Short running title

Far-red light regulates floral induction network

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

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ABSTRACT

Light is one of the most important signals that regulate flowering through quality, quantity and duration. The low red to far-red ratio accelerates flowering in a wide range of species. The central gene pathways for controlling flowering time, identified in arabidopsis, appear to be largely conserved in legumes. However, there are numerous examples 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 whole-genome transcriptome profiling 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 CCT motif related to *CONSTANS* and a *FLOWERING LOCUS T b2* like protein, and their active downstream cascade. The earlier down-regulation of these genes observed under blue compared to far-red -enriched light may explain their role in floral induction. We also found contrasting responses to light quality related to reproduction and defence mechanisms. These results will contribute to a better understanding of the molecular basis of flowering in response to different light quality in long-day plants.

KEYWORD INDEX

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

INTRODUCTION

Flowering behaviour is modified by environmental cues. Light is one of the most important signals that regulate flowering through quality, quantity and duration (Thomas 2006). The genetic mechanisms involved in this response have been widely studied, but questions remain. Plants perceive subtle changes in light composition, duration and direction through photoreceptor pigments, which result in physiological and morphological modifications necessary for adaptation to environmental changes (Rajapakse & Shakak 2007). In higher plants, the 'shade avoidance syndrome' is an adaptive developmental strategy mediated by the perception of light spectral quality (in particular the red to far-red ratio, R: FR), which acts as an early warning of potential shading (Salter *et al.* 2003; Ballaré & Pierik 2017). In arabidopsis, the most noticeable shade avoidance responses include rapid internode elongation and accelerated floral initiation at the expense of leaf expansion and chlorophyll synthesis reduction (Devlin *et al.* 1999). Similarly, research in the model long-day species pea (*Pisum sativum*) demonstrated that FR-enriched light, with an R: FR ratio below 3.5, is most effective for early floral induction (Runkle & Heins 2001; Cummings *et al.* 2007; Croser *et al.* 2016; Ribalta *et al.* 2017).

Changes in light quality are detected in the leaf by the action of a family of plant photoreceptors, including phytochromes (R and FR light receptors) and cryptochromes (blue light receptors) and involve complex gene regulatory networks (Andrés & Coupland 2012; Viczián *et al.* 2017). In *Arabidopsis thaliana* (arabidopsis), significant progress has been made toward understanding the role light quality plays on floral initiation pathways. The genes *GIGANTEA* (*GI*), *FLAVON KELCH F BOX* (*FKF1*), *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) have major regulatory roles in the promotion of flowering in response to photoperiod (Thomas 2006; Jiao *et al.* 2007). In particular, *CO* has been described as a network hub for the integration of internal and external signals into the

63 photoperiodic flowering pathway to induce *FT* expression (Wong *et al.* 2014; Shim *et al.*
64 2017). *FT* acts as a mobile flower-promoting signal that integrates day length, light quality,
65 circadian clock, temperature and vernalisation inputs (Turck *et al.* 2008). In legumes, the
66 basic genes and gene families central to pathways controlling flowering time in arabidopsis
67 appear to be largely conserved. However, there are numerous examples of gene duplication
68 and loss (Weller & Ortega 2015). In pea, the conserved role of the arabidopsis genes *GI*,
69 *EARLY FLOWERING 3* and *EARLY FLOWERING 4* in the regulation of *FT* genes has been
70 demonstrated (Hecht *et al.* 2007; Liew *et al.* 2009; Weller *et al.* 2012). Orthologous
71 arabidopsis genes play a part in the photoperiodic flowering pathway in legumes. However,
72 the role of *CONSTANS-LIKE* (*COL*) genes as integrators of the photoperiod response has
73 been questioned in several dicot species including legumes (Blackman 2017). Recent studies
74 in *Medicago truncatula* (Medicago) revealed that none of the *COL* genes identified was
75 functionally equivalent to *CO*, with respect to inducing *FT* expression (Wong *et al.* 2014).
76 These findings support the idea of *CO*-independent pathways involved in flowering induction
77 in legumes.

78 An improved understanding of the gene networks underlying flowering induction in
79 response to light quality will require better characterisation of the transcriptome. The recent
80 development of deep-sequencing technologies, such as RNA-Seq, has enabled the generation
81 of a high-resolution global view of the transcriptome and its organisation for some species
82 and cell types (Wang *et al.* 2009). Whole-transcriptome sequencing using RNA-Seq is a
83 convenient and rapid means to study gene expression at the whole-genome level and define
84 putative gene function (Wang *et al.* 2009; Ozsolak & Milos 2011; Singh *et al.* 2013;
85 Hirakawa *et al.* 2016; Kaur *et al.* 2017). Rapid advances have been made toward
86 understanding the transcriptional regulation of specific developmental processes in legumes
87 (Benedito *et al.* 2008; Libault *et al.* 2008; Severin *et al.* 2010); we now seek to apply whole-

88 transcriptome sequencing to characterise the genetic regulatory mechanisms underlying the
89 induction of flowering in legumes in response to changes in the R: FR ratio using light
90 emitting diodes (LED).

