* **14**, 2366–2376.

Liao Y., Smyth G.K. & Shi W. (2014) featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923–930.

Libault M., Thibivilliers S., Bilgin D.D., Radwan O., Benitez M., Clough S.J. & Stacey G. (2008) Identification of four soybean reference genes for gene expression normalization. *Plant Genome* **1**, 44–54.

Liew L.C., Hecht V., Weeden N.F. & Weller J.L. (2009) Isolation of Pseudo Response Regulator genes and evaluation as candidate genes for photoperiod response loci. *Pisum Genetics* **41**, 21–25.

511 Ma H., Yanofsky M.F. & Meyerowitz E.M. (1991) AGL1-AGL6, an Arabidopsis gene
512   family with similarity to floral homeotic and transcription factor genes. *Genes &*
513   *Development* **5**, 484–495.
514 Moon J., Suh S., Lee H., Choi K., Hong C.B., Paek N., …, Lee I. (2003) The SOC1 MADS-
515   box gene integrates vernalization and gibberellin signals for flowering in Arabidopsis.
516   *Plant Journal* **35**, 613–623.
517 Ozsolak F. & Milos P.M. (2011) RNA sequencing: advances, challenges and opportunities.
518   *Nature Reviews Genetics* **12**, 87–98.
519 Pazos-Navarro M., Castello M., Bennett R.G., Nichols P. & Croser J. (2017) *In vitro*-assisted
520   single seed descent for breeding cycle compression in subterranean clover (*Trifolium*
521   *subterraneum* L.). *Crop & Pasture Science* **68**, 958 – 966.
522 Pelaz S., Ditta G.S., Baumann E., Wisman E. & Yanofsky M.F. (2000) B and C floral organ
523   identity functions require SEPALLATA MADS-box genes. *Nature* **405**, 200–203.
524 Pin P.A. & Nilsson O. (2012) The multifaceted roles of FLOWERING LOCUS T in plant
525   development. *Plant, Cell & Environment* **35**, 1742–1755.
526 Rajapakse N.C. & Shahak Y. (2007) Light quality manipulation by horticulture industry. In
527   Annual Plant Reviews Volume 30: Light and Plant Development (eds G. C. Whitelam
528   & K. J. Halliday), pp. 290–312. Blackwell Publishing Ltd, Oxford.
529 Ribalta F.M., Pazos-Navarro M., Nelson K., Edwards K., Ross J.J., Bennett R.G., …, Croser
530   J.S. (2017) Precocious floral initiation and identification of exact timing of embryo
531   physiological maturity facilitate germination of immature seeds to truncate the lifecycle
532   of pea. *Plant Growth Regulation* **81**, 345–353.
533 Robinson M.D., McCarthy D.J. & Smyth G.K. (2010) edgeR: a Bioconductor package for
534   differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–
535   140.
536 Ruijter J. M., Ramakers C., Hoogaars W. M. H., Karlen Y., Bakker O., van den Hoff M. J. B.
537   & Moorman A. F. M. (2009) Amplification efficiency: linking baseline and bias in the
538   analysis of quantitative PCR data. *Nucleic Acids Research* **37**, e45.
539 Runkle E.S. & Heins R.D. (2001) Specific functions of red, far red, and blue light in
540   flowering and stem extension of long-day plants. *Journal of the American Society for*
541   *Horticultural Science* **126**, 275–282.
542 Saitoh A., Takase T., Kitaki H., Miyazaki Y. & Kiyosue T. (2015) Gene expression profile of
543   zeitlupe/lov kelch protein1 T-DNA insertion mutants in *Arabidopsis thaliana*:
544   Downregulation of auxin-inducible genes in hypocotyls. *Plant Signaling & Behavior*
545   **10**. doi: 10.1080/15592324.2015.1071752
546 Salter M.G., Franklin K.A. & Whitelam G.C. (2003) Gating of the rapid shade-avoidance
547   response by the circadian clock in plants. *Nature* **426**, 680–683.
548 Schmittgen T.D. & Livak K. J. (2008) Analysing real-time PCR data by the comparative C_T
549   method. *Nature protocols* **3**, 1101–1108.
550 Scofield S., Dewitte W. & Murray J.A.H. (2007) The KNOX gene SHOOT
551   MERISTEMLESS is required for the development of reproductive meristematic tissues
552   in *Arabidopsis*. *Plant Journal* **50**, 767–781.
553 Severin A.J., Woody J.L., Bolon Y.T., Joseph B., Diers B.W., Farmer A.D., …, Shoemaker
554   R.C. (2010) RNA-Seq atlas of *Glycine max*: a guide to the soybean transcriptome. *BMC*
555   *Plant Biology* **10**, 160.
556 Shim J.S., Kubota A. & Imaizumi T. (2017) Circadian clock and photoperiodic flowering in
557   Arabidopsis: CONSTANS is a hub for signal integration. *Plant Physiology* **173**, 5–15.
558 Singh A.M., Chappell J., Trost R., Lin L., Wang T., Tang J., …, Dalton S. (2013) Cell-cycle
559   control of developmentally regulated transcription factors accounts for heterogeneity in
560   human pluripotent cells. *Stem Cell Reports* **1**, 532–544.

