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Running head: Photoperiodic control of defense

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Long-day photoperiod enhances jasmonic acid-related plant defense

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One-sentence summary: Long days perceived by photo-sensory receptors enhance jasmonic acid-dependent resistance in Arabidopsis.

ABSTRACT

Agricultural crops are exposed to a range of daylengths, which act as important environmental cues for the control of developmental processes such as flowering. To explore the additional effects of daylength on plant function, we investigated the transcriptome of Arabidopsis plants grown under short days (SD) and transferred to long days (LD). Compared to that under SD, the LD transcriptome was enriched in genes involved in jasmonic acid-dependent systemic resistance. Many of these genes exhibited impaired expression induction under LD in the *phytochrome A (phyA)*, *cryptochrome 1 (cry1)*, and *cry2* triple photoreceptor mutant. Compared to that under SD, LD enhanced plant resistance to the necrotrophic fungus *Botrytis cinerea*. This response was reduced in the *phyA cry1 cry2* triple mutant, in the *constitutive photomorphogenic 1 (cop1)* mutant, in the *myc2* mutant and in mutants impaired in DELLA function. Plants grown under SD had an increased nuclear abundance of COP1 and decreased DELLA abundance, the latter of which was dependent on COP1. We conclude that growth under LD enhances plant defense by reducing COP1 activity and enhancing DELLA abundance and *MYC2* expression.

INTRODUCTION

A given crop species can typically be exposed to a range of different photoperiods, the nature of which depend on sowing date, duration of the cycle, and latitude. Daylength profoundly affects the timing of key developmental transitions, including flowering in many species, tuberisation in potato, and bud set and growth cessation in trees (Jackson, 2009). The ability to respond specifically to current daylength helps to reduce the risk of plants being exposed to severe stressful conditions (Casal et al., 2004). Response to daylength can also enhance the tolerance to seasonal abiotic stress. Short days (SD) anticipate the cold temperatures of winter and increase freezing tolerance (Alonso-Blanco et al., 2005; Lee and Thomashow, 2012). Long days (LD) can induce antioxidative capacities in plants (Becker et al., 2006) and mimic plant acclimation to high light intensities (Lepistö and Rintamäki, 2012) that is typical of summer.

In Arabidopsis, growth under LD maintains the activity of phytochrome A (phyA), cryptochrome 1 (cry1), and (cry2) photoreceptors, which promote flowering (Andrés and Coupland, 2012). These photoreceptors stabilise CONSTANS (CO; Valverde et al., 2004) by reducing the activity of the CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)–SUPRESSOR OF PHYA-105 1 (SPA1)–SPA3–SPA4 complex (Liu et al., 2008). Growth under LD also enhances the expression of *CO* (Sawa et al., 2007) and the stability of CO protein (Song et al., 2012) via the action of the FLAVIN-BINDING, KELCH REPEAT, F-BOX (FKF1) photoreceptor (Lee et al., 2017). In turn, CO enhances the expression of *FLOWERING LOCUS T* (*FT*), which promotes flowering (Andrés and Coupland, 2012). The phyB photoreceptor, PHYTOCHROME INTERACTING FACTOR 4 (PIF4), and PIF7 play important roles in repressing the C-repeat binding factor (CBF) pathway and freezing tolerance under LD (Lee and Thomashow, 2012). These examples illustrate that different photoreceptors and downstream pathways mediate diverse outputs of photoperiodic signals.

The aim of this work was to explore the occurrence of additional responses to photoperiod mediated by phyA, cry1, and cry2 and to elucidate their key signalling components. To identify and prioritise these responses, we analysed the transcriptome of plants grown under either SD or LD and tested biological responses guided by overrepresented GO terms. Our results show that growth under LD compared to growth under SD enhances the expression of defense-related genes and plant resistance to the necrotrophic pathogen *Botrytis cinerea.* Growth under LD does not increase jasmonic acid (JA) levels; however, plants grown in LD had enhanced JA-induced defense by increasing the expression of *MYC2* and reducing COP1 nuclear activity, which in turn allowed for increased stability of DELLA proteins (Lorenzo et al., 2004; Wild et al., 2012; Chico et al., 2014).