91 Despite the substantial number of legumes with their genome sequenced and/or
92 significant genomic resources such as pea, soybean (*Glycine max*), Medicago and *Lotus*
93 *japonicus*, no legume species has emerged as a predominant model in the study of flowering
94 time (Weller & Ortega 2015). Recently, we established subterranean clover (*Trifolium*
95 *subterraneum* L.) as a reference species for genetic and genomic studies within the genus
96 *Trifolium* which seems to respond well to changes in the R: FR ratio using LED.
97 Subterranean clover is diploid (2n = 16), predominantly inbreeding, and has a well-assembled
98 and annotated genome with a tissue type transcriptome atlas available (Hirakawa *et al.* 2016;
99 Kaur *et al.* 2017). Understanding the genetics of important traits in this model species can
100 provide a pathway to understanding the genetic mechanisms in other genetically complex
101 species.

102 Within this research, we used deep-sequencing technologies to investigate
103 transcriptional activity under different light spectra at three time-points in the long-day plant,
104 subterranean clover. We have previously shown the accelerated onset of flowering in a range
105 of leguminous species under FR-enriched LED light spectra and have thus adopted this
106 wavelength profile and compared it with a blue-enriched LED light spectra (Croser *et al.*
107 2016; Pazos-Navarro *et al.* 2017; Ribalta *et al.* 2017). We expect that the FR-enriched spectra
108 will accelerate the up-regulation of genes related to floral initiation. Our results will enable us
109 to understand better the effect of FR-enriched light spectra on the gene networks regulating
110 time to flowering.

111

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 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

137 environments were at the third-leaf stage), 42 growing days for TP2, and 47 growing days for
138 TP3. Leaf tissue (75–100 mg FW) was harvested and snap-frozen in liquid nitrogen and
139 stored at –80 °C until RNA extraction.

140 **RNA isolation and library preparation**

141 Total RNA from all tissue samples was extracted using the Spectrum™ Plant Total RNA Kit
142 (Sigma-Aldrich, St Louis, USA) following the manufacturer's instructions. Aliquots of
143 purified RNA were stored at −80 °C. The concentration of RNA was confirmed using a Qubit
144 fluorometer with the Qubit RNA assay kit (Life Technologies, Carlsbad, USA). The integrity
145 of total RNA was determined by electrophoretic separation on 1.2% (w/v) denaturing agarose
146 gels. Sequencing libraries were constructed using 500 ng of total RNA with a TruSeq®
147 Stranded Total RNA Sample Prep Kit with Ribo-Zero (Illumina Inc., San Diego, USA)
148 following the manufacturer's instructions. Concentrations of libraries were measured using
149 the Qubit fluorometer with the Qubit dsDNA HS assay kit (Life Technologies, Carlsbad,
150 USA) and Agilent high-sensitivity DNA chips (Agilent Technologies, Santa Clara, USA).
151 The amplified libraries were pooled in equimolar amounts, and quality was assessed with
152 Agilent high-sensitivity DNA chips (Agilent Technologies, Santa Clara, USA). All reads
153 were pair-end sequenced using the HiSeq 2000 platform (Illumina Inc., San Diego, USA).

154 **Differential gene expression analysis**

155 The sequencing quality of the Illumina reads was assessed using FastQC (Andrews 2010).
156 The reads were quality filtered using Trimmomatic (Bolger *et al.* 2014) to remove adapter
157 sequences. Filtered reads were mapped to the advanced genome assembly (Tsub_Refv2.0)
158 (Kaur *et al.* 2017) using TopHat v2.1.1 (Trapnell *et al.* 2009) with default parameters and
159 passing the reference annotation with the-G option. Paired-end and single-end reads were
160 mapped separately, and the BAM files were used to calculate the read counts for each gene

using featureCounts (Liao *et al.* 2014) (Table S1.1 and Table S1.2). For each sample, the sum of read counts at each gene locus was taken by adding the read counts from the paired-end BAM file and single-end BAM files. Differential expression analysis was carried out on the matrix of read counts for each gene (rows) and sample (columns) using the perl script run_DE_analysis.pl from the Trinity v2.2.0 (Haas *et al.* 2013) suite of programs based on the edgeR method (Robinson *et al.* 2010). Volcano plots were generated for each time-point and differentially expressed genes with false discovery rate (FDR) less than 5% were output for each time-point.

Functional characterisation and GO enrichment analyses

Gene ontology (GO) enrichment was performed using the Fisher exact test as implemented in R using the topGO package (Alexa & Rahnenfuhrer 2010) with method 'weight01' used to adjust for multiple comparisons. Up- and down-regulated DEGs (logFC>2 and FDR<0.05) were considered for the analysis.

RESULTS

Far-red enriched light accelerates floral onset

To study the effect of light quality on time to flowering, plants were grown simultaneously in controlled environments under the same temperature (24/20 °C day/night) and photoperiod (20 h) regime, but different light spectra. Environment 1 (E1) consisted of an R: FR ratio of 2.89 (FR-enriched light) and Environment 2 (E2) consisted of an R: FR ratio of 5.86 (blue-(B) enriched light). We observed a clear effect of light spectra on floral onset in subterranean clover. Time to flowering was significantly reduced ($P < 0.001$) under FR-enriched light by 14 days compared to B-enriched light (Fig. 1, A and B).

Gene response to different light spectra

Given the clear effect of the FR-enriched spectrum on the acceleration of floral onset, we aimed to identify the influence of light on gene expression related to flowering induction.