561 Song Y.H., Ito S. & Imaizu T. (2013) Flowering time regulation: photoperiod- and
562   temperature-sensing leaves. *Trends in Plant Science* **18**, 575–583.
563 Strayer C., Oyama T., Schultz T.F., Raman R., Somers D.E., Más P., …, Kay S.A. (2000)
564   Cloning of the Arabidopsis clock gene TOC1, an autoregulatory response regulator
565   homolog. *Science* **289**, 768–771.
566 Tapia-L☐pez R., Garcia-Ponce B., Dubrosky J.G., Garay-Arroyo A., Perez-Ruiz R.V., Kim
567   S., …, Alvarez-Buylla E.R. (2008) An AGAMOUS-related MADS-box gene, XAL1
568   (AGL12), regulates root meristem cell proliferation and flowering transition in
569   Arabidopsis. *Plant Physiology* **146**, 1182–1192.
570 Thomas B. (2006) Light signals and flowering. *Journal of Experimental Botany* **57**, 3387–
571   3393.
572 Trapnell C., Pachter L. & Salzberg S.L. (2009) TopHat: discovering splice junctions with
573   RNA-Seq. *Bioinformatics* **25**, 1105–1111.
574 Turck F., Fornara F. & Coupland G. (2008) Regulation and identity of florigen:
575   FLOWERING LOCUS T moves center stage. *Annual Review of Plant Biology* **59**, 573–
576   594.
577 Viczián A., Klose C., Ádám É. & Nagy F. (2017) New insights of red light-induced
578   development. *Plant, Cell & Environment* doi: 10.1111/pce.12880
579 Wang Y., Liu K., Liao H., Zhuang C., Ma H. & Yan X. (2008) The plant WNK gene family
580   and regulation of flowering time in Arabidopsis. *Plant Biology* **10**, 548–562.
581 Wang Z., Gerstein M. & Snyder M. (2009) RNA-Seq: a revolutionary tool for
582   transcriptomics. *Nature Reviews Genetics* **10**, 57–63.
583 Weller J.L., Liew L.C., Hecht V.F., Rajandran V., Laurie R.E., Ridge S., …, Lejeune-Hénaut
584   I. (2012) A conserved molecular basis for photoperiod adaptation in two temperate
585   legumes. *Proceedings of the National Academy of Sciences of the United States of
586   America* **109**, 21158–21163.
587 Weller J.L. & Ortega R. (2015) Genetic control of flowering time in legumes. *Frontiers in
588   Plant Science* **6**, 207.
589 Wang Y., Liu K., Liao H., Zhuang C., Ma H. & Yan X. (2008) The plant WNK gene family
590   and regulation of flowering time in *Arabidopsis*. *Plant Biology* **10**, 548 – 562.
591 Wong A.C.S., Hecht V.F.G., Picard K., Diwadkar P., Laurie R., Wen J., …, Weller J.L.
592   (2014) Isolation and functional analysis of CONSTANS-LIKE genes suggests that a
593   central role for CONSTANS in flowering time control is not evolutionary conserved in
594   *Medicago truncatula*. *Frontiers in Plant Science* **5**, 486.
595 Ye J., Coulouris G., Zaretskaya I., Cutcutache I., Rozen S. & Madden T. L. (2012) Primer-
596   BLAST: A toll to design target-specific primers for polymerase chain reaction. *BMC
597   Bioinformatics* **13**, 134.
598

599   **Table 1.** LogFC > 2 [FRD < 0.05] of up- and down-regulated flowering-related genes

600   identified at each time-point when comparing blue-enriched with far-red enriched lights. ns =

601   not significant

Gene ID	Functional annotation	logFC > 2 [FRD < 0.05]		
		Time-point 1	Time-point 2	Time-point 3
TSub_g7401	CCT motif protein: conserved domain related to *CONSTANS* and *CONSTANS*-like proteins	–3.576	ns	ns
TSub_g11956	With no lysine (K) kinase 5 (*WNK5*): It may regulate flowering time by modulating the photoperiod pathway	–2.773	ns	ns
TSub_g12538	*FLOWERING LOCUS T b2* (*FTb2*): probable floral promoter	–4.54	ns	ns
TSub_g25875	*AGAMOUS*-like 20 (*AGL20*): Transcription activator active in flowering time control	–2.977	ns	ns
TSub_g15483	*AGAMOUS*-like 8 (AGL8): Probable transcription factor that promotes early floral meristem identity	–3.47	ns	ns
TSub_g21607	*AGAMOUS*-like 4 (AGL4): Probable transcription factor that could be involved in genes regulation during floral meristem development	–7.996	ns	ns
TSub_g3280	*AGAMOUS*-like 6 (AGL6): Probable transcription factor that could be involved in genes regulation during floral meristem development	–6.786	ns	ns
TSub_g20101	*AGAMOUS*-like 12 (AGL12): Probable transcription activator that may act as promoter of the flowering transition through up-regulation of *AGL20*, *FT* and *LEAFY*	–7.263	3.103	ns
TSub_g491	Homeobox protein knotted-1-like 1 (*KNAT1*): It may play a role in meristem function for transition from vegetative to reproductive development	–2.268	ns	ns
TSub_g22633	*GIBBERELLIN 20 OXIDASE 2* (*GAS20OX2*): It is a key oxidase enzyme in the biosynthesis of gibberellin involved in the promotion of the floral transition	–2.538	ns	ns
TSub_g15042	*SUCROSE- PROTON SYMPORTER 2* (*SUC2*): Responsible for the transport of sucrose into the cell, with the concomitant uptake of protons.Related to the florigen	–3.32	ns	ns
TSub_g30315	Flowering promoting factor	ns	ns	3.833
TSub_g30317	Flowering promoting factor	ns	ns	4.291