RESULTS

Transcriptome responses to LD

Wild-type (WT) plants of Arabidopsis Landsberg *erecta* and of all the possible combinations among the photoreceptor mutants *phyA*, *cry1*, and *cry2* (Mazzella et al., 2001) were grown under SD (8 h white light) for 3 weeks. On day 21, some plants remained under white light beyond the time when the night started in previous days and were harvested when the photoperiod reached 16 h, i.e. at the end of the first LD (SD→LD). A control group remained under the SD regime and was harvested simultaneously with SD→LD-treated plants but under dim green light (protocol in Supplemental Fig. S1A). Transcriptome analysis revealed that 749 genes showed significant responses to changes in photoperiod, which were grouped in three major clusters (Fig. 1A, Supplemental Table S1).

Cluster 1 (163 genes) showed higher expression under SD→LD than that under SD. The response to LD was reduced in *cry2*, *cry1*, and *cry1 cry2,* but in these backgrounds, the *phyA* mutation restored (or overcompensated) the LD response. Exactly the same pattern had been observed for flowering in these mutants under the same light conditions (Mazzella et al., 2001). The enhanced response to LD in the *phyA* background might reflect the activity of phyB, which can be reduced by phyA (Krzymuski et al., 2014).

Cluster 2 (265 genes) showed higher expression under SD→LD than that under SD. The response to LD was significantly reduced in the *phyA cry1 cry2* triple mutant but not in the single mutants (for some genes the *phyA* and *cry2* mutants actually showed enhanced response) indicating redundancy among phyA, cry1, and cry2.

Cluster 3 (321 genes) showed reduced expression under SD→LD compared to that under SD, but in this cluster, all the mutants showed largely WT responses.