Transcriptome dynamics in response to light spectra

RNA-Seq technology was used to analyse variations in gene expression related to changes in the R: FR ratio across three time-points (TP) based on the attainment of precise developmental milestones under E1 growing conditions (low R: FR ratio). This correlated to 14 growing days for TP1 (third-leaf stage), 42 growing days for TP2 (pre-flowering stage under FR-enriched light) and 47 growing days for TP3 (flowering stage under FR-enriched light). From a total of 31,272 protein-coding genes identified in the subterranean clover advanced assembly Tsub_Refv2.0 (Kaur *et al.* 2017), 644 genes were differentially expressed in response to different light spectra in at least one of the three time-points analysed (Table S2). Clear differences in the number of differentially expressed genes (DEGs) were observed in response to the light treatments (Fig. 2B; Fig. 3, A and B; Table S2). The highest number of DEGs was found at TP1 with a total of 448 genes, of which 418 were exclusively differentially expressed at this stage (93%; Table S3). Of the 448 DEGs at TP1, 241 were up-regulated and 207 were down-regulated in B-enriched light compared to FR-enriched light. At TP2, we found 156 DEGs (135 up- and 21 down-regulated) with 122 of them exclusively differentially expressed at this point (78%; Table S4). At TP3, we identified 80 DEGs (16 up- and 64 down-regulated), with 65 of them exclusively differentially expressed at this point (81%; Table S5).

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

To identify the major functional biological categories represented by DEGs in response to light spectra, we performed gene ontology (GO) enrichment analysis. Changes in gene expression were seen in the three different time-points in GO terms, suggesting biological processes are differentially affected by light quality. A total of 459 (71.3%) of all DEGs were assigned GO categories corresponding to several aspects of metabolic and cellular processes, response to stress, plant defence, ion localization and transport, and flowering-related processes among others (Table S6a–c).

When analysing DEGs in both environments at TP1, the most significantly enriched GO categories with respect to up-regulated genes (p-value < 0.05) were related to defence response, mainly to biotic stimuli such as bacteria or fungus (Table S6a). Some of those genes were related to flavonoid pathways (Tsub_g874 and Tsub_g13273) and LRR disease resistance related proteins (Tsub_g14165, Tsub_g23725 and Tsub_g6245; Table S3). On the other hand, the GO terms related to 'ion transport' and floral development were predominantly associated with down-regulated genes in TP1 (Table S6a). Related to the red light signalling category, a CCT motif protein (TSub_g7401) was down-regulated under B-enriched light. Most of the genes identified in the floral development categories were MAD-box proteins (TSub_g15483, TSub_g25875, TSub_g21607 and TSub_g3280; Table S3).

At TP2, biological processes related to nodulation (GO: 0009877), defence response (GO: 0006952), and vegetative to reproductive transition (GO: 00110228) were up-regulated in B-enriched light compared to FR-enriched light. In contrast, genes involved in the secondary compound biosynthesis of isoflavonoids and flavonoids (GO: 0009717 and GO: 0009813) and anthocyanin-containing compounds (GO: 0009718) were down-regulated (Tables S4 and S6b).

232 At TP3, genes associated with biological processes related to photoperiodism,
233 flowering (GO: 2000028 and GO: 0048573) and developmental vegetative growth (GO:
234 0080186) were up-regulated under B-enriched light, and therefore their expression was lower
235 under FR-enriched light (Table S6c). We found that TSub_g17978 was the same gene
236 involved in those biological processes (Table S5). On the other hand, genes associated with
237 the response to stress (GO: 0006950) were down-regulated. Additionally, we found other
238 categories related to stress response: high light intensity (GO: 0009644), response to
239 hydrogen peroxide (GO: 0042542), L-proline biosynthetic process (GO: 0055129), and
240 detection of visible light (GO: 0009584; Table S6c).

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

242 The expression of genes related to flowering promotion across species occurred at an
243 earlier stage of plant development under FR-enriched light than B-enriched light. We
244 identified 13 DEGs related to light signalling, meristem identity and flowering promotion, of
245 which ten were exclusively expressed at TP1, one at TP2 and two at TP3. One of the DEGs
246 was shared between TP1 and TP2 (Table 1). All of the flowering-related DEGs identified at
247 TP1 were down-regulated under B-enriched light, as their expression was higher under FR-
248 enriched light. Of those, we found a gene-coding protein related to light signalling, a CCT
249 motif protein (TSub_g7401), and an uncharacterised protein identified as a probable
250 serine/threonine-protein kinase (with no lysine kinase 5-*WKN5*; TSub_g11956). Additionally,
251 we observed five different MADS-box transcription factors involved in floral promotion
252 (TSub_g15483, TSub_g21607, TSub_g3280, TSub_g25875 and TSub_g20101; Tables I and
253 Table S2). The floral promoter TSub_g12538, which is a possible pea *FLOWERING LOCUS*
254 *T b2* (*FTb2*), was among the down-regulated genes. Gene-coding proteins potentially linked
255 with floral promotion were also identified: an uncharacterised protein (TSub_g22633), a
256 possible *GIBBERELLIN 20 OXIDASE 2* (*GAS20OX2*) involved in the promotion of floral

transition, a sugar transporter (TSub_g15042), possible *SUCROSE-PROTON SYMPORTER 2* (*SUC2*), and a homeobox related protein that may be related to meristem identity (TSub_g491; Table 1 and Table S2).

