**Title page**

# Light spectra trigger divergent gene expression in barley cultivars

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Date of submission: 3-February-2021

Number of tables and figures: 1 table, 9 figures.

Word count: 5277

Running title: Light spectra affects barley gene expression

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Highlight

Development genes were affected by light quality in the barley varieties tested. Different grades of sensitivity were related to the expression of transcription factors, senescence, light signaling and cold-regulated genes.

Highlight

## 1   Abstract

2   Light spectra influence barley development, causing a diverse range of responses among

3   cultivars that are poorly understood. Here, we exposed three barley genotypes with

4   different light sensitivities to two light sources: fluorescent bulbs, over-representing green

5   and red wavebands, and metal halide lamps, with a more balanced spectrum. We used RNA

6   sequencing to identify the main genes and pathways involved in the different responses,

7   and RT-qPCR to validate the expression values. Different grades of sensitivity to light

8   spectra were associated with transcriptional reprogramming, plastid signals, and

9   photosynthesis. The genotypes were especially divergent in the expression of genes

10   regulated by transcription factors from MADS-box, WRKY, and NAC families, and in

11   specific photoreceptors such as phytochromes and cryptochromes. Variations in light

12   spectra also affected the expression of circadian clock, flowering time, and frost tolerance

13   genes, among others, resembling plant responses to temperature. The relation between

14   *PPD-H1*, *HvVRN1,* and *HvFT1* expression might explain genotypic differences. Light-

15   sensitive genotypes experienced a partial reversion of the vernalization process and

16   senescence-related stress under the less favorable light quality conditions. The observed

17   light-quality sensitivities reveal a complex mechanism of adaptation to regions with

18   specific light quality features and/or possible regulation of light spectra in plant

19   development during early spring.

## 20   Keywords

21   Barley, cold-regulated genes, development, light quality, RNA-seq, senescence, signaling,

22   transcription factors.

23

## Abbreviations

25    DE, differentially expressed; DEV, developmental stage; F, fluorescent; LD, long day; M,

26    metal halide; TF, transcription factor.

27

## Introduction

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29 As sessile organisms, plants have evolved adapting and surviving in a wide variety of

30 environments. One of the main developmental triggers is light, whose features regulate

31 growth and determine the adaptation to changing environments with different light

32 duration, quantity, and quality (Franklin, 2009; Ugarte *et al.*, 2010).

33 Like day length, light quality and intensity are not constant in natural environments, as

34 revealed by the different spectra that occur in different moments of the day, seasons,

35 climates, and atmospheric conditions (Holmes and Smith, 1977; Smith, 1982). Although

36 the responses of plants to some light features have been thoroughly analyzed (Franklin,

37 2009; Ugarte *et al.*, 2010; Monostori *et al.*, 2018), there is a gap in the study of natural

38 genetic variation in crop plants and its possible effect on crop development and adaptation.

39 For instance, light quality effects have been widely studied in *Arabidopsis* (Adams *et al.*,

40 2009), but only to a lesser extent in cereals (Ugarte *et al.*, 2010).

41 Differential regulation of genes whose expression levels are affected by light quality signals

42 may reflect different abilities to compete in diverse species or crop varieties. The

43 concentration and efficiency of photoreceptors that control the dynamics of red/far-red

44 (R/FR) light signaling are highly variable in different species and crop varieties (Merotto Jr.

45 *et al.*, 2009). Higher plants possess two types of signal-transducing photoreceptors:

46 phytochromes (in cereals PhyA, PhyB, and PhyC) absorbing principally in the 600-800 nm

47 waveband, and cryptochromes (Cry1, Cry2), absorbing only in the 300-500 nm band

48 (Smith 1982; Casal 1993). Phytochrome proteins are characterized by a red/far-red

49 photochromicity, changing their spectral absorbance properties upon light absorption. Their

5

50    biologically inactive form activates after absorbing R light and reverts to inactive after

51    absorbing FR light (Rockwell *et al.*, 2006). As dimeric proteins, the role of homo and

52    heterodimers is still an open area of research but it has been proven that both *PhyB* and

53    *PhyC* genes are required for the induction of wheat flowering under long photoperiods

54    (Pearce *et al.*, 2016). Cryptochromes have been found to regulate photomorphogenesis and

55    the expression of genes involved in blue light signaling and stress response (Kleine *et al.*

56    2007). Both phytochrome dimers and cryptochromes interact with transcription factors (TF)

57    known as Phytochrome Interacting Factors (PIFs) (Leivar and Monte, 2014; Pedmale *et al.*,

58    2016), regulating clock and flowering time genes (Oakenfull & Davis 2017), and are at the

59    top of fundamental light-driven processes.

60    Together with light, crops responses to temperature are receiving increasing attention, and

61    more efforts are being dedicated to unraveling the catalog of cross-talk and nodes at which

62    both signals converge (Franklin *et al.*, 2014). Both are particularly important in winter

63    cereals, which need to satisfy cold needs (called *vernalization*) before the spring when long

64    days (LD) mark the signal to flower. Thus, perception of photoperiod and cold are critical

65    to enabling flowering timely. Two main genes control the vernalization response in winter

66    cereals. In barley, these genes are *HvVRN1,* an *AP1*-like MADS-box TF, and promoter of

67    flowering, and *HvVRN2,* also known as *ZCCT-Ha-c*, a zinc-finger and CCT domain-

68    containing repressor protein that belongs to the *CONSTANS*-like family (Trevaskis *et al.*,

69    2003; Yan *et al.*, 2004). Both interact with the floral pathway integrator *HvFT1* (Yan *et al.*,

70    2006). When the cold requirement has not been satisfied, long-days promote *HvVRN2*

71    expression, repressing *HvFT1*, and delaying flowering until plants complete vernalization

72    (Trevaskis *et al.*, 2006; Hemming *et al.*, 2008). In temperate cereals, other members of the

73    MADS-box TF family genes, such as the *Flowering Locus C* (FLC)-clade member *OS2*

74    (*ODDSOC2*) and the *Short Vegetative Phase* (*SVP*)-clade member, *VRT2*, cause a delay

75    until experiencing enough cold (Kane *et al.*, 2005; Greenup *et al.*, 2010; Xie *et al.*, 2019).

76    Cold induces *HvVRN1*, which then represses *HvVRN2*, and together with the influence of

77    LD, allows the expression of the flowering integrator *HvFT1*. During the vernalization of

78    winter genotypes, the expression of the *SVP*-clade MADS-box TFs (*HvVRT2*, *HvBM1,* or

79    *HvBM10*) is up-regulated during the vegetative phase (Trevaskis *et al.*, 2007). Particularly,

80    VRT2 participates in regulating the vernalization flowering pathway, interacting and

81    cooperating with VRN1 (Xie *et al.*, 2019), and after the transition to the reproductive phase,

82    its expression declines (Kane *et al.*, 2005). The connection between photoreceptor and

83    photoperiod pathways has been attributed to PhyC, which activates the long-day

84    photoperiod response gene, *PPD-1* in LD (Nishida *et al.*, 2013; Chen *et al.*, 2014; Pankin *et*

85    *al.*, 2014; Woods *et al.*, 2014). Both *PhyC* and *PhyB* promote flowering under LD,

86    although *PhyB* regulates more genes than *PhyC* (Kippes *et al.*, 2020), particularly those

87    involved in vegetative development, hormone biosynthesis, and signaling, shade avoidance

88    response, abiotic stress tolerance (Pearce *et al.*, 2016), and cold tolerance (Franklin and

89    Quail, 2010; Novák *et al.*, 2016).

90    Motivated by the lack of studies on natural genetic variation in crop plants and its possible

91    effect on crop development and adaptation, we explored the phenotypic variability for plant

92    growth, in response to different light quality environments, in barley (*Hordeum vulgare*,

93    L.). Plants were exposed to two light sources: fluorescent light, which presents high peaks

94    at the 550-650 nm regions, corresponding to green and red wavebands, and metal halide

95    bulbs, which yield a more balanced spectrum. Although all genotypes had been vernalized

96  before the start of the experiment and temperature, photoperiod and light intensity were

97  identical and inductive to promote flowering, plants grown under fluorescent light showed

98  delayed development compared to those under metal halide light (Monteagudo *et al*. 2020).