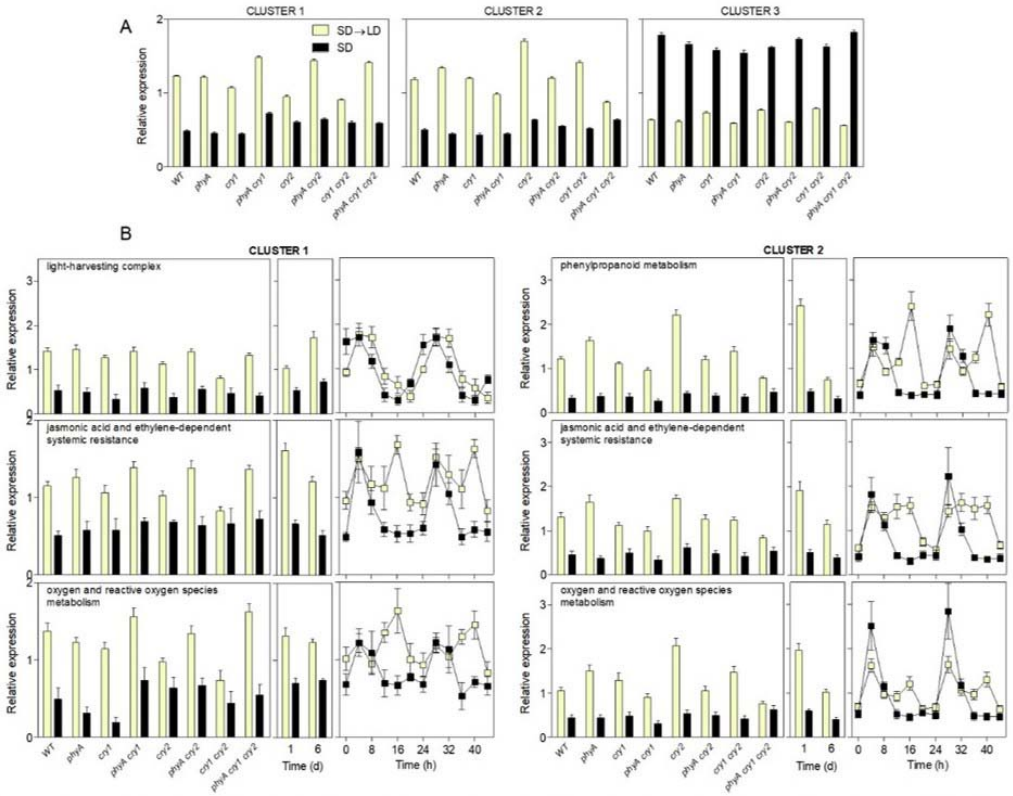

Figure 1. Robust responses of the transcriptome to initial LD exposure. A, Three major clusters grouped 749 genes showing statistically significant responses to photoperiod. Plants of Arabidopsis accession Landsberg *erecta* were grown under SD for three weeks, then transferred to LD and harvested after the end of the first LD photoperiod (Experiment 1, Supplemental Fig. S1A). The expression of each gene was normalised to the average for that gene across the genotypes and treatments and then the cluster average and SE was calculated for each genotype and condition. B, For each overrepresented GO term and cluster, average normalised expression and SE for each genotype and condition is shown in boxes corresponding to (left to right): Experiment 1, Experiment 2 (Supplemental Fig. S1A, independent experiment with accession Columbia following the same protocol as experiment 1 but followed up for 6 d), and publicly available data (Supplemental Fig. S1B, time course corresponding to plants of accession Landsberg *erecta* grown continuously under either SD or LD (Michael et al., 2008)).

To validate the list of genes identified in the WT as responsive to LD we conducted a fully independent experiment using the same light protocol in a different growth chamber and plants of the accession Columbia (protocol in Supplemental Fig. S1A). The strong correlation observed between the SD→LD/SD expression ratios of both experiments demonstrates the robustness of the gene expression responses to LD across two different growth conditions and accessions (Supplemental Fig. S2A, Supplemental Table S2).

To test whether these genes also respond in a coordinated manner under different scenarios, we analysed their expression across samples involving multiple

developmental stages and conditions (Obayashi et al., 2011). We observed that the expression of transcription factors present in clusters 1 and 2 tended to positively correlate with the expression of other genes present in these clusters, whereas there was a negative correlation with the expression of genes present in cluster 3 (Supplemental Fig. S3). Conversely, the expression of the transcription factor genes present in cluster 3 positively correlated with the expression of other genes present in cluster 3, and there was a negative correlation of these genes with those present in clusters 1 and 2 (Supplemental Fig. S3). This pattern indicates that changes in photoperiod affect the expression of a set of genes that are part of a robust network.

Daily gene expression responses to LD

To investigate whether the gene expression responses observed initially under LD are largely a transient reaction to the change or represent a daily difference between LD and SD, we compared the SD→LD/SD gene expression ratio of Columbia plants transferred from SD to LD for 1 or 6 d (Supplemental Fig. S1A, Supplemental Table S2). A highly significant correlation indicated that the genes that respond to the first day of exposure to LD tend to respond daily to LD compared to that under SD (Supplemental Fig. S2B).

To challenge the above conclusion, we compared the SD→LD/SD gene expression ratio of our Landsberg *erecta* plants exposed to a single LD with the LD/SD gene expression ratio calculated for a publicly available time-course data set that was generated using samples from Landsberg *erecta* plants grown for 7 d under either LD or SD and harvested 16 h after the beginning of the photoperiod (Michael et al., 2008; Supplemental Fig. S1B). The highly significant correlation confirmed and extended the validity of the gene list, thus further supporting the idea that the genes that respond to the first day of exposure to LD tend to respond daily to LD compared to SD after prolonged exposures to the different photoperiods (Supplemental Fig. S2C).