602

603

604   **Figure 1.** A. Light spectrum profiles (wavelength (nm) vs. photon flux density (PFD, μmol

605    $m^{-2} s^{-1}$). B. Time to flowering of *Trifolium subterraneum* plants and spectral characteristics

606   (PFD, μmol $m^{-2} s^{-1}$) of the environments used in this study. E1: far-red enriched light (AP67

607   LED lights, Valoya, Finland); E2: blue-enriched LED light (108D18-V12 tubes, S-Tech

608   Lighting, Australia). The red to far-red ratio calculations followed the method of Runckle and

609   Heins (2001): photon irradiance between 655 and 665 nm/ photon irradiance between 725

610   and 735 nm.

611   **Figure 2.** A. Time-points (TP) selected for this study based on when plants in the E1

612   environment attained the following developmental milestones: third-leaf stage (TP1),

613   appearance of the first flower bud (TP2), open flower (TP3). B. Volcano plots for the

614   differentially expressed genes. The red dots represent genes with logFC > 2 [FDR < 0.05]

615   whereas the green dots represent genes logFC > 4 [FDR < 0.05].

616   **Figure 3.** A. The number of genes differentially regulated depending on the light provided.

617   Red represents genes up-regulated and blue represents genes down-regulated under blue-

618   enriched lights at the different time-points (TP) selected in this study when plants in the E1

619   environment attained the following developmental milestones: third-leaf stage (TP1),

620   appearance of the first flower bud (TP2), open flower (TP3). B. Gene expression differences

621   for genes with FDR < 0.05 and logFC > 2. The Venn diagram presents the number of genes

622   that are exclusively differentially expressed at each developmental stage, with the

623   overlapping regions showing the number of genes that are differentially expressed at two or

624   more developmental stages.

625   **Figure 4.** Differentially expressed genes related to flowering induction by different light

626   spectra. A Genes identified at time-point 1 (TP1) when plants reached third-leaf stage, these

627   genes were down-regulated under blue-enriched light (high R: FR). B. Genes identified at

628   time-points 2 (TP2) and 3 (TP3), these genes were up-regulated under blue-enriched light

629   (high R: FR). *CRY:* cryptochrome, *PHYA:* phytochrome *A*, *PHYB:* phytochrome B, CCT:

630   CCT motif protein, *KNAT1:* homeobox protein knotted-1-like 1, *AGL8: AGAMOUS*-like 8,

631   *FTb2: FLOWERING LOCUS T b2, SUC2:* sucrose-proton symporter 2, *AGL4: AGAMOUS*-

632   like 4, *AGL6*: *AGAMOUS*-like 6, *AGL20: AGAMOUS*-like 20, *AGL12: AGAMOUS*-like 12,

633   *WNK5:* with no lysine (K) kinase, *GAS20OX2*: gibberellin 20 oxidase 2, FP1 and FP2:

634   flowering promoters.

635   **Figure 5.** Relative gene expression of *TSub_g7401* (CCT motif protein), *TSub_g3280*

636   (*AGL6*: *AGAMOUS*-like 6), *TSub_g25875* (*AGL20: AGAMOUS*-like 20), *TSub_g20101*

637   (*AGL12: AGAMOUS*-like 12), *TSub_g15483* (*AGL8: AGAMOUS*-like 8), *TSub_g12538*

638   (*FTb2: FLOWERING LOCUS T b2), TSub_g11956* (*WNK5:* With no lysine (K) kinase) and

639   *TSub_g21607* (*AGL4: AGAMOUS*-like 4) in response to Far Red-enriched (FR) and Blue-

640   enriched (B) lights. Gene expression was quantified by qPCR at third-leaf stage (14 d after

641   sowing). Data is represented as expression of the gene of interest relative to *Pisum sativum* L.

642   (Alaska) ubiquitin (PUB3) gene L81141.1 as reference gene using the $2^{-\Delta Ct}$ method

643   (Schmittgen & Livak 2008). Values are means ± SE (n = 3 replicates of 2 plants).