99  We thus observed an effect of light quality on the expression of flowering time genes,

100  opening new questions about the regulation of photoperiod and vernalization pathways in

101  different barley varieties.

102  Here, we exposed three of the previously assessed genotypes to the same two contrasting

103  light spectral conditions to further investigate their gene expression patterns: an insensitive

104  line (minor response to light quality), Esterel, and two sensitive lines (major growth

105  differences between light quality conditions), Price (sensitive-intermediate) and WA1614-

106  95 (sensitive-extreme). The aims of this study were: a) to identify genes that explain the

107  different sensitivities of barley lines to light spectral quality, and b) to gain further

108  knowledge on the influence of light quality on development and on the expression of

109  flowering time genes. Here we provide evidence of the molecular mechanisms affected by

110  light quality. We identified key flowering pathway regulators, and other important genes

111  involved in development whose expression was affected in all varieties. Genotypic

112  differences in the expression of light signaling and cold responses were also found, which

113  might be related to barley adaptability to a wide range of environments and to an additional

114  regulation mechanism of plant development during early spring.

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## Materials and Methods

### Plant material and phenotyping

117    We selected varieties Esterel, Price, and WA1614-95 (Supplementary Table S1), from a set

118    of 11 winter or facultative barley varieties described in a previous study (Monteagudo *et*

119    *al.*, 2020) that revealed different responses to light environments (see Figure 1).

120    The experiment was carried out at the phytotron facilities of the Agricultural Research

121    Institute of the Hungarian Academy of Sciences, Martonvásár (Hungary), using Conviron

122    PGR-15 growth chambers (Conviron Ltd., Canada). Two light treatments were established

123    in independent growth chambers. Two lamp types were used: Sylvania cool white

124    fluorescent (F) and Tungsram HGL-400 metal halide (M) light bulbs. The height of the

125    lamps in the F chamber was adjusted once per week to 1.4 m above the canopy, to match

126    the light intensity of the M chamber, in which the lights were set at a fixed height. The

127    conditions in both chambers were set to long photoperiod (16 h light/8h night), and $18 \pm$

128    $1°C$ constant temperature, and light intensity of 250 µmol $m^{-2}$ $s^{-1}$. Temperature was

129    continuously monitored through an air-sampling channel, located in the middle of the

130    cabinet at canopy level. This system of temperature control eliminated the possibility that

131    plants experienced different temperatures at both chambers.

132    For phenotypic measurements, four seeds per genotype and treatment were sown in

133    individual pots (12 x 18 cm, 1.5 kg). Additionally, 20 seeds per genotype and treatment,

134    sown in groups of 5 plants/pot, were used for destructive samplings to record apex

135    development stage, and for gene expression studies. All plants were fully vernalized ($5 \pm$

136    $2°C$ for 52 days under 8h light/16 h night, low-intensity metal-halide light bulbs) before

137    entering the light quality chambers, to synchronize the development of the three genotypes.

138    Plant development was monitored twice a week, checking for first node appearance (plant

139    developmental stage 31, or DEV31) and appearance of the awns just visible above the last

140    leaf sheath (DEV49). All these data were defined based on stages of the Zadoks's scale

141    (Zadoks *et al.*, 1974), following the description of Tottman *et al.* (1979). Apex dissection

142    was carried out 23 days after the end of the vernalization period in 3 plants per variety and

143    treatment. Phenotyping consisted of recording apex stage following the Waddington's scale

144    (Waddington *et al.*, 1983). Plants were grown to full maturity.

145    ## RNA extraction and transcriptome sequencing

146    Three genotypes were used for transcriptome analysis. Three biological replicates per

147    genotype and treatment were produced. Each biological replicate pooled the last expanded

148    leaves of the main tillers from two different plants. Leaves were sampled in the middle of

149    the light cycle, 20 days after the end of the vernalization period, and immediately frozen in

150    liquid $N_2$. Leaf samples for Real-time PCR quantification (qRT-PCR) validation were

151    obtained in an independent experiment, as reported by Monteagudo *et al.* (2020).

152    Total RNA was isolated with TRIzol (Thermo Fisher Scientific, Ltd.) followed by the

153    Qiagen RNeasy plant mini kit, following the manufacturer's instructions (Qiagen, Ltd.).

154    Then, the material was extracted in the QIAcube equipment (Qiagen, Ltd.) with an extra

155    step of DNase treatment programmed. RNA quality was assessed with a NanoDrop 2000

156    spectrophotometer (Thermo Fisher Scientific, Ltd.) at ATK (Hungary).

157    RNAseq was performed by Novogene (HK) Co. Ltd. (China) after quality controls by

158    agarose gel electrophoresis and Bioanalyzer 2100 (Agilent, USA; RIN $\geq$ 6.3). Library

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159  construction was developed from enriched RNA, using oligo(dT) beads. Then, mRNA was

160  randomly fragmented, followed by cDNA synthesis using random hexamers and reverse

161  transcriptase. After the first-strand synthesis, a custom second-strand synthesis buffer

162  (Illumina, USA) was added, with dNTPs, RNase H, and *Escherichia coli* polymerase F to

163  generate the second strand by nick-translation, and AMPure XP beads were used to purify

164  the cDNA. The final cDNA library was ready after a round of purification, terminal repair,

165  A-tailing, ligation of sequencing adapters, size selection, and PCR enrichment. Library

166  concentration was first quantified using a Qubit 2.0 fluorometer (Life Technologies) and

167  then diluted to 1 ng/μl before checking insert size on an Agilent 2100 and quantifying to

168  greater accuracy by quantitative PCR (library activity > 2 nM). Eighteen barcoded libraries

169  were multiplexed and sequenced, 2x150 bp paired-end reads, in an Illumina HiSeq$^{TM}$ 2500

170  sequencer, yielding on average 50 Million reads per sample. The whole dataset consisted of

171  18 samples, i.e. 3 biological replicates, from 3 varieties and 2 light conditions.

172  Raw reads were processed with Illumina CASAVA v1.8 (Illumina, USA). Low-quality

173  reads (reads with more than 50% low-quality base ($Q \leq 20$)) were removed. Reads from the

174  three genotypes were jointly assembled *de novo*, with the software Trinity (Haas *et al.*,

175  2013) (Figure 2). The obtained assembled transcripts had a median length of 366 bp. Raw

176  transcripts of all three genotypes were combined, followed by a step of hierarchical

177  clustering. Then, the longest transcripts were kept and unigenes were called with Corset

178  v1.05 (-m 10 to remove redundancy, Davidson and Oshlack, 2014). The median unigene

179  length was 779 bp. Raw reads of the sequencing experiments (accessions ERR3763262-

180  ERR3763279) and assembled *de novo* transcripts (ERZ1264422) have been submitted to

181  the European Nucleotide Archive.

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## Quantification of gene expression and differential expression analysis

There are several barley assembly references available. We tested eight references and chose the most suitable for each purpose (Supplementary Information). We mapped clean reads against each reference and quantified transcript abundance as Transcripts Per Million (TPM) using Kallisto (Bray *et al.*, 2016). We used the R functions 'heatmap' and 'hclust' (R Core Team, 2020), to cluster the gene expression patterns from the experimental replicates, from the three genotypes, under the two light conditions. The resulting clusters were used to assess the expression quantification with several mapping references (see Supplementary Information). The gene sets which grouped more biological replicates together were selected for gene expression quantification. The barley reference genome from cultivar Morex (named **"Morex CDS sequences"**) (Mascher *et al.*, 2017) was used for all downstream analyses except GO enrichment, as these sequences lacked relevant GO terms related to light responses. For this reason, assembled Zangqing320 PacBio reads, from here on noted as **"Tibetan transcripts"** (Dai *et al.*, 2018), and the assembly generated in this work (**"*de novo* assembly"**), were chosen for conducting the GO enrichment analysis (Figure 2).