All of the DEGs related to flowering induction identified at TP2 and TP3 were up-regulated under B-enriched light. At TP2, one DEG was identified, a MADS-box protein (TSub_g20101). Subsequently, at TP3, we found two DEGs associated with flowering promotion factors (TSub_g30315 and TSub_g30317; Fig. 4 and Table 1).

DISCUSSION

For the first time, we have shown that growing plants under FR light accelerates the up-regulation of genes related to floral initiation pathways using the long-day plant subterranean clover as a model. We identified novel genes which link photoperiod and FR-light signalling in addition to the up-regulation of genes related to plant defence under B-enriched light.

RNA-Seq time-course analysis identified 13 DEGs related to light signalling, meristem identity and flowering promotion. There was clear evidence in the transcriptome of up-regulation of gene networks related to flowering at a very early stage of plant growth (TP1, third-leaf stage) when plants were grown under FR-enriched light. Under B-enriched light, the up-regulation of this network was not expressed until TP2 and TP3 when the expression of *AGL12* and floral promoting factors was identified. These results are in accordance with the acceleration of time to flowering observed under FR-enriched light (low R: FR ratio; 14 days faster) compared to B-enriched light (high R: FR ratio).

The genetic link for the integration of photoperiod, light perception and circadian clock pathways has been well characterised in arabidopsis and involves the B-box transcription factor *CO*. The floral promoter *CO* protein is up-regulated by long-day growing

281 conditions and its expression is stabilised by FR light through *PHYTOCHROME A* activity
282 (Kim *et al.* 2008; Pin & Nilsson 2012; Song *et al.* 2013). For temperate legumes (such as
283 subterranean clover), it has been suggested that the integration of responses to day length and
284 light quality may not be regulated by *CO*-like (*COL*) genes (Wong *et al.* 2014; Weller &
285 Ortega 2015). These findings support our results as we identified a CCT motif protein
286 (TSub_g7401) at an early growing stage (TP1) under FR-enriched light. The presence of a
287 conserved domain within the *CO* family of transcription factors in arabidopsis (Strayer *et al.*
288 2000; Datta *et al.* 2006) suggests that the same protein is present in subterranean clover from
289 the *COL* family. In addition, we identified a serine/threonine-protein kinase (TSub_g11956),
290 *WNK5* involved in regulating time to flowering in response to photoperiod in arabidopsis
291 (Wang *et al.* 2008). Our results, therefore, suggest that floral initiation under FR-enriched
292 light is mediated by *CO*-like and *WNK5*-like proteins.

293 The *CO* protein has a role in activating the expression of *FT* (Kim *et al.* 2008). *FT*-
294 like proteins from several species function similarly to *FT* with respect to induction of
295 flowering, transport in phloem, and interaction with *FLOWERING lOCUS D*-like proteins
296 (Hecht *et al.* 2011). In our experiment, at TP1 under FR-enriched light, we found a gene-
297 coding protein (TSub_g12538) homologous of a pea *FTb2*. This protein is expressed
298 specifically in pea and Medicago leaves under long days and meets the characteristics of the
299 classical 'florigen' (Weller & Ortega 2015). Based on our results, it is not clear if the
300 promoting effect of the *WNK5*-like protein on *FTb2* is direct or indirect through *COL* and
301 further work is suggested in this area.

302 Downstream in the photoperiod pathway, we identified four MADS-box transcription
303 factors exclusively expressed at TP1 under FR-enriched light (TSub_g15483, TSub_g21607,
304 TSub_g3280, and TSub_g20101). We also identified one MAD-box TF (AGL12) that was

up-regulated at TP1 under FR-enriched light and not identified until TP2 under B-enriched light (TSub_g25875). These TFs have been identified as key components of the networks that control the transition from vegetative to flowering and flower development in arabidopsis (Tapia-López *et al.* 2008). These flowering promoters are homologous to *AGAMOUS-LIKE* (*AGL*) MADS-box proteins: *AGL4, AGL6, AGL8, AGL12* and *AGL20*. *AGL8* promotes floral determination in response to FR-enriched light (Hempel *et al.* 1997). *AGL20* has been described as an integrator of the vernalisation, autonomous and photoperiod pathways controlling flowering in arabidopsis (Lee *et al.* 2000). It has also been associated with gibberellins in the induction of flowering (Moon *et al.* 2003). *AGL12* has been described as an important floral promoter through the up-regulation of A*GL20* (Tapia-López *et al.* 2008). The expression of *AGL4* and *AGL6* is associated with gene regulation during floral meristem and floral organ development, with both found mainly in flowers (Ma *et al.* 1991; Pelaz *et al.* 2000; Dreni & Zhang 2016). The fact that *AGL12* was found at TP1 under FR-enriched light and at TP2 under B-enriched light provides further evidence that FR-enriched spectra accelerate flowering induction.