196

197 **Specificity of the gene expression responses to LD**

198

199 Although statistically significant, the correlation observed between the response of the
200 749 gene set to the first LD and to the first light exposure of fully dark-grown seedlings
201 (Peschke and Kretsch, 2011) was modest (Supplemental Fig. S4A). The list of genes
202 whose expression was at least doubled in both cases was enriched in light-harvesting
203 complexes (10^{-7}) and phenylpropanoid metabolism (10^{-9}). The 749 gene set failed to
204 show correlation between their response to LD and to the transfer of low-light-grown
205 plants to high light (Rossel et al., 2002; Kleine et al., 2007; Supplemental Fig. S4B).
206 Therefore, the gene expression response to LD is specific, with restricted similarity to
207 the response to light during de-etiolation or during high-light stress.

208

209 **GO terms overrepresented among the genes responding to LD**

210

211 The GO terms enriched (Vandepoele et al., 2009) among the genes that increased
212 their expression in response to LD included light-harvesting complexes (mainly cluster
213 1), phenylpropanoid metabolism (mainly cluster 2), JA and ethylene-dependent
214 systemic resistance (clusters 1 and 2), and oxygen and reactive oxygen species
215 metabolism (clusters 1 and 2, Supplemental Table S3). The average expression
216 patterns of these genes in the Landsberg *erecta* and Columbia SD→LD transition
217 experiments and in the continuous SD or LD time-course experiment demonstrates that
218 their response to LD is robust (Fig. 1B). Furthermore, in all cases the enhanced
219 expression occurred during the portion of the day when the plants were exposed to
220 light (under LD) versus darkness (i.e. 8–16 h, Fig. 1B).

221

222 **Analysis of plant physiological outputs and resistance to *B. cinerea***
223 **under LD**

We investigated whether the observed changes in gene expression under LD correlated with rapid changes in physiology (Protocol in Supplemental Fig. S1A). No significant differences in leaf chlorophyll or anthocyanin levels were observed 3 d after the SD→LD transfer compared to that in the SD controls (Supplemental Fig. S5). In accordance with other reports (Bermúdez et al., 2010), only a weak increment in oxidative stress was observed after the SD→LD transfer, as indicated by the small differences in the levels of Malondialdehyde (MDA) and the lack of response of catalase activity (Supplemental Fig. S6), which are biological markers of oxidative stress.

Using reverse transcription quantitative PCR (RT-qPCR) based on independent samples, we confirmed the expression response to SD→LD compared to that under SD of 12 genes present in clusters 1 and 2 and corresponding to the GO term JA and ethylene-dependent systemic resistance (Supplemental Table S4). Guided by these results, we conducted experiments to test the effects of growth under LD on plant resistance to the necrotrophic fungus *B. cinerea*. LD significantly reduced the area infected by *B. cinerea* compared to that in SD-grown plants (Fig. 2). Compared to continuous darkness, a 12-h photoperiod and continuous light also reduce the lesion areas caused by *B. cinerea* (Canessa et al., 2013). The pathogen-resistance response to LD was not observed in the *phyA cry1 cry2* photoreceptor mutant, indicating that extended light acted more as a signal perceived by photoreceptors than as a source of energy via photosynthesis or through alterations of oxidative stress metabolism (Rossi et al., 2017). The *phyA cry1 cry2* mutant showed no difference in *B. cinerea* resistance compared to that in WT under SD (Fig. 2), which is consistent with previous reports showing no effects of either lowering blue light or using a *cry1* mutant on *B. cinerea* resistance under SD (Cerrudo et al., 2012). As a negative control, we used the *myc2* mutant that is known to have enhanced resistance to *B. cinerea* (Lorenzo et al., 2004). It must be noted that MYC2 has a dual role as a positive regulator of JA-dependent