For ensuring the quality of the biological replicates, we calculated Pearson correlation coefficients across the transcript abundance of biological samples and replicates derived from reads mapped to **"Morex CDS sequences"**, with the R package "corrplot" (Wei and Simko, 2017). Independently validated RNA-seq expression values were obtained through qRT-PCR (ABI 7500, Applied Biosystems), performed on biological replicates for the same varieties and conditions, grown in an independent experiment. Expression values of 10 genes (Supplementary Table S2) were calculated relative to *Actin*

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205    (*HORVU5Hr1G039850.3*), taking into account the efficiency of each pair of primers. Each

206    PCR reaction contained 5 µl of PowerUp SYBR Green Master Mix (Applied Biosystems),

207    0.5 µM of each primer, and 250 ng of cDNA in a volume of 10 µl. Reactions were run with

208    the following conditions: 2 min activation at 50°C, 10 min of pre-denaturation at 95ºC;

209    followed by 44 cycles of 15 s denaturation at 95ºC, 50 s annealing at 60ºC, and 45 s of

210    extension step at 72ºC, ending with a melting curve 60º–95ºC default ramp rate. The same

211    normalization (relative to *Actin*) was accomplished for the number of TPM values and

212    compared for the same genes and treatments in the RNA-seq. This procedure was carried

213    out with a total of 60 points (average of 3 biological replicates per treatment and variety, in

214    10 genes).

215    We used Sleuth (Pimentel *et al.*, 2017) for calculating differential expression (DE) of the

216    genes from the three barley varieties. Nine DE analyses (M vs F for each one of three

217    genotypes, and comparisons between pairs of genotypes for each light quality condition)

218    were achieved for each mapping reference. However, here we only report results on the DE

219    genes calculated from the three aforementioned references (Figure 2 and Supplementary

220    Information). We validated the Kallisto/Sleuth methodology by comparing it to the RSEM

221    (Li and Dewey, 2011) and DESEQ (Anders and Huber, 2010) pipelines, originally used by

222    Novogene (Supplementary Information).

223    DE isoforms were detected using False Discovery Rate (FDR) adjusted p-values (named

224    hereby "q-values"), setting the threshold at 0.05. As plants responded better to metal halide

225    light, such condition was considered as control. Thus, DE genes are expressed in terms of

226    being up- or down-regulated under fluorescent light.

227    We used the DE genes calculated with Morex CDS reference for describing the patterns of

228    expression of individual genes. To reduce the number of DE genes to a workable number

229    and increase confidence, we only focused on DE genes with q-value < 0.01 ("Key genes")

230    for producing Venn diagrams and the identification of individual genes affected by light

231    quality. All the key genes were referenced to the barley reference genomes Morex v1

232    (Mascher *et al.*, 2017), and Morex v2 (Monat *et al.*, 2019).

233    ## GO enrichment analysis

234    We used DE genes (q-value < 0.05) calculated from the "Tibetan transcripts" and "*de novo*

235    assembly" references in three within genotype comparisons for the GO enrichment tests.

236    We mapped the three sets of DE genes calculated from both references against the Morex

237    genome assembly WGS (Mayer *et al.*, 2012) in PlantRegMap (Jin *et al.*, 2017), which uses

238    reciprocal best BLAST hits to assign Morex gene *id*s to query sequences. Matched genes

239    received the GO terms from the Morex reference. Enrichment analysis was calculated by

240    Fisher's exact test, with the complete gene set of Morex as control. GO terms with a q-

241    value < 0.05 were considered enriched. GO term enrichment analysis was performed

242    independently for the three sets of DE genes derived from the two mapping references; we

243    considered GO terms enriched in both references for each of the three within genotype

244    comparisons as highly reliable.

245    ## Clusters of DE genes

246    From within genotype comparisons in the two light conditions, we selected DE genes

247    obtained using the Morex CDS sequences as mapping reference. TPMs values were

248    clustered by the k-means method using the 'eclust' function from the 'factoextra' R

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249    package (Kassambara and Mundt, 2020), which separates the points into a defined k

250    number of groups and returns the total within-cluster sum of squares. The optimal number

251    of clusters was calculated minimizing and stabilizing that term.

## Motif discovery

253    We manually selected some clusters based on their different expression patterns between

254    light conditions in sensitive and insensitive varieties. To retrieve promoter sequences of the

255    corresponding clustered DE genes, we extracted upstream sequences (−500, +200

256    nucleotides around annotated Transcription Start Sites) for each gene, from the server

257    http://plants.rsat.eu (Nguyen *et al.*, 2018) and performed the motif discovery protocol

258    described in Contreras-Moreira *et al.* (2016) and Ksouri *et al.* (2021). For each cluster

259    analyzed, 50 clusters of the same size made by random picking upstream barley sequences

260    were used as negative controls for assessing the significance of motifs found (parameters

261    MAXSIGGO=60 MAXSIG=10 MINCOR=0.7 MINNCOR=0.5). The resulting motifs were

262    compared to motifs annotated in the footprintDB database (Sebastian and Contreras-

263    Moreira, 2014). The complete motif discovery results are available at

264    http://rsat.eead.csic.es/plants/data/light_report.

## Results

## Diversity in the response to different light sources

267    The three varieties differ in growth habit. The *HvVrn2* gene is present in Esterel and Price

268    and absent in WA1614-95 (Supplementary Table S1), and all three genotypes have a winter

269    allele at *HvVrn1*. Therefore, Esterel and Price are winter varieties, whereas WA1614-95 is a

270    facultative variety. The vernalization treatment placed the three lines at a similar

271    developmental stage (between Z11 and Z12) at the beginning of the light treatments. Under

272    M conditions, development was accelerated, compared to F, as revealed by the more

273    developed apices in 23-day old plants (Figure 1A). All three varieties flowered earlier in M

274    than in F. However, Esterel showed the least differences between treatments in days to the

275    appearance of the first node (DEV31, Figure 1B) and days to awns appearance (DEV49,

276    Figure 1C, and D). WA1614-95 presented the largest differences, with Price in an

277    intermediate position (Monteagudo *et al.*, 2020).

278    RNA-seq performance

279    Sampling for RNA-seq took place three days before the examination of the apices shown in

280    Figure 1. At that time, all three varieties had started the reproductive phase, or at least were

281    very close to reaching that point. Sequencing cDNA of 18 samples produced a total amount

282    of 1.92 billion paired-end reads. The joint *de novo* assembly for the three genotypes

283    contained 375,488 isoforms, from which we obtained 181,337 unigenes. We benchmarked

284    different barley references for mapping reads to transcripts (Supplementary Information).

285    As Morex is the most widely used barley reference, performs well in our reference

286    benchmarking, and has been functionality annotated by the community over the years,

287    "Morex CDS sequences" were chosen to calculate gene expression values and calling DE

288    genes (Figure 2). However, because of the incomplete GO annotation of the Morex 2017

289    genome (for instance, several GO terms involved in light stress were missing), we also

290    calculated DE genes using two of the best references according to our benchmarks

291    (Supplementary Information): our assembly ("*de novo* assembly" DE genes) and the

292    "Tibetan transcripts" (Dai *et al.*, 2018). Note that DE genes calculated from these two

293    references were only used for GO enrichment analysis.

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294     We used correlation coefficients between genes, estimated counts across the three

295     biological replicates, as a sample quality control. Esterel_F2 showed lower correlation

296     coefficients with the other two biological replicates than the remaining samples

297     (Supplementary Figure S1B). Consequently, replicate Esterel_F2 was discarded from

298     downstream analyses. Additionally, it is remarkable that Price and WA1614-95 expression

299     patterns were highly correlated in M conditions, consistently for all replicates

300     (Supplementary Figure S1A), but showed lower correlation coefficients in F conditions

301     (Supplementary Figure S1B), indicating similar responses of these two genotypes to M

302     light, but variable responses when exposed to fluorescent light. Esterel (insensitive

303     genotype) showed lower expression level correlation coefficients with the other genotypes

304     in both conditions.