In addition to the DEGs related to the photoperiod flowering pathway and downstream cascade, we identified a further three DEGs related to flowering at TP1 under FR-enriched light: TSub_g491, TSub_g22633 and TSub_g15042. The TSub_g491 gene-coding protein is a homolog of the *KNAT1* protein and involved in the development of both vegetative and reproductive meristems (Scofield *et al.* 2007; Aguilar-Martinez *et al.* 2015). The TSub_g22633 is homologous to *GAS20OX2,* which is involved in the promotion of floral transition in arabidopsis (Andrés *et al.* 2014). The TSub_g15042 is a sugar transporter, *SUC2* involved in the transport of *FT* through phloem companion cells in leaves to the meristem for the induction of floral organ formation (Corbesier *et al.* 2007). At the later growing time-points (TP2 and TP3), only three genes were exclusively differentially expressed under B-

enriched light. At TP2, we identified a gibberellin-related protein (TSub_15579) homologous to *AT5G14920*, which is thought to be related to flowering induction in arabidopsis over-expressing the *ZEITLUPE/LOV KELCH PROTEIN 1*, a blue light photoreceptor (Saitoh *et al.* 2015). At TP3, we found two genes (TSub_g30315 and TSub_g30317) described as homologs of flowering-promoting factor-like proteins in Medicago. The fact that genes related to reproductive processes were identified at TP1 under FR-enriched light and at TP2 and TP3 under B-enriched light is in accordance with the accelerated flowering observed under a low R: FR ratio.

Interestingly, we found contrasting responses to light quality related to reproduction and defence mechanisms. In our experiment, at TP1 and TP2, growing plants under B-enriched light (high R: FR ratio) enhanced the expression of genes involved in flavonoid and anthocyanins pathways. At TP3, we found down-regulation of genes related to L-proline biosynthetic process, which is involved in the response to abiotic stress (Devlin 2016). This is in agreement with studies indicating that plant health responses are modulated by B-enriched and FR-enriched light. For example, B-enriched light can enhance the production of secondary metabolites like flavonoids and anthocyanins, which are defensive compounds against fungus, bacteria and environmental stresses (Johkan *et al.* 2010). Similarly, plants grown under FR-enriched light (low R: FR ratio) can express a weak defence phenotype (Cerrudo *et al.* 2012; Ballaré 2014). Our findings provide further support that the stress response is active under FR-enriched light, which may be due to the preferential allocation of resources to reproduction over defence.

The results from this study will contribute to a better understanding of the molecular basis of flowering and the possible negative correlation between reproductive and defence mechanisms in response to different light quality in long-day plants. Using RNA-Seq and

354 tightly controlled light spectra, we have identified key genes which link photoperiod and FR-
355 light signalling coding for a CCT motif and an *FTb2*-like protein, and the active downstream
356 cascade. The earlier down-regulation of these genes observed under B-enriched light
357 compared to FR-enriched light may explain their role in the acceleration of floral onset.

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534

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

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

537 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

538

539

Figure 1. A. Light spectrum profiles (wavelength (nm) vs. photon flux density (PFD, µmol m^{-2} s^{-1}). B. Time to flowering of *Trifolium subterraneum* plants and spectral characteristics (PFD, µmol m^{-2} s^{-1}) of the environments used in this study. E1: far-red enriched light (AP67 LED lights, Valoya, Finland); E2: blue-enriched LED light (108D18-V12 tubes, S-Tech Lighting, Australia). The red to far-red ratio calculations followed the method of Runckle and Heins (2001): photon irradiance between 655 and 665 nm/ photon irradiance between 725 and 735 nm.

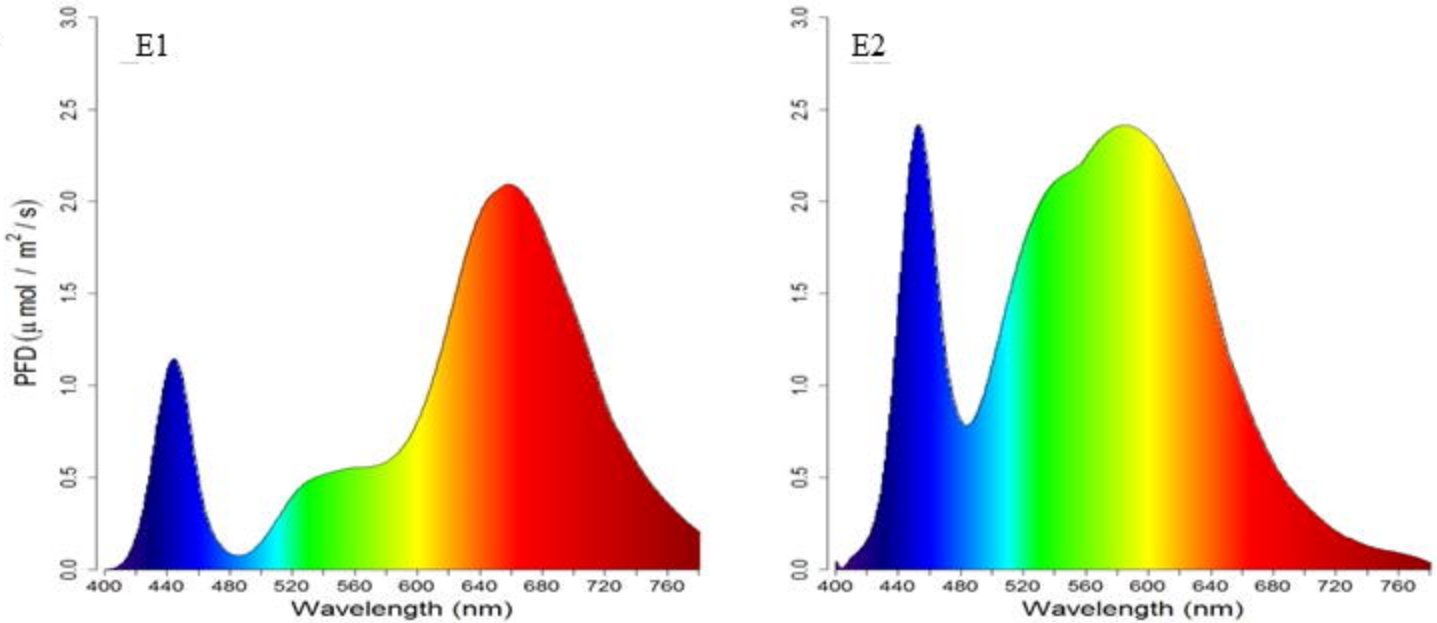
Figure 2. A. Time-points (TP) selected for this study based on when plants in the E1 environment attained the following developmental milestones: third-leaf stage (TP1), appearance of the first flower bud (TP2), open flower (TP3). B. Volcano plots for the differentially expressed genes. The red dots represent genes with logFC > 2 [FDR < 0.05] whereas the green dots represent genes logFC > 4 [FDR < 0.05].