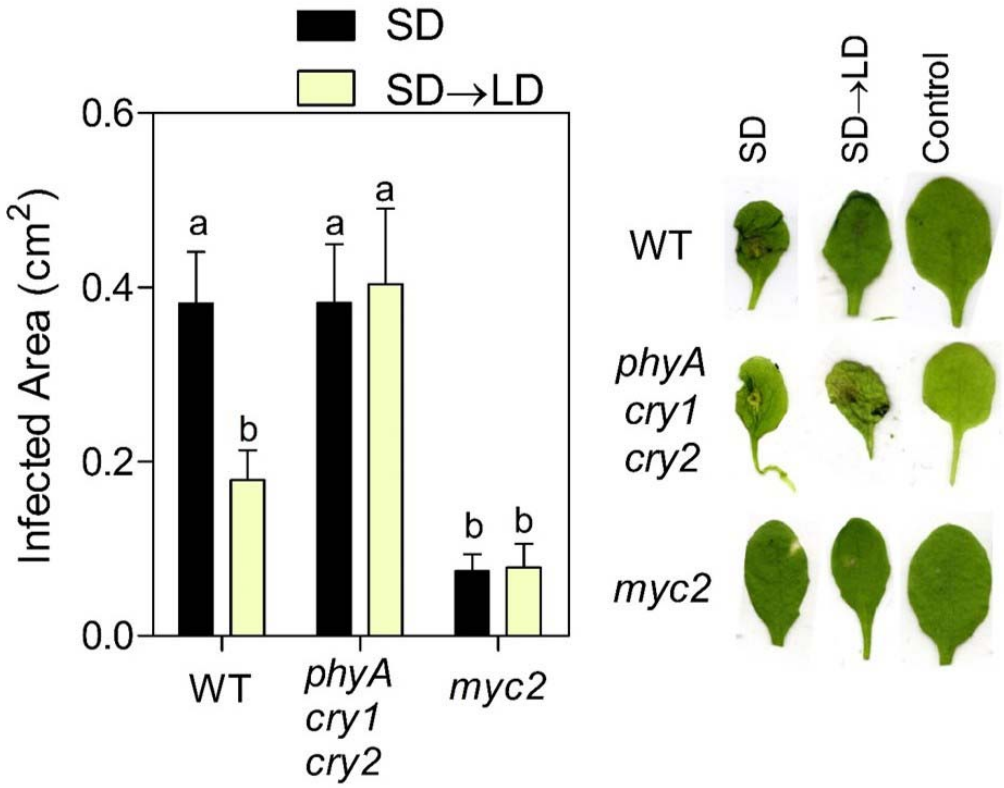

Figure 2. LD enhances resistance to *B. cinerea*. Plants of Arabidopsis accession Columbia were grown under SD for three weeks and inoculated at 7 h of day 21. One group was transferred to LD while the other remained under SD, and leaves were harvested 48 h after inoculation. Data are means and SE of at least 11 plants. Different letters indicate significant differences ($P < 0.05$) among means determined using Bonferroni post hoc test s. Leaves were photographed individually and a composite image was produced with representative cases.

252 responses and a negative regulator of ethylene signalling, which in turn regulates
253 resistance to necrotrophic fungi synergistically with JA (Song et al., 2014). Due to
254 functional redundancy with MYC3 and MYC4, the phenotype of the *myc2* single mutant
255 was dominated by the released repression of the ethylene pathway.
256
257 **JA signalling and absolute levels under LD**
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259 Since 12 out of 13 genes within the GO term JA and ethylene-dependent systemic
260 resistance also corresponded to response to JA stimulus (Supplemental Table S3), we