305     ## Expression analysis of key genes

306     To unravel differential responses of the three genotypes to light quality, we focused on DE

307     genes occurring within each genotype across light treatments. Price, WA1614-95, and

308     Esterel initially showed 2,869, 4,218, and 3,591 DE genes with q-value < 0.05, listed in

309     Supplementary Datasets S3, S4, and S5. To focus on genes most likely affected by light

310     conditions, we further filtered the list considering only genes with q-value < 0.01 for

311     subsequent analysis, and denoted them as "key genes". Key DE genes in Esterel were

312     predominantly down-regulated (in F compared to M), whereas Price showed more up-

313     regulated than down-regulated genes, and WA1614-95 showed a similar number of up- and

314     down-regulated DE genes (Figure 3).

315     The intersection of key genes for the three genotypes comprised 17 sequences (Table 1).

316     Among them, *HvBM3* (*Barley MADS-box 3*), *HvBM8*, *PPD-H1* (*PSEUDO RESPONSE*

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317     *REGULATOR 7*, *HvPRR37*, Turner *et al.*, 2005), *HvFT1* (*FLOWERING LOCUS T*-like,

318     Yan *et al.*, 2006) were down-regulated under fluorescent light conditions in the three

319     genotypes, whereas *HvVRT2* (*VEGETATIVE TO REPRODUCTIVE TRANSITION 2*) and

320     *RVE7*-like (*EARLY PHYTOCHROME RESPONSIVE 1/REVEILLE7*) were up-regulated in

321     fluorescent light in the three genotypes. *RVE7*-like and *HvVRT2* were expressed at higher

322     levels in WA1614-95, whereas *HvBM3*, *HvBM8*, and *HvFT1* showed higher expression in

323     the insensitive line, Esterel (Figure 4). Two genes were annotated as *HvFT1*, aligned to

324     chromosomes 3H and 7H, with the same expression levels. As *HvFT1* is located

325     exclusively on 7H (Yan *et al.*, 2006), we believe the hit on 3H probably comes from a

326     duplication/misassembly in the Morex genome assembly v1, which contains a very large

327     number of fragmented genes (Beier *et al.*, 2017; Prade *et al.*, 2018). After referencing key

328     genes to the reference Morex v2 (Monat *et al.*, 2019), we found that *HvBM8* was also

329     duplicated (*HORVU2Hr1G063800* and *HORVU2Hr1G063810*, Supplementary Dataset

330     S1), thus leaving 15 single copy key DE genes in the three lines (Figure 3). The 15 key

331     genes were grouped in 14 high confidence (HC) and 1 low confidence (LC) genes

332     according to their annotation. Only the LC gene (*Ethylene-responsive transcription factor*)

333     followed different expression patterns among genotypes (up-regulated in F in the sensitive

334     genotypes, Price and WA1614-95 and down-regulated in the insensitive genotype Esterel).

335     This gene lacks a functional annotation in Morex v1 (only 159 bp were found in common

336     between the 2017 reference and the RNA-seq sequences). Instead, in Morex v2 the gene

337     has a larger coverage (539 bp). The remaining DE genes showed the same expression

338     directions in all three genotypes.

339    When we looked into key DE genes shared by just two varieties (Supplementary Dataset

340    S1), Price and WA1614-95 (both considered sensitive lines to light quality) had the largest

341    number (125, 66 up-regulated and 59 down-regulated in F, all in the same direction for both

342    genotypes). In the set of DE genes shared by Esterel and Price, 20 genes showed

343    differential expression with the same sign (14 up and 6 down), and 3 genes were different

344    (up-regulated in Price and down-regulated in Esterel). Among the DE gene set shared by

345    Esterel and WA1614-95, there were 13 genes with similar, and 29 with opposite trends.

346    WA1614-95 had more up- (35) than down-regulated (7) genes, whereas Esterel had more

347    down- (32) than up-regulated (10).

348    In the intersection between Price and WA1614-95, several transcripts coding for jasmonate-

349    induced proteins were commonly up-regulated in condition F. When focusing on the DE

350    genes in the intersection between WA1614-95 and Esterel, two WRKY family TFs with q

351    value<0.01 were identified (Supplementary Dataset S1). With the initial q-value threshold

352    of 0.05, naturally more genes were found (Supplementary Dataset S2), mostly up-regulated

353    in F in the sensitive genotype WA1614-95 and down-regulated in the insensitive Esterel.

354    Also, MADS-box TFs appeared frequently in the list of DE genes (as mentioned

355    previously, *HvVRN1*, *HvVRT2*, *HvBM3*, *HvBM8*, *HvBM10*, *HvOS2*). Among genes

356    annotated as MADS-box TFs, *HORVU3Hr1G095090* displayed the most extreme

357    contrasting pattern of expression between sensitive (up-regulated) and insensitive

358    (unchanged) varieties. BLASTN searches against NCBI nt and Ensembl Plants (Howe *et*

359    *al.*, 2020) databases revealed high similarity of this gene with a member of the wheat *FLC*

360    subclade (*TaFLC-A4-2,* Schilling *et al.*, 2020), and with *HvOS2* (*HORVU3Hr1G095240*), a

361    neighbor gene on chromosome 3H. The latter has similar expression patterns (see section

19

362 *Relevant light-response and developmental genes*), which might indicate a tandem

363 duplication of *FLC*-like genes.

## GO enrichment analysis

365 We carried out a GO enrichment analysis to find functional commonalities among the

366 genes present in each of the three sets of within genotype DE genes through shared GO

367 terms. Within genotype DE genes calculated were independently subjected to GO

368 enrichment analysis with "*de novo* assembly" and "Tibetan transcripts" as references. For

369 robustness, the intersection of the resulting terms was declared as the final set of enriched

370 GOs for each within genotype comparison (Supplementary Dataset S6). The main

371 functional terms associated with DE genes are represented in Figure 5, with those involved

372 in responses relevant for this study summarized in Supplementary Figure S3. Overall, the

373 GO terms of DE genes suggest that major changes among varieties are related to translation

374 and diverse metabolic processes. Those terms were associated with genes up-regulated in

375 WA1614-95 and down-regulated in Price and Esterel (Figure 5A). The generic term

376 "translation" also belongs to many biological processes already represented among the

377 most significant GOs (organic substance metabolism process, cellular protein metabolic,

378 cellular macromolecule biosynthetic, amide biosynthesis process, etc.). Thus, translation

379 seems to be the main process underlying the extreme sensitivity of WA1614-95. Genes

380 annotated with this GO category (n=153), mostly up-regulated, were related to ribosomal

381 proteins in WA1614-95 (Supplementary Dataset S4), contrasting with the 37 and 121 DE

382 genes found in Price and Esterel, respectively, mostly down-regulated in fluorescent light

383 (Supplementary Datasets S3 and S5). Furthermore, the responses to light spectra seem

384 variety-specific. Down-regulated DE genes in F were specifically associated with

20

385  photosynthesis and responses to light stimulus and radiation in WA1614-95, to chloroplast

386  organization in Price, and to organic substances metabolism and vacuolar activity in Esterel

387  (Figures 5A and 5B).

388  We then relaxed the selection criteria of GO enrichment (p-value < 0.05), to have a wider

389  look at categories involving developmental and light-specific responses (Supplementary

390  Figure S3). Photosynthesis and responses to radiation, and abiotic stimulus, among others,

391  were up-regulated in F in the insensitive genotype and down-regulated in the sensitive

392  genotypes. Responses to starvation (down-regulated in F) and reproductive development

393  (up-regulated in F) were only enriched in Esterel, whereas the response to red or far-red

394  light appeared solely up-regulated in F in Price, and responses to UV-A and oxidation, and

395  photosynthesis GOs appeared exclusively in WA1614-95 (down-regulated in F).