644

A

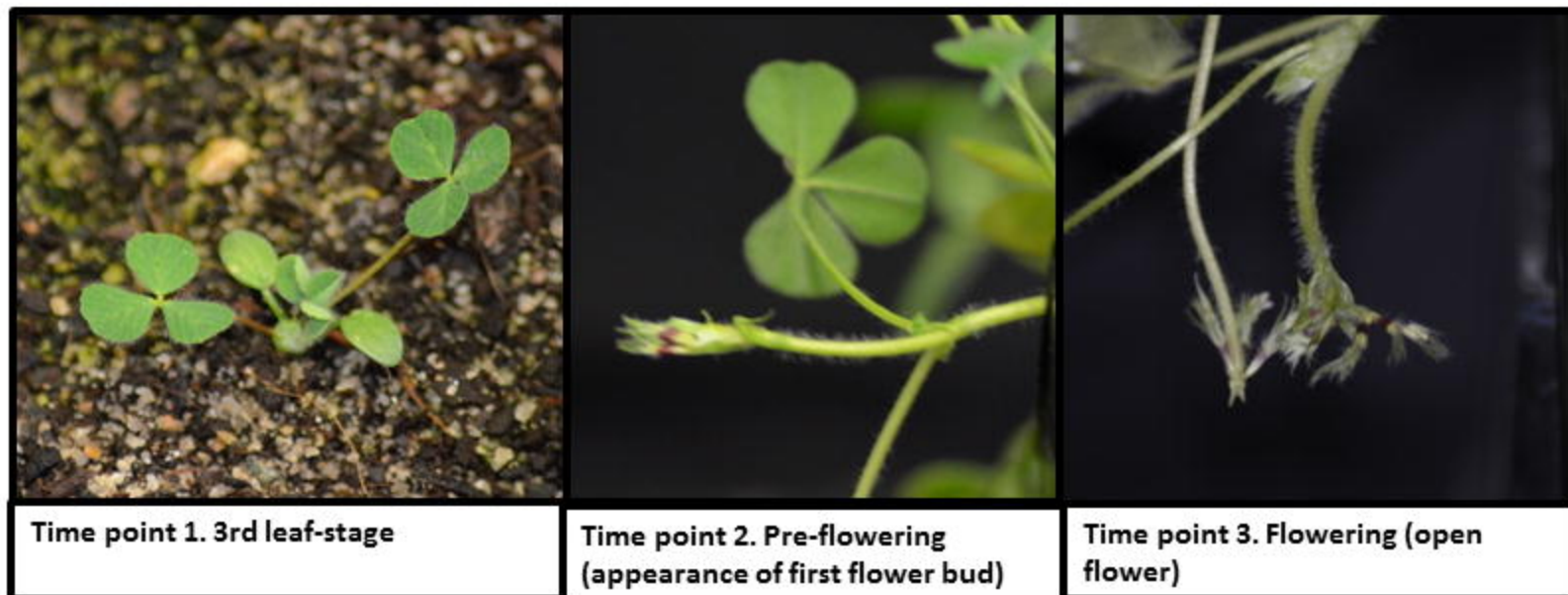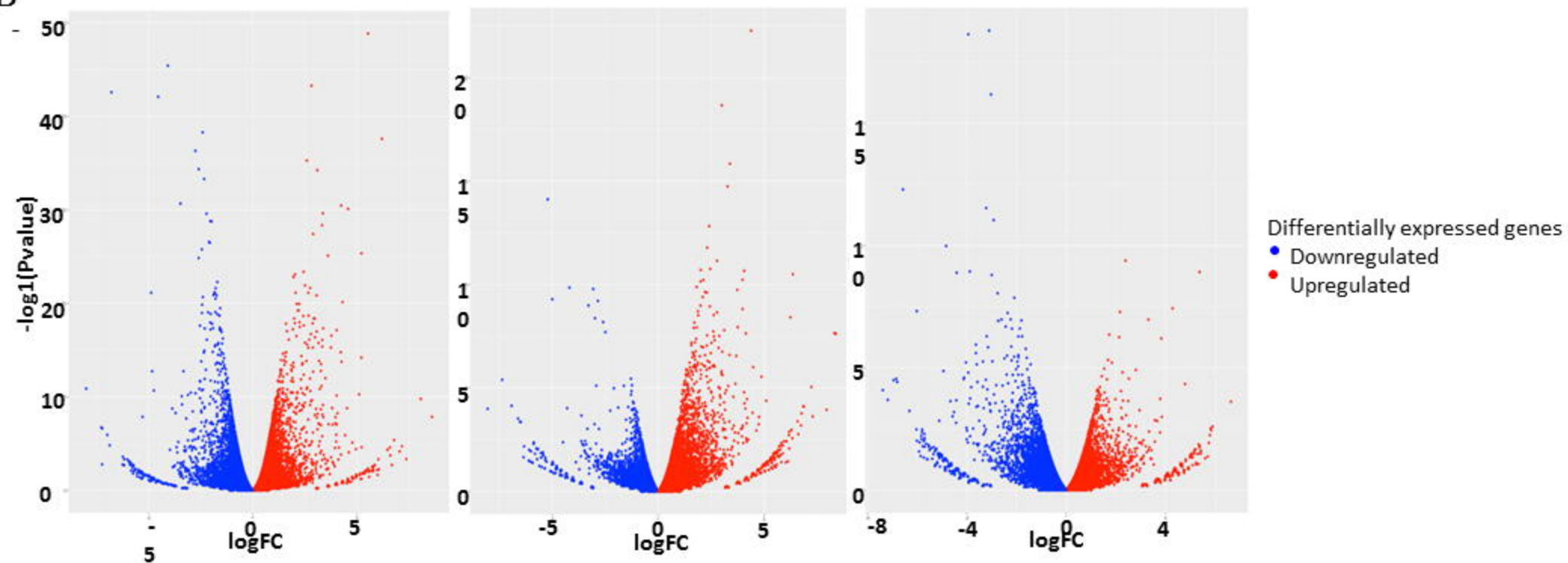| GeneID	Functional annotation	FR-enriched light		B-enriched light		Fold change due to treatment (FR vs B)
		$2^{(-\Delta Ct)}$	SE	$2^{(-\Delta Ct)}$	SE	
TSub_g7401	CCT motif protein: conserved domain related to CONSTANS and CONSTANS-like proteins	0.0193	0.0037	0.0013	0.0002	-14.16
TSub_g3280	AGAMOUS-like 6 (AGL6): Probable transcription factor that could be involved in genes regulation during floral meristem development	0.1782	0.0064	0.0087	0.0010	-20.79
TSub_g25875	AGAMOUS-like 20 (AGL20): Transcription activator active in flowering time control	0.2880	0.0264	0.1794	0.0100	-1.60
TSub_g20101	AGAMOUS-like 12 (AGL12): Probable transcription activator that may act as promoter of the flowering transition through up-regulation of AGL20, FT and LEAFY	0.0523	0.0012	0.0053	0.0004	-9.83
TSub_g15483	AGAMOUS-like 8 (AGL8): Probable transcription factor that promotes early floral meristem identity	0.3450	0.0053	0.1335	0.0065	-2.59
TSub_g12538	FLOWERING LOCUS T b2 (FTb2): probable floral promoter	0.1798	0.0207	0.0525	0.0132	-4.09
TSub_g11956	With no lysine (K) kinase 5 (WNK5): It may regulate flowering time by modulating the photoperiod pathway	1.7416	0.1345	0.7418	0.0222	-2.25
TSub_g21607	AGAMOUS-like 4 (AGL4): Probable transcription factor that could be involved in gene regulation during floral meristem development	1.7759	0.4321	0.0162	0.0017	-104.28