Figure 3. A. The number of genes differentially regulated depending on the light provided. Red represents genes up-regulated and blue represents genes down-regulated under blue-enriched lights at the different time-points (TP) selected in this study when plants in the E1 environment attained the following developmental milestones: third-leaf stage (TP1), appearance of the first flower bud (TP2), open flower (TP3). B. Gene expression differences for genes with FDR < 0.05 and logFC > 2. The Venn diagram presents the number of genes that are exclusively differentially expressed at each developmental stage, with the overlapping regions showing the number of genes that are differentially expressed at two or more developmental stages.

Figure 4. Differentially expressed genes related to flowering induction by different light spectra. A Genes identified at time-point 1 (TP1) when plants reached third-leaf stage, these genes were down-regulated under blue-enriched light (high R: FR). B. Genes identified at

564 time-points 2 (TP2) and 3 (TP3), these genes were up-regulated under blue-enriched light
565 (high R: FR). *CRY:* cryptochrome, *PHYA:* phytochrome *A*, *PHYB:* phytochrome B, CCT:
566 CCT motif protein, *KNAT1:* homeobox protein knotted-1-like 1, *AGL8: AGAMOUS*-like 8,
567 *FTb2: FLOWERING LOCUS T b2*, *SUC2:* sucrose-proton symporter 2, *AGL4: AGAMOUS*-
568 like 4, *AGL6*: *AGAMOUS*-like 6, *AGL20: AGAMOUS*-like 20, *AGL12: AGAMOUS*-like 12,
569 *WNK5:* with no lysine (K) kinase, *GAS20OX2*: gibberellin 20 oxidase 2, FP1 and FP2:
570 flowering promoters.