396  ## Clusters of DE genes

397  To determine genes with possible common regulation, the Morex CDS DE genes (q-

398  value<0.05), were grouped based on their expression patterns (see Figure 2). We created

399  three sets of clusters, one for each of the three 'within genotype' DE gene sets. The optimal

400  number of clusters was 39 for Price, 30 for Esterel, and 37 for WA1614-95. The clusters

401  revealed different patterns of expression (Figure 6). Among them, some grouped genes with

402  marked downregulation in F exclusively in the most sensitive variety WA1614-95 (Figure

403  6A, 6B). Other clusters grouped genes up-regulated in F in the sensitive varieties while

404  showing stable expression in the insensitive (Figure 6C and 6D).

405  Cluster 16 from the Price DE genes showed a common up-regulation in F in the three

406  varieties (Figure 6E). The 5 clusters highlighted (Clusters 10, 33, 36, 37 in WA1614-95 and

21

407  Cluster 16 in Price) were subjected to a motif discovery analysis. Upstream sequences of

408  genes within clusters 16 and 33 showed enriched DNA conserved motifs similar to

409  *Arabidopsis* ANAC092 (Figure 6F), suggesting that a barley homolog of ANAC092 could

410  be coordinating the expression of the genes within these clusters.

411  ## Relevant light-response and developmental genes

412  A large number of DE genes were found (Supplementary Datasets S3, S4, and S5) beyond

413  those in Table 1. We narrowed down the list focusing on genes known to be involved in

414  light perception (phytochromes, cryptochromes), circadian clock, flowering initiation, and

415  development (Figure 7). Among these, we found that *HvPhyC* was up-regulated in F in the

416  three genotypes, whereas *HvPhyB* and *HvCry2* were only differentially expressed in Price

417  (up-regulated in M), whilst no differences were found for *HvPhyA* or *HvCry1a*

418  (Supplementary Figure S2). Two TFs that act downstream in the photoreception machinery

419  and the light-signal transduction, *HvPIF5,* and *HvHY5,* were expressed with the same

420  pattern as *HvPhyC*.

421  The three genotypes showed reduced transcription levels of *PPD-H1*, *HvFT1* (Figure 4),

422  and *HvVRN1* (Figure 7) in fluorescent conditions, consistent with the delayed plant

423  development. Esterel showed higher expression levels than the sensitive genotypes for

424  *HvFT1* and *HvVRN1*, in accordance with its accelerated development in both conditions.

425  Besides, the three genotypes showed increased transcript levels of the flowering repressors

426  *HvOS2* (Figure 7), *HvVRT2,* and an orthologue of *RVE7*-like in wheat under fluorescent

427  light (Figure 4). WA1614-95, which was the latest flowering genotype, showed the highest

428  transcript levels of these repressor genes. On the other hand, *HvFPF1*-like (*Flowering*

429  *Promoting Factor 1*) transcripts were up-regulated in Price and WA1614-95 under M.

22

430     We also identified some differentially expressed genes, mainly in Price and some of them

431     in WA1614-95, that encode components of the circadian clock: *HvCCA1*, *HvLUX,* and

432     *HvPRR73* up-regulated in F; *HvGI*, *HvTOC1,* and *HvPRR95*, down-regulated in F; and

433     clock output genes, as *HvCO1*, up-regulated in F.

434     Two members of the *C-REPEAT/DREB BINDING FACTOR* (CBF) family (*HvCBF14*,

435     *HvCBF4a*), one member of the *COLD-RESPONSIVE* (COR) family (*WCOR15A*) and one

436     from the *INDUCER of CBF EXPRESSION* (*ICE*), an ortholog of rice (*ICE-like* annotated as

437     *metacaspase I*), all relevant in the acquisition of freezing tolerance, were up-regulated in

438     fluorescent light, mainly in the sensitive genotypes (Figure 8).

439     The RNA-seq results were validated through qRT-PCR analysis using 10 genes responsive

440     and non-responsive to light quality conditions (Figure 9). Samples were extracted from the

441     same genotypes, conditions, and age in an independent experiment. We obtained a positive

442     Pearson correlation ($r = 0.70$).

443     **Discussion**

444     Light quality affects to development genes

445     Delayed development of fluorescent light-grown plants was paralleled by dramatic changes

446     in the expression of development-related genes, which were overrepresented among the

447     14+1 key DE genes. Among them, the flowering repressors *RVE7-like*, *VRT2*, and *ICE-like*

448     were up-regulated in fluorescent light, whereas *AP1* MADS-box photoperiod responsive

449     genes *HvBM3* and *HvBM8*, and *HvFT1* were down-regulated. The large reduction of

450     expression of *PPD-H1* could explain the downregulation on these last three genes because

451     it mediates the long-day induction of *HvFT1* (Turner *et al.*, 2005), and several studies have

452    reported the *PPDH1*-dependent up-regulation of *HvBM3* and, *HvBM8* during development

453    (Digel *et al.*, 2015, 2016; Ejaz and von Korff, 2017).

454    GO analysis of DE genes did not suggest the existence of general responses to light spectra.

455    Differences in overrepresented GO terms among the three varieties indicated the presence

456    of different light quality responses. For instance, translation and diverse metabolic

457    processes were associated with up-regulated genes in the sensitive-extreme variety and

458    down-regulated in the other two (Figure 5A). Ribosomal protein genes were

459    overrepresented in the sensitive-extreme variety, indicating a transcriptional

460    reprogramming or translational regulation only in this genotype, as found in other species

461    subjected to biotic and abiotic stresses (Solano-De la Cruz *et al.*, 2019). Furthermore, genes

462    down-regulated in F were associated with chloroplast and plastid organization and

463    photosynthesis responses in the sensitive varieties only, and not in Esterel (Figure 5;

464    Supplementary Figure S3). Therefore, the fluorescent light spectrum might alter the

465    photosynthetic electron transport chain differently in these barley varieties. In conclusion,

466    the different grades of sensitivity to light spectra were associated with transcriptional

467    reprogramming, plastid signals, and photosynthesis. Interestingly, plant development

468    reprogramming in response to high light intensity in *Arabidopsis thaliana* has been related

469    to epigenetic changes involving *FLC* activity (Feng *et al.*, 2016). Fittingly, *HvOS2*, the

470    barley *FLC* orthologous gene, was up-regulated in F in both sensitive varieties.

24

471    ## Sensitive varieties experienced partial reversion of vernalization and

472    ## displayed cold tolerance responses

473    Sensitive and insensitive varieties showed strikingly different patterns of DE genes.

474    However, there was a remarkable similarity in the sets of DE genes for sensitive varieties

475    WA1614-95 and Price.

476    TFs seemed overrepresented among the DE genes. Although some MADS-box TFs were

477    equally affected across varieties (*HvBM3*, *HvBM8*, *HvVRT2*), other MADS-box genes

478    showed contrasting patterns following the varieties' sensitivity. This was the case of *FLC*-

479    like *HvOS2* and its paralog *HORVU3Hr1G095090*. In wheat, the duplication within the

480    *FLC*-clade has been related to adaptation (Schilling *et al.*, 2020). *HvOS2* represses the

481    expression of *Flowering Promoting Factor1*-like genes (Greenup et al. 2010; Hemming et

482    al. 2012), which also appear differentially expressed in our study (Figure 7). *HvOS2*

483    expression responds to cold, mediated by *HvVRN1*, which was also differentially expressed

484    only in the sensitive varieties. This gene should have been fully induced after vernalization

485    in all three varieties (even more so in WA1614-95, which needs little vernalization), but it

486    was less induced in F light in Price and WA1614-95, not different from a light-mediated

487    de-vernalization.

488    The high expression of *HvOS2* and its nearby paralog, as well as other genes related to cold

489    acclimation (*HvCBF14* and *WCOR15a*) under F light in sensitive varieties, bodes well with

490    their reduced *HvVRN1* expression because all these genes are VRN1 targets in barley

491    (Deng *et al.*, 2015). Cold-acclimation responses elicited by light are not a new finding.