B

A
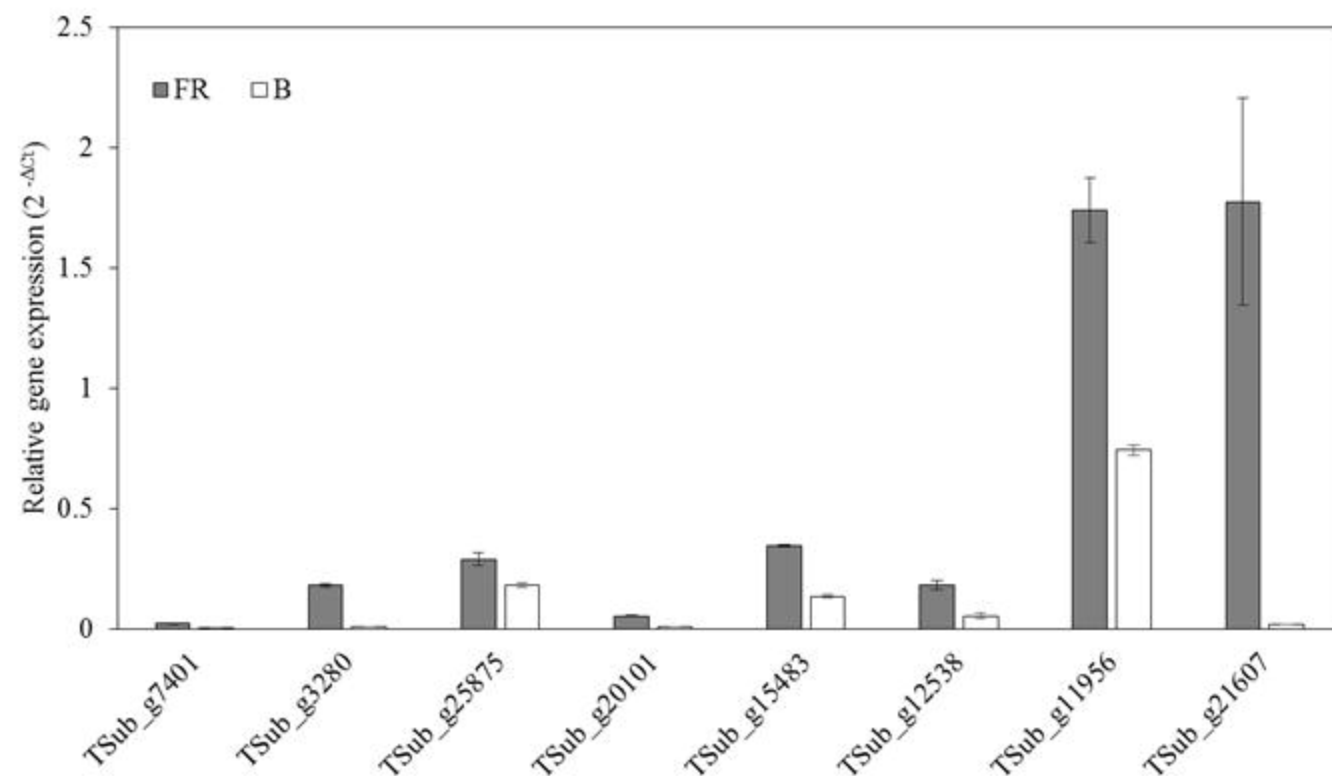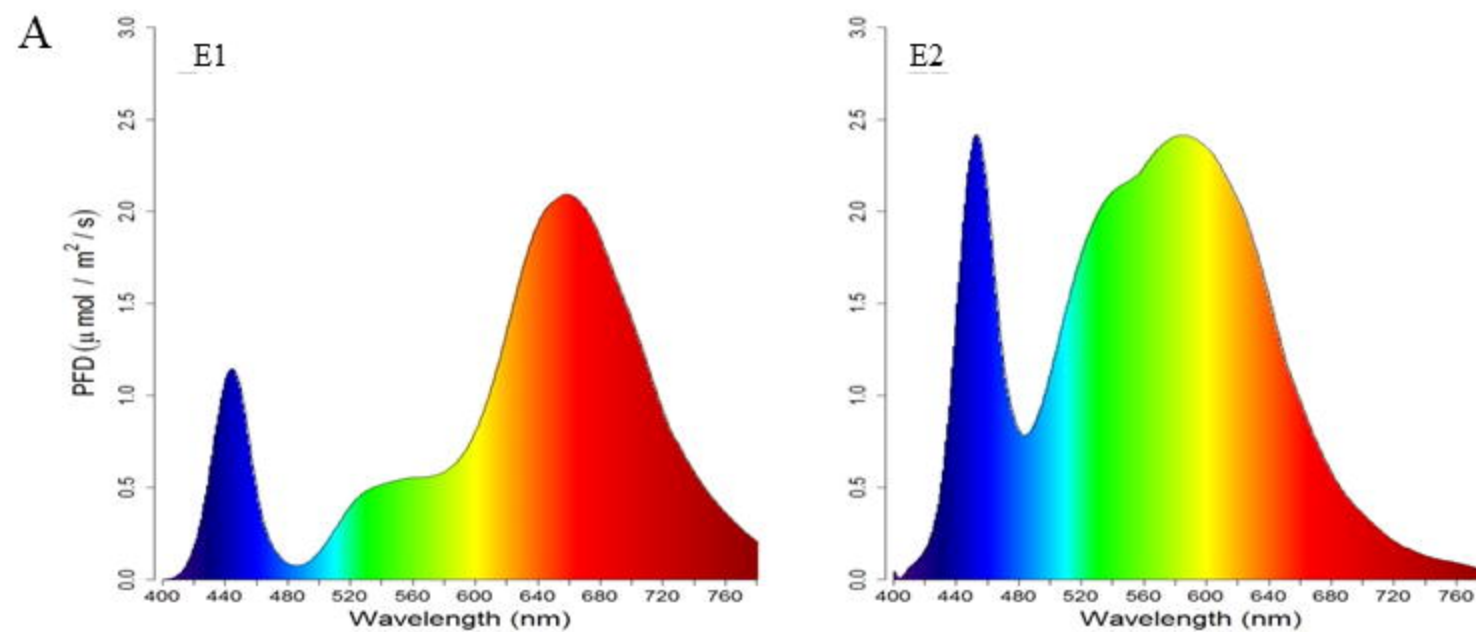
B

	E1	E2
Blue	38.1	99.7
Red	168.3	129.6
Far red	52.9	12.5
Total intensity	310	450
Red: Far red ratio	2.89	5.86
Time to flowering	46.5 ± 1.4	60.7 ± 2.2
F value**	582.69***	

*One-way ANOVA, F value and significance for flowering time at **$P \leq 0.001$

A

Time point 1. 3rd leaf-stage

Time point 2. Pre-flowering (appearance of first flower bud)

Time point 3. Flowering (open flower)