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 ≤ 0.001

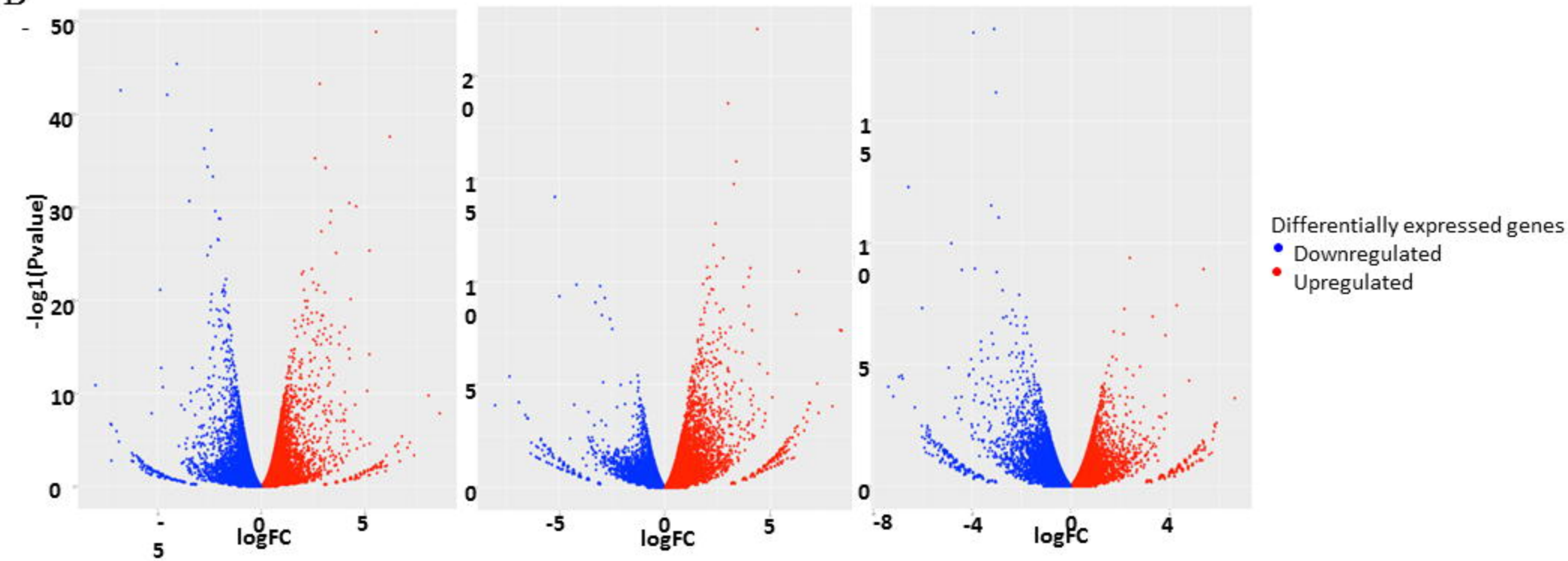
A

Time point 1. 3rd leaf-stage

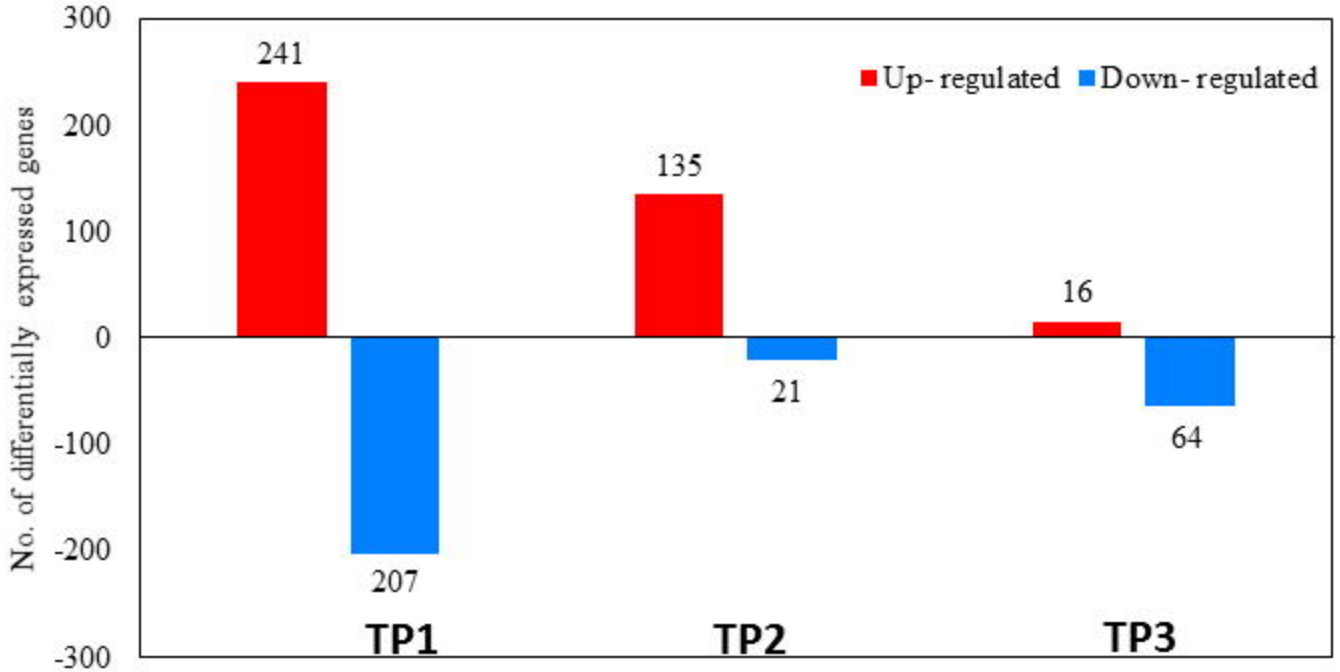
Time point 2. Pre-flowering (appearance of first flower bud)

Time point 3. Flowering (open flower)