492    Novák *et al.* (2016) reported that barley plants grown under fluorescent light supplemented

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493    with far-red light presented high *HvCBF14* induction, increasing their freezing tolerance,

494    but these results were found in plants during the hardening process, not in fully vernalized

495    plants grown under inductive conditions, as was the case here. We also found two members

496    of the CBF-clade (*HvCBF14* and *HvCBF4a*, Skinner *et al.*, 2005) up-regulated in

497    fluorescent conditions. A relationship between regulation of the *CBF* regulon and light (low

498    R:FR ratio), mediated through phytochromes, and under higher temperatures than those that

499    confer cold acclimation, was reported by Franklin and Whitelam (2007). In a related

500    manner, freezing tolerance genes were affected in *phyB*-null mutants in wheat (Pearce *et*

501    *al.*, 2016), rice (He *et al.*, 2016), and *Arabidopsis* (Franklin and Whitelam, 2007), causing

502    downregulation of a member of the *INDUCER of CBF EXPRESSION* (*ICE*) gene family

503    (Badawi *et al.*, 2008). All these pieces of evidence strongly suggest a role of *PhyB* in light-

504    mediated activation of cold acclimation pathway, and our results support this hypothesis.

505    Adding to the cold-like effect of the fluorescent light, genes related with cold acclimation

506    as *VRT2* (Kane *et al.*, 2005), a homolog of *RVE-7,* and an *ICE*-like protease showed

507    consistent higher expression under fluorescent light. We hypothesize that upregulation of

508    repressors and cold-induced genes under fluorescent light in fully-vernalized plants and LD

509    indicate that these plants are not sensing the favorable conditions, and remain in the cold

510    acclimation phase, eliciting cold-related responses, particularly in the sensitive varieties.

511    ### Sensitive varieties experienced phenomena related to senescence

512    Among the highly significant DE genes, the only one showing opposite directions between

513    insensitive Esterel (down) and the sensitive varieties (up) was an *ethylene-responsive*

514    *transcription factor*. Genes encoding for jasmonate-induced proteins were up-regulated in

515    fluorescent light in the sensitive varieties. Jasmonate and ethylene are hormones involved

516    in plants' responses to a wide range of abiotic stresses, and the latter is also involved in

517    senescence. This might be connected with *hemoglobin 1*, a key gene up-regulated under

518    fluorescent light in the three varieties. Non-symbiotic hemoglobins are involved in abiotic

519    stress responses, with a purported role in protecting cells from dehydration by modulating

520    nitric oxide concentration (Rubio *et al.*, 2019; Becana *et al.*, 2020). Sensitive varieties were

521    suffering from abiotic stress under fluorescent light, and these pathways are good

522    candidates to explain the phenotypic reactions of sensitive and insensitive varieties.

523    The clusters of DE genes up-regulated in fluorescent light in sensitive varieties were

524    enriched in regulatory motifs similar to *ANAC092*, a regulator of senescence, belonging to

525    the *NAC* family (Balazadeh *et al.*, 2010). NAC TFs control multiple processes, although

526    they are mainly associated with senescence and response to abiotic stresses, integrating also

527    cold signals and flowering (Yoo *et al.*, 2007). The set of DE genes shared by WA1614-95

528    and Esterel showed mostly opposite trends of expression, in accordance with their

529    sensitivities. Among them, there were several encoding for WRKY TF. These TFs are

530    involved in the regulation of transcriptional reprogramming associated with plant abiotic

531    and biotic stress responses (Bakshi and Oelmüller, 2014) and, at least in grapevine, WRKY

532    TFs trigger cell wall modifications to block the entrance of UV light into the cell

533    (Lesniewska *et al.*, 2004).

534    Interestingly, a link between cold acclimation, senescence, and flowering delay was

535    described by Wingler (2011) for *Arabidopsis thaliana* and barley. Through a promoter

536    motif analysis, this author found that flowering and senescence regulation are closely

537    associated with light and stress signaling and that cold-responsive genes were induced in

538    plants with delayed senescence.

27

## Signaling pathways affected by light quality

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540    The variability of expression patterns found for genes related to response to light and

541    specific spectra regions (red, far-red, UV) indicates that the treatments affected the function

542    of light receptors and photomorphogenesis. We found higher expression levels of *HvPhyC*

543    under fluorescent light in all three genotypes, and an opposite trend for *HvPhyB* and

544    *HvCry2* (consistent with their antagonist role, reported by Más *et al.*, 2000). According to

545    our results, the fluorescent light caused a strong imbalance of the expression of these three

546    signaling genes, which could lead to a disruption of 1) the balance of active and inactive

547    forms of phytochromes, 2) patterns of occurrence of homo- and heterodimers, and 3)

548    phytochromes to cryptochromes ratios. These imbalances could be at the top of the cascade

549    of changes in gene expression found in downstream pathways.

550    Both PhyC and PhyB are required for the photoperiodic induction of flowering in wheat

551    (Pearce *et al.*, 2016; Kippes *et al.*, 2020). Null wheat mutants of either *PhyC* or *PhyB*

552    produced late-flowering plants, with altered vegetative development. Up-regulation of

553    *HvPhyC* in F light resembled the phenotype of lacking a functional gene in wheat.

554    PhyC signal may have cascaded down through PPD-H1, as it is known to activate PPD-1 in

555    LD (Nishida *et al.*, 2013; Chen *et al.*, 2014; Woods *et al.*, 2014). In our study, there were

556    opposite patterns of expression between the DE genes *HvPhyC* and *PPD-H1* in the three

557    genotypes, supporting their close relation. The altered phytochrome expressions may have

558    shifted their proportions from the optimum, reducing *PPD-H1* induction in fluorescent

559    light.

28

560   Also, several signaling and clock genes were DE in the sensitive lines. In *A. thaliana*,

561   Franklin and Whitelam (2007) observed that the phytochrome signaling was mediated by

562   the circadian clock. Thus, the complex relation between photoreceptors and the clock might

563   provide a reason for the differences in downstream development genes, and in the

564   phenotype. It is remarkable that *HvPhyB*, *HvCry2,* and most of the clock genes represented,

565   were only differentially expressed in cultivar Price.

### Why would an annual crop develop mechanisms of adaption to light quality?

567   We have revealed the presence of phenotypic variation of barley in response to light

568   quality. Could this variation be adaptive? Barley spread from its cradle at 35º-40ºN to

569   latitudes beyond 60ºN involving well-known adaptation mechanisms like insensitivity to

570   day length (Jones *et al.*, 2008). Adaptations to other possible light-related factors for crop

571   species have been largely overlooked. If factors other than day length underlie light

572   adaptation of crops, this area deserves urgent attention. Climate change is already causing

573   latitudinal shifts of variety distribution and plant breeders should know how to cope with

574   possible light-related genetic effects other than photoperiodic response (Hunt *et al.*, 2019).

575   Light quality effects have received more attention in perennials. In a recent report, Chiang

576   *et al.* (2019) found that light quality affects tree growth showing a wide natural variation.

577   This is caused by the latitudinal change in the duration of periods under low solar angles

578   and by variation of overcast conditions (that implies variable R:FR ratios). They suggested

579   that trees that originated at high latitudes are more sensitive to light quality, compared to

580   those from lower latitudes. The barley lines tested in our experiment are autumn sown, and

581   much of their growth period occurs in winter, the period of the lowest solar angle. It is

582   conceivable that annual crops also took advantage of mutations to optimize their growth in

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583    regions with light quality features different from the ones found at their center of origin. In

584    addition, the solar angle in early spring is still relatively low and shows a large latitudinal

585    variation. This may have resulted in the evolvement of additional regulation mechanisms of

586    plant development at the higher latitudes, preventing the precocious initiation of stem

587    elongation in fully vernalized plants.

588    To conclude, we have found considerable variability among barley genotypes regarding

589    light quality sensitivity, involving different molecular mechanisms, akin to other abiotic

590    stress responses. Further research is needed to pin down their molecular bases and, in

591    particular, the extent of their role under natural conditions, with possible repercussions on

592    crop breeding.