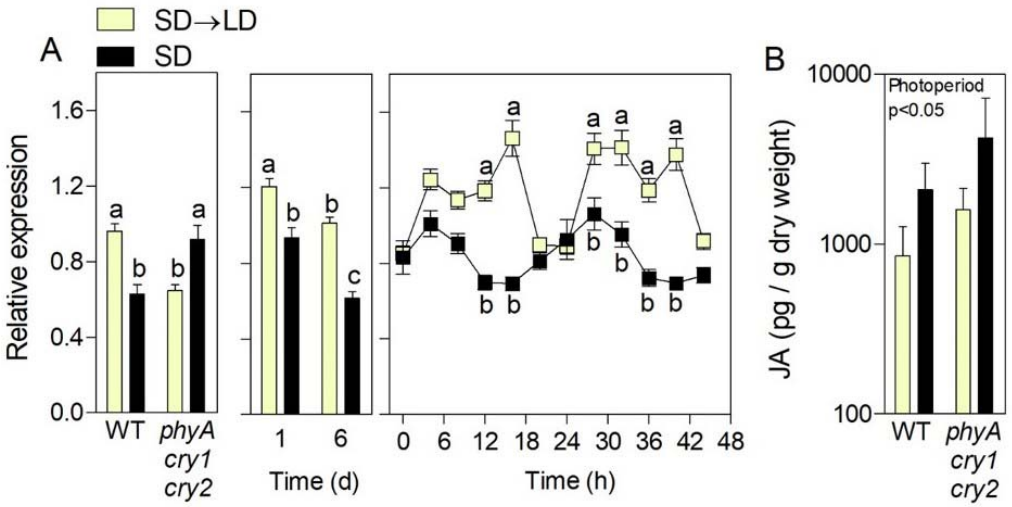

Figure 3. LD enhances JA signalling but not JA levels. A, Expression of a set of 100 genes whose expression is promoted by JA (Goda et al., 2008) was used as a proxy for JA signalling. Left: Experiment 1. Middle: Experiment 2. Right: published data (Michael et al., 2008). B, JA levels in plants exposed to SD→LD. Data are averages ± SE. Different letters indicate significant differences ($P < 0.05$) among means determined using Bonferroni post hoc tests and the significant effect of photoperiod in factorial ANOVA is shown in B.

therefore focused on JA signalling. We analysed the expression of a set of 100 genes that are known to respond positively to JA (Goda et al., 2008) as a proxy for JA signalling intensity. The index indicated that LD enhanced JA signalling (Fig. 3A). This response to LD could in principle be the result of enhanced levels of JA, however, measurements of hormone levels did not support this hypothesis (Fig. 3B). The transcription factor gene *MYC2*, which is involved in JA signalling (Lorenzo et al., 2004; Chico et al., 2014), showed enhanced expression (cluster 2), and the CACGTG motif, which is the main binding site of MYC2 (Yadav et al., 2005; Dombrecht et al., 2007; Fernández-Calvo et al., 2011), was overrepresented (O'Connor et al., 2005) mainly in cluster 2 ($P < 10^{-10}$) but also in the three clusters analysed as a single group ($P < 10^{-10}$).

Correlation between the effects of COP1 and MYC transcription factors on gene expression

Considering that the LD-specific regarding the genes related to plant defense requires cry1, cry2, and in some cases phyA (Fig. 1A), and that COP1 is a target of these photoreceptors (Lau and Deng, 2012), we investigated the expression of 12 genes present in clusters 1 and 2 that also corresponded to the GO term JA and ethylene-dependent systemic resistance in *cop1* mutant plants (Supplemental Table S4). Compared to that in WT, the impact of the *cop1* mutations on the expression of 11 of these genes under SD showed a significant inverse correlation with the impact of the *myc2 myc3 myc4* mutations (Fernández-Calvo et al., 2011; Fig. 4). The exception was *CORI3* that responded more significantly to the *cop1* mutations (Supplemental Table S4) than could be predicted by the *my2 myc3 myc4* mutant phenotype. These observations suggest that the effect of photoperiod may be mediated by COP1 regulation of MYC2, MYC3, and/or MYC4 activity. Since only the *MYC2* gene responded to photoperiod (Supplemental Table S1, cluster 2) and this response was unaffected by the *cop1* mutations (Supplemental Table S4), such COP1-mediated regulation of MYC2, MYC3, and/or MYC4 activity likely occurs at the post-transcriptional level.