B

Differentially expressed genes

- Downregulated
- Upregulated
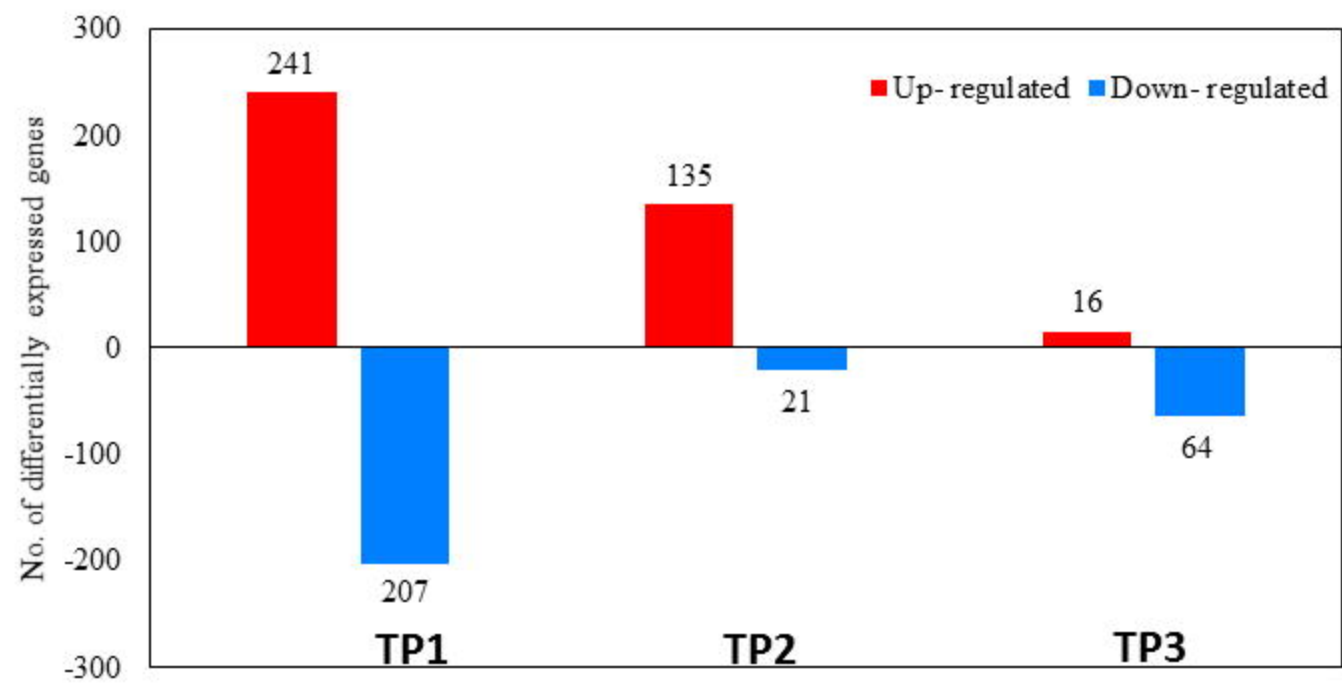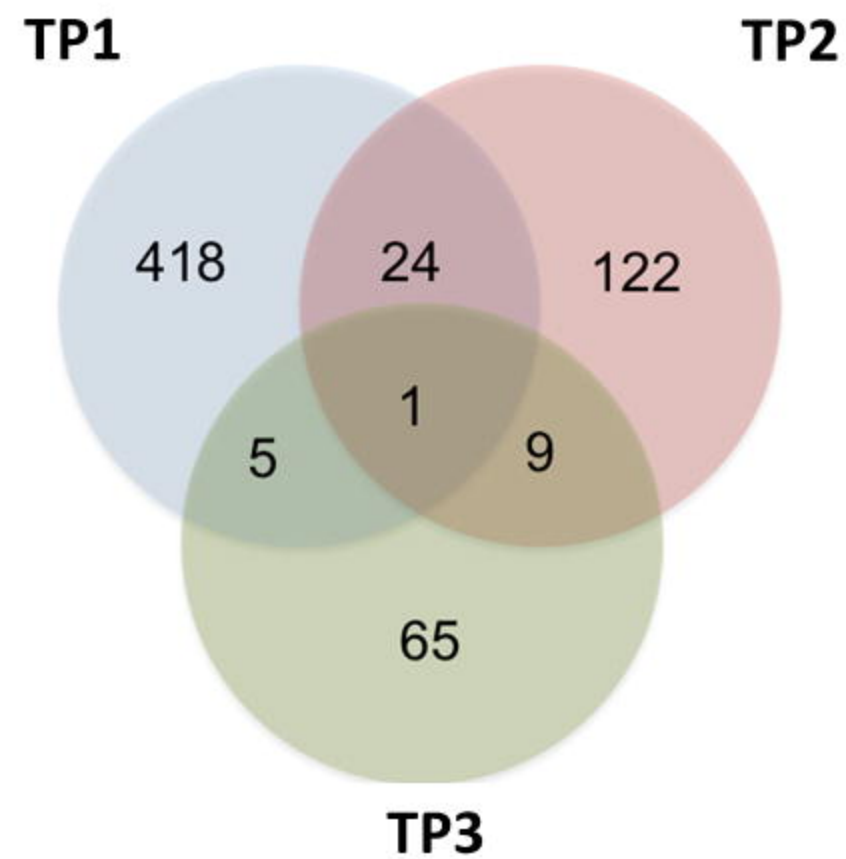
A — Down-regulated genes by Blue-enriched light