593    ## Supplementary data

594    **Supplementary Tables and Figures**

595    **Supplementary Table S1.** List of the barley genotypes examined and allelic variants for

596    the major genes of flowering time.

597    **Supplementary Table S2**. Primer sequences for qRT-PCR assay.

598    **Supplementary Figure S1**. Correlation of transcript abundances across biological

599    replicates.

600    **Supplementary Figure S2**. Selection of flowering related genes (photoreceptors, circadian

601    clock and development).

602    **Supplementary Figure S3**. Enriched GO terms relevant for plant responses to light quality

603    conditions.

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604  **Supplementary Information.**

605  **Supplementary Information S1.** Summary of the DE experimental design and results of

606  DE genes from within line comparison.

607  **Supplementary Information S2**. Number of differentially expressed genes (DE) in each

608  of the six between line comparisons.

609  **Supplementary Information S3**. Number of biological samples correctly clustered in the

610  hierarchical clustering generated for the nine comparisons.

611  **Supplementary Dataset.**

612  **Supplementary Dataset S1.** List of Key DE genes (q-value< 0.01) shared between

613  varieties.

614  **Supplementary Dataset S2.** List of DE genes (q-value < 0.05) shared between varieties.

615  **Supplementary Dataset S3.** List of DE genes in Price, comparison between M and F

616  conditions.

617  **Supplementary Dataset S4.** List of DE genes in WA1614-95, comparison between M and

618  F conditions.

619  **Supplementary Dataset S5.** List of DE genes in Esterel, comparison between M and F

620  conditions.

621  **Supplementary Dataset S6.** GO enrichment.

## Acknowledgments

## Author contributions

AC, BC-M, EI, IK: Conception and design.

BC-M, AR-R: Methodology

AR-R, AM: Formal analysis, Writing - Original draft.

AM: Visualization

TK, MM, IK: Acquisition of data

AC, EI: Writing – Review & Editing, Project administration, Funding acquisition

AC, EI, BC-M: Supervision

## Data availability

The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB35759 (https://www.ebi.ac.uk/ena/browser/view/PRJEB35759). Additionally, motif discovery analysis of selected clusters of differentially expressed genes is available at rsat.eead.csic.es/plants/data/light_report/.

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# Tables

**Table 1**. List of DE genes (q-value < 0.01) shared by three barley varieties studied. We show up-regulated (u) and down-regulated (d) genes in fluorescent light for each genotype.

| Target ID Morex v1.0[a] | Target ID Morex v2.0[b] | Description[b] | Gene | Cite | Price | WA1614-95 | Esterel |
|---|---|---|---|---|---|---|---|
| HORVU0Hr1G003020.3 | HORVU.MOREX.r2.2HG0105150.1 | MADS box transcription factor | HvBM3 | 1, 2 | d | d | d |
| HORVU2Hr1G063800.7 | HORVU.MOREX.r2.2HG0129220.1 | MADS box transcription factor | HvBM8 | 1, 2 | d | d | d |
| HORVU2Hr1G063810.1 | | | | | d | d | d |
| HORVU7Hr1G036130.1 | HORVU.MOREX.r2.7HG0551090.1 | MADS box transcription factor | HvVRT2 | 3, 4 | u | u | u |
| HORVU7Hr1G083670.3 | HORVU.MOREX.r2.7HG0591700.1 | Cytochrome P450 family protein | | | u | u | u |
| HORVU2Hr1G013400.32 | HORVU.MOREX.r2.2HG0088300.1 | Pseudo-response regulator | HvPRR37 (PPD-H1) | 5 | d | d | d |
| HORVU4Hr1G090860.12 | HORVU.MOREX.r2.4HG0348610.1 | Metacaspase-1 | cell death | | u | u | u |
| HORVU5Hr1G071940.2 | HORVU.MOREX.r2.5HG0405790.1 | Glycosyltransferase | | | u | u | u |
| HORVU2Hr1G024120.10 | HORVU.MOREX.r2.2HG0097130.1 | Terpene synthase | | | d | d | d |
| HORVU0Hr1G038850.2 | HORVU.MOREX.r2.6HG0516180.1 | Protein kinase | | | u | u | u |
| HORVU3Hr1G111550.2 | HORVU.MOREX.r2.3HG0270770.1 | Ethylene-responsive transcription factor | | | u | u | d |
| HORVU3Hr1G021880.1 | HORVU.MOREX.r2.3HG0198440.1 | Glycosyltransferase | Glycosyl-transferase | | d | d | d |
| HORVU3Hr1G087100.1 | HORVU.MOREX.r2.7HG0542540.1 | Flowering locus T | HvFT1 (VRN-H3) | 6, 7 | d | d | d |
| HORVU7Hr1G024610.1 | | | | | d | d | d |
| HORVU5Hr1G029260.1 | HORVU.MOREX.r2.5HG0372130.1 | Protein kinase family protein | | | d | d | d |
| HORVU2Hr1G104580.2 | HORVU.MOREX.r2.2HG0162680.1 | Homeodomain-like superfamily protein | RVE7-like | | u | u | u |
| HORVU1Hr1G076460.3 | HORVU.MOREX.r2.1HG0062670.1 | Hemoglobin | | 8 | u | u | u |

[a] Morex reference genome v1.0 (Mascher *et al.*, 2017)

[b] Morex reference genome v2.0 (Monat *et al.*, 2019)

1, Schmitz *et al.* (2000); 2, Ejaz and von Korff (2017); 3, Kane *et al.* (2005); 4, Szucs *et al.* (2006); 5, Turner *et al.* (2005); 6, Yan *et al.* (2006); 7, Casas *et al.* (2011); 8, Becana *et al.* (2020).

# Figure legends

**Figure 1.** Phenotypic differences between varieties.

A) Apex development in plants dissected 23 days after the end of the vernalization treatment. WD, Waddington stage. B) and C) Days to first node (DEV31) and awn appearance (DEV49), expressed in days from the end of the vernalization treatment, measured in 4 biological replicates. Vertical black lines represent the days of difference between fluorescent and metal halide light conditions. D) Plants photographed 58 days after the end of the vernalization treatment.

**Figure 2.** Pipeline of the RNA-seq analysis.

Because of the incomplete GO annotation of the Morex 2017 genome (several GO terms involved in light stress are missing), we also calculated DE genes using the two best references according to our benchmarking, for performing the GO enrichment analysis.

**Figure 3.** Differentially expressed (DE) genes in the three genotypes (q-value < 0.01).

A) Clustering of DE genes with Morex CDS sequences used as reference. Colour code: red, upregulated in fluorescent light; yellow, downregulated. Three biological replicates per variety and condition are represented, except for Esterel in fluorescent light, from which a replicate was discarded. F, fluorescent; M, metal halide. B) Venn diagram showing the intersection in the number of DE genes among genotypes. Blue and red arrows indicate tthe number of DE genes upregulated or down regulated in fluorescent conditions in each genotype. The intersection between the three varieties indicates the number of DE genes that are annotated as High Confidence (14) and Low Confidence (1) genes in the Morex genome v2 (Monat *et al.* 2019).

**Figure 4.** Expression levels of selected key genes belonging to flowering pathways, with similar expression patterns in the three genotypes.

Two asterisks of the same colour indicate that differences are significant at q<0.01. Genes are separated in two groups: upregulated (left) and downregulated (right) under fluorescent conditions. Genotypes are coded as green (Esterel), red (Price) and blue (WA1614-95), in fluorescent (F) or metal halide conditions (M).

**Figure 5.** Enriched GO terms (q-value < 0.05), selected with different criteria.

A) 10 most significant GO terms for each set of up- and down-regulated genes, ordered by increasing q-value in *de Novo* reference. B) Relevant GO terms for plant responses to light quality conditions. In both cases, only enriched GO terms corresponding to biological process (P) were selected. X-axis represents the q-value of the GO terms when taking the "Tibetan" reference, in Esterel, Price and WA1614-95 for each comparison: upF (upregulated in fluorescent conditions) downF, (downregulated in fluorescent conditions). Dot size is proportional to the number of transcript counts annotated to that GO term in the correspondent DE list.