Nuclear abundance of COP1 under LD and its effect on *B. cinerea* resistance

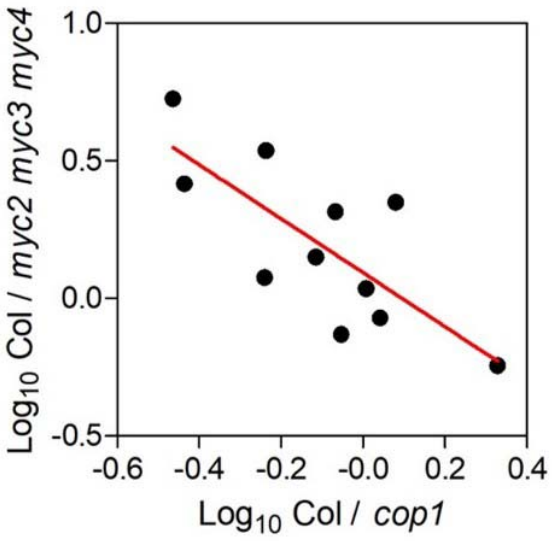

Figure 4. Negative correlation between the impact of the *myc2 myc3 myc4* (Fernández-Calvo et al., 2011) and *cop1* mutations (Data in Supplemental Table S4) compared to the WT. Regression: P <0.01.

Based on the above observations, we investigated whether LD repressed COP1 activity compared to that under SD. One of the regulatory features of COP1 activity is its nuclear abundance (Lau and Deng, 2012), which is rapidly reduced by dark to light transitions (Pacín et al., 2014). Prolonged light exposure under SD→LD reduced the nuclear abundance of COP1 compared to that under SD (Fig. 5A). The expression of *COP1* was unaffected by daylength (SD: 792 ± 116; SD→LD: 822 ± 144). Of note, the *cop1* mutant showed reduced damage by *B. cinerea* under SD and failed to respond to LD (Fig. 5B). Furthermore, the COP1 overexpressor showed increased damage by *B. cinerea* under LD and also failed to respond to LD compared to the response of its Nossen WT (Fig. 5B).

COP1-dependent DELLA accumulation under LD

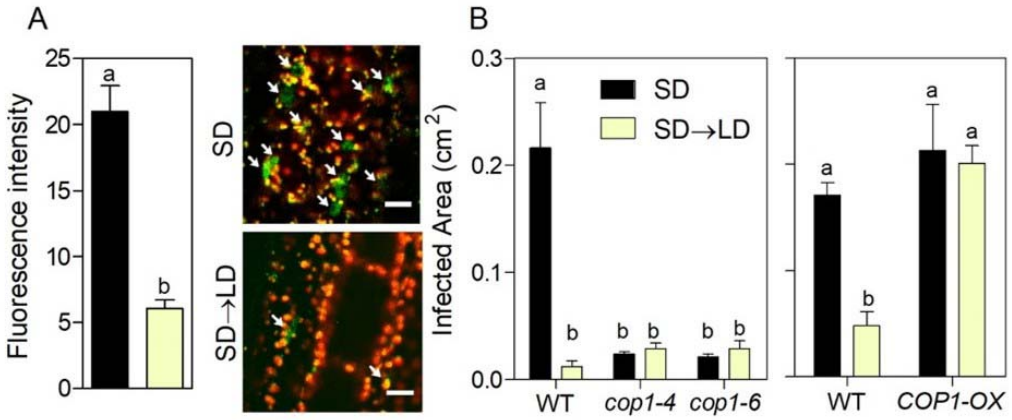

Figure 5. COP1 increases the lesions inflicted by *B. cinerea* under SD, whereas LD reduces COP1 nuclear abundance. A , Nuclear abundance of YFP-COP1 at the end of the first photoperiod under LD and in SD controls. Data are means ± SE of 8–9 plant replicates and representative images (arrows point to nuclei with detectable YFP-COP1, size bar= 20 µm). B, Resistance to *B. cinerea* in *cop1* mutants and the COP1 overexpressor (*COP1-OX*) under SD and LD represented by relative lesion size. Data are means ± SE of 13 plant replicates. Different letters indicate significant differences (P <0.05) determined using Student's *t*-test (A) or Bonferroni post hoc tests (B).

The activity of MYC transcription factors is enhanced by DELLA proteins, which bind JA ZIM-DOMAIN (JAZ) proteins that are negative regulators of MYC2 (Wild et al., 2012). We therefore investigated whether COP1 affects the abundance of the DELLA protein REPRESSOR OF *ga1-3* (RGA). Confocal microscopy revealed that fluorescence resulting from the *pRGA:GFP-RGA* transgene increased under SD→LD compared to that under SD in the WT background in a COP1-dependent manner (Fig. 6). Moreover, the expression of *RGA* was unaffected by daylength (SD: 1231 ± 243; SD→LD: 965 ± 128).

Function of DELLA proteins in the response to photoperiod

Considering that LD reduces the susceptibility to *B. cinerea* (Fig. 2) and increases the abundance of RGA (Fig. 6), and that DELLAs are positive regulators of defense against *B. cinerea* (Wild et al., 2012), we investigated whether the effects of photoperiod on

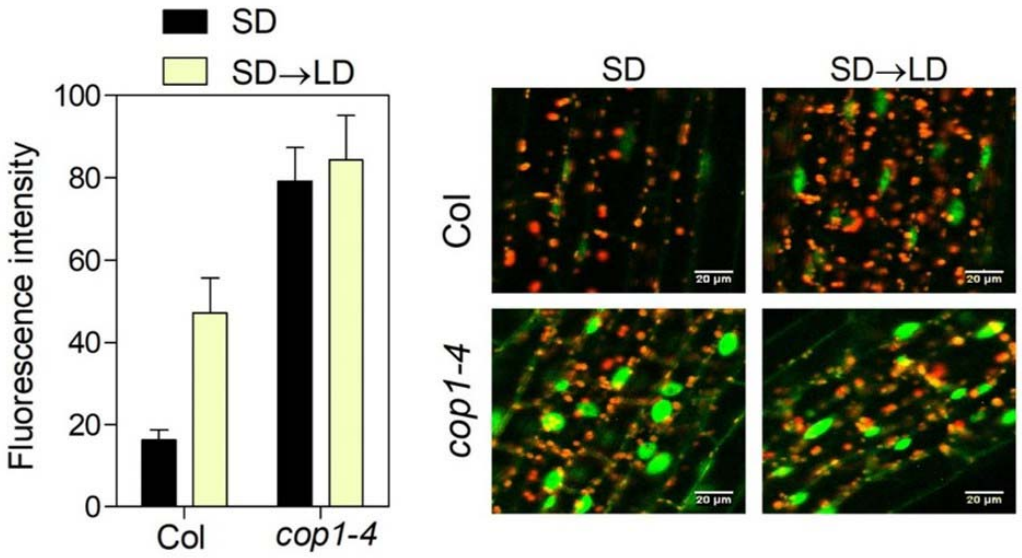

Figure 6. LD increases RGA abundance in a COP1-dependent manner. Data are means ± SE of 6 plant replicates and representative images are shown (size bar= 20 µm). Different letters indicate significant differences ($P < 0.05$) among means determined using Bonferroni post hoc tests.

fungal resistance depended on DELLAs. We considered the infected area as a proportion of the total leaf area to compare genotypes of different leaf size. Compared to that in Columbia WT, the *gai rga* double mutant (lacking two DELLAs) of the same background showed increased infection under SD→LD and no response to photoperiod (Fig. 7A). Similarly, compared to that in Landsberg *erecta* WT, the *rgl1, gai rgl2*, *gai rga rgl1*, and *gai rga rgl1 rgl2* (lacking one-four DELLAs) mutants of the same background showed increased infection under SD→LD, whereas a *gai* gain-of-function allele showed reduced infection. None of these genotypes responded to photoperiod (Fig. 7B). Of note, even a single loss-of function mutation resulted in almost the full leaf area affected by the lesion, leaving no room for additional effects in multiple mutants. In other experiments, the *cop1* phenotype under SD was partially rescued by the *gai rga* double mutation (Fig. 7C). This observation provided genetic evidence supporting that the effects of COP1 on the susceptibility to *B. cinerea* are at least partially mediated by