Light input

TP1

CRY
PHYA
PHYB
CCT
WNK5
FT
SUC2
AGL8
AGL6
AGL12
AGL20
GA20OX1
KNAT1
AGL4

Light signalling; phytochromes and cryptochromes

Blue-light

Far red-light

Red-light

Known gene interactions

Hypothetical gene interactions
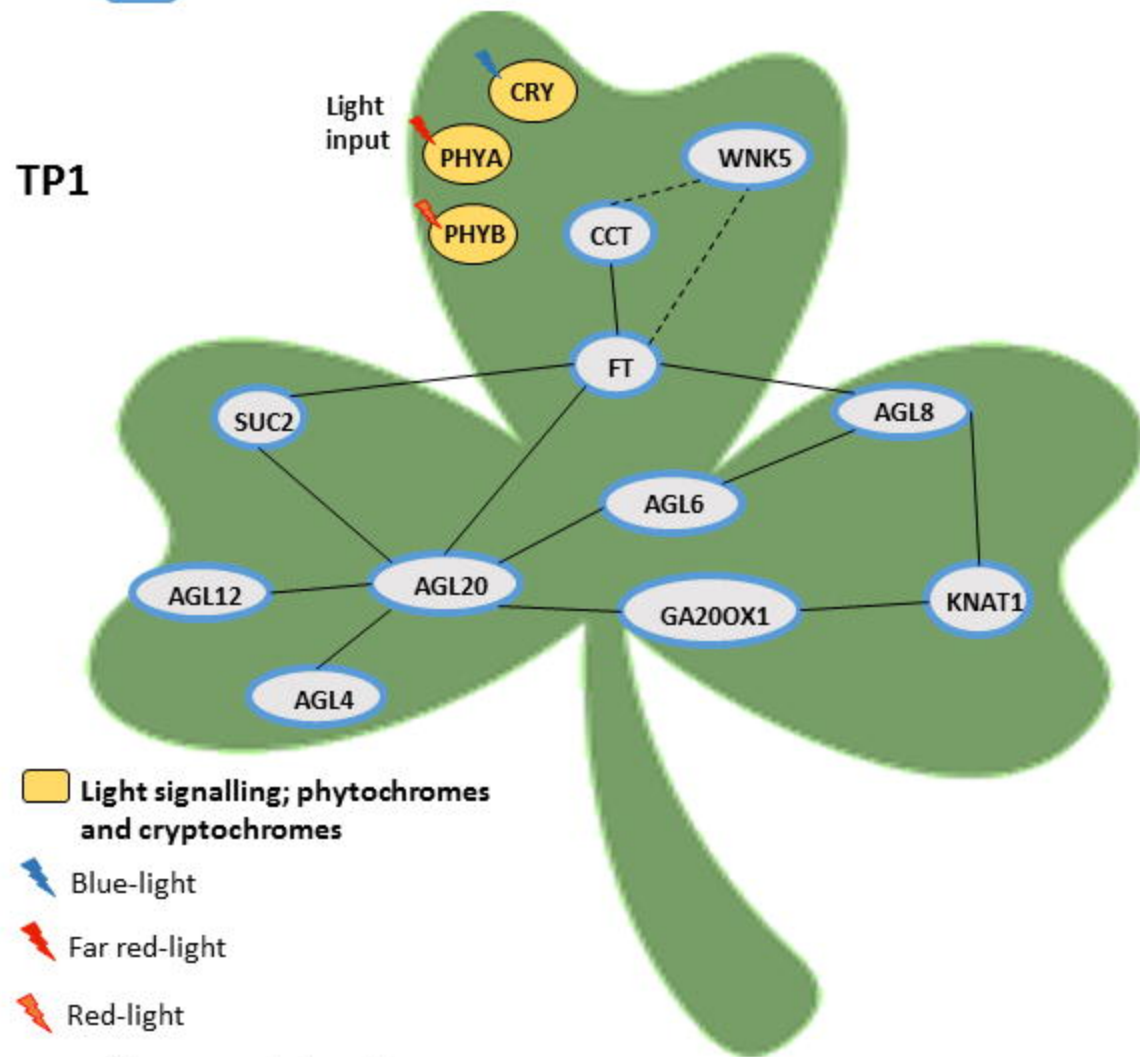
B — Up-regulated genes by Blue light- enriched light

Light input

CRY
PHYA
PHYB

TP2

AGL12

TP3

FP1
FP2