**Figure 6.** Relevant clusters of DE genes sharing expression patterns under fluorescent (F) and metal halide (M) conditions.

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Each grey line corresponds to the expression value of one gene across the different lines and conditions. Red lines indicate the average expression value of the sequences. (A, B) Clusters showing expression differences across light treatments in the most sensitive variety in F (WA1614-95). (C, D) Clusters showing expression differences across light conditions in sensitive (WA1614-95 and Price) varieties. (E) Cluster of genes showing a concurrent pattern of expression across light conditions in the three varieties. The number of genes within each cluster is indicated above each plot. (F) Common motif domain overrepresented in genes belonging to clusters 33 and 16. The consensus sequence was discovered in an upstream region covering [-500, 200] bp. The significance of the motif is proportional to the height of the letters. The consensus sequences found are similar to that of *Arabidopsis thaliana* ANAC092 annotated in footprintDB.

**Figure 7.** Expression levels of flowering-related genes (photoreceptors, circadian clock and development) in the three genotypes.
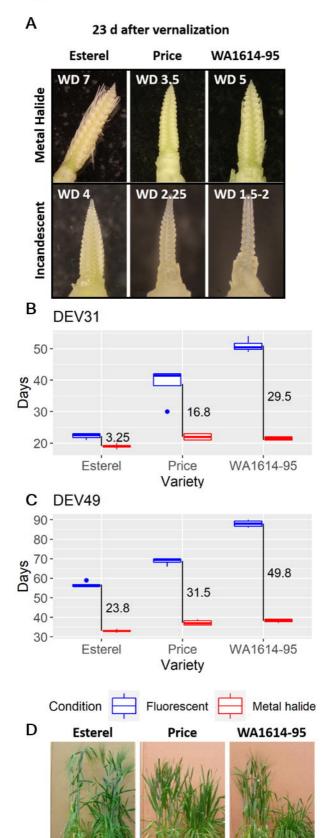
Genes are separated in two groups: upregulated (left) and downregulated (right) under fluorescent conditions. F, fluorescent conditions; M, metal halide conditions. Genotypes coded as in Figure 4. Differences between treatments are significant at q-value < 0.05 (*) or q-value<0.01 (**).

**Figure 8.** Expression levels of genes related to frost response in the three genotypes.

Genes are separated in two groups: upregulated (left) and downregulated (right) under fluorescent conditions. F, fluorescent conditions, M, metal halide conditions. Genotypes coded as in Figure 4. Differences between treatments are significant at q-value < 0.05 (*) or q-value <0.01 (**).

**Figure 9.** Correlation between RNA-seq and qRT-PCR expression values.

Values obtained after normalization (Log2 of gene expression relative to *Actin*). The dotted line corresponds to a linear regression. $R^2$, coefficient of determination.
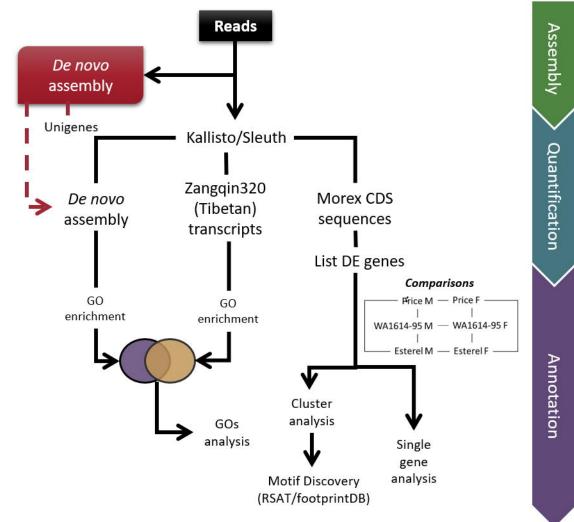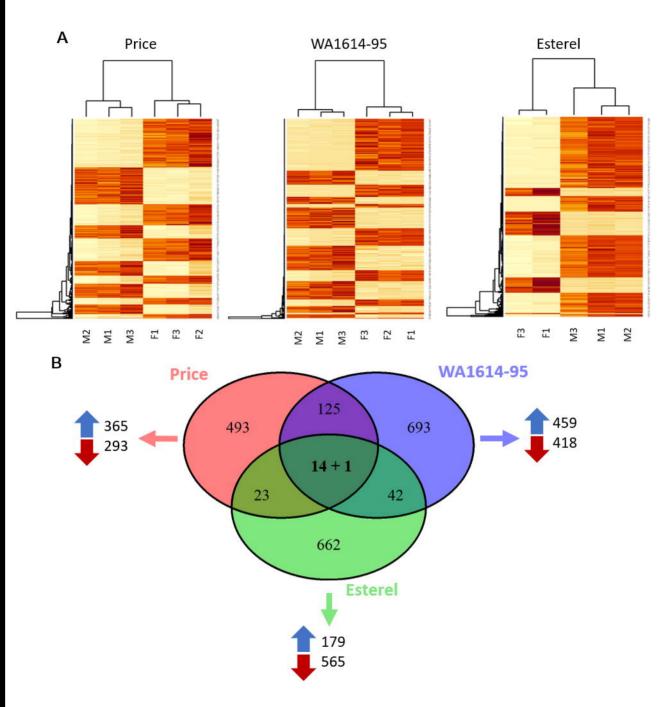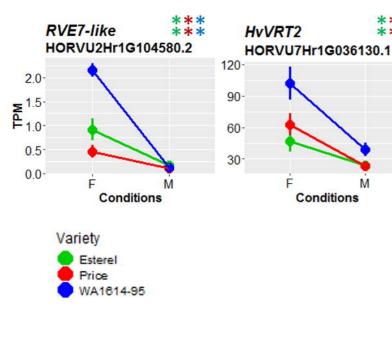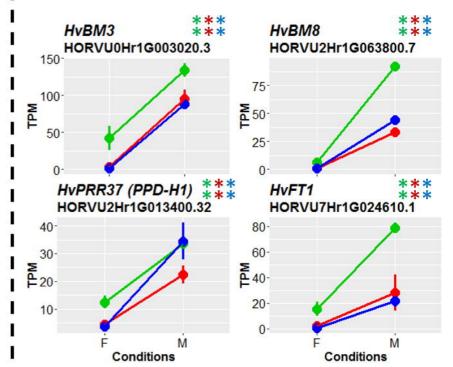
# Figure 1



A

**23 d after vernalization**

| | Esterel | Price | WA1614-95 |
|---|---|---|---|
| Metal Halide | WD 7 | WD 3.5 | WD 5 |
| Incandescent | WD 4 | WD 2.25 | WD 1.5-2 |

B **DEV31**

Days (y-axis): 20, 30, 40, 50

Esterel 3.25, Price 16.8, WA1614-95 29.5

Variety

C **DEV49**

Days (y-axis): 30, 40, 50, 60, 70, 80, 90

Esterel 23.8, Price 31.5, WA1614-95 49.8

Variety

Condition — Fluorescent — Metal halide

D

| Esterel | Price | WA1614-95 |
|---|---|---|
| M    I | M    I | M    I |

# Figure 2

# Figure 3

Figure 4

# Figure 5

# Figure 6



**A** WA1614-95 Cluster10 — N.transcripts = 172

**B** WA1614-95 Cluster36 — N.transcripts = 155

**C** WA1614-95 Cluster33 — N.transcripts = 234

**D** WA1614-95 Cluster37 — N.transcripts = 160

**E** Price Cluster16 — N.transcripts = 165

**F** ANAC092

Arabidopsis NAC domain containing protein

wt**ACACGCAAC**ck

...5-7nt_m5 oligos_5-7nt_m5oligos_5-7nt_m5

CaCAcGCAc

43 sites

# Figure 7

# Figure 8

# Figure 9



Validation

y = 0.528x + 2.2673
R² = 0.5032

log (RNASeq)

log (qRT-PCR)