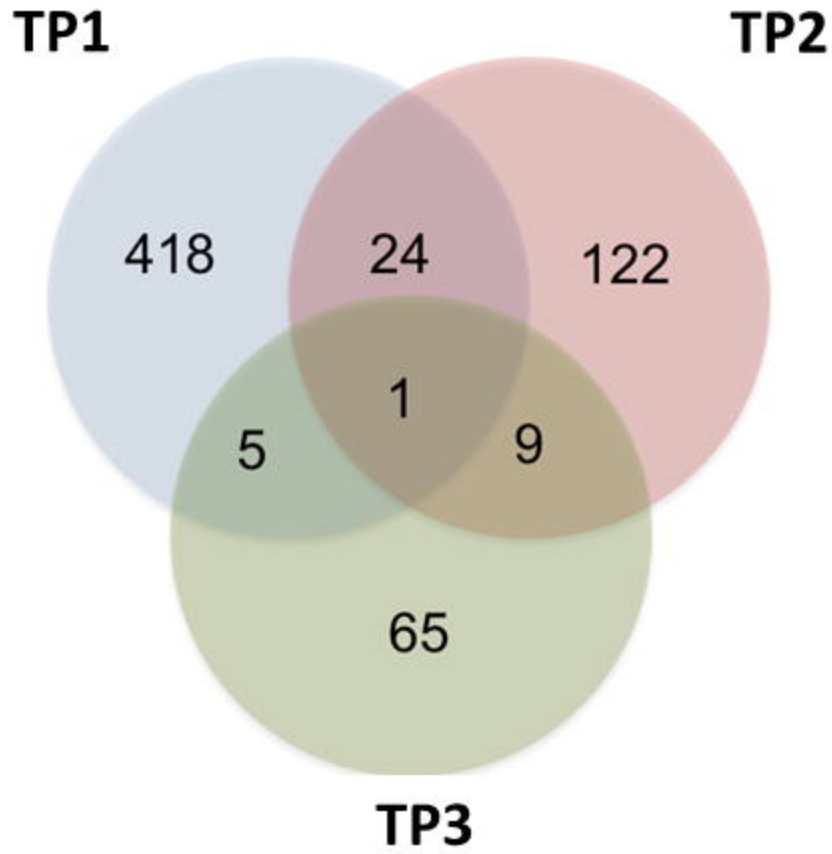
B

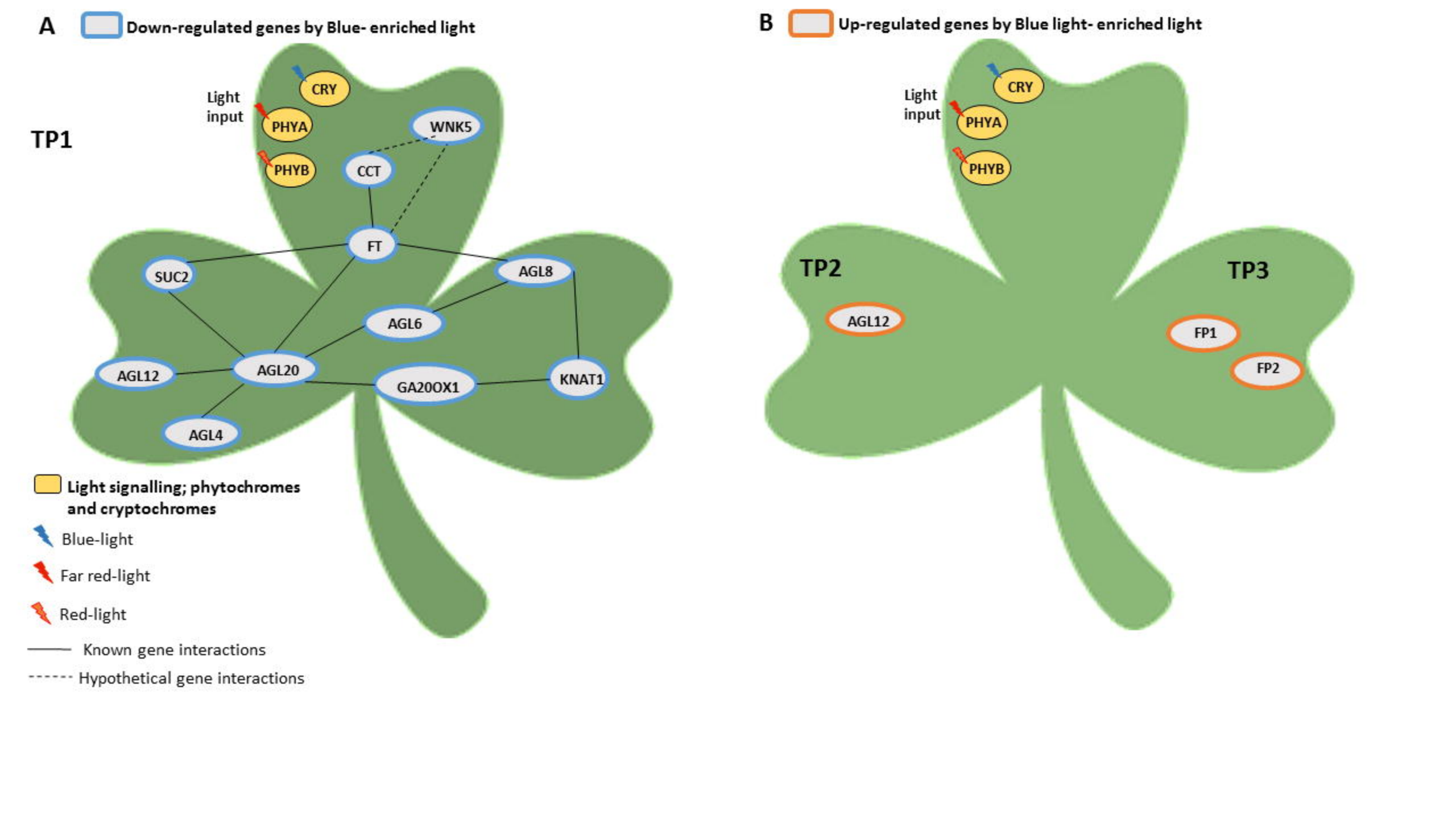


A


300
200
100
0
-100
-200
-300
No. of differentially expressed genes
Up- regulated
Down- regulated
241
207
135
21
16
64
TP1
TP2
TP3

B


TP1
TP2
418
24
122
1
5
9
65
TP3


A
Down-regulated genes by Blue- enriched light
Light input
CRY
PHYA
PHYB
WNK5
CCT
FT
SUC2
AGL8
AGL6
AGL12
AGL20
GA20OX1
KNAT1
AGL4
TP1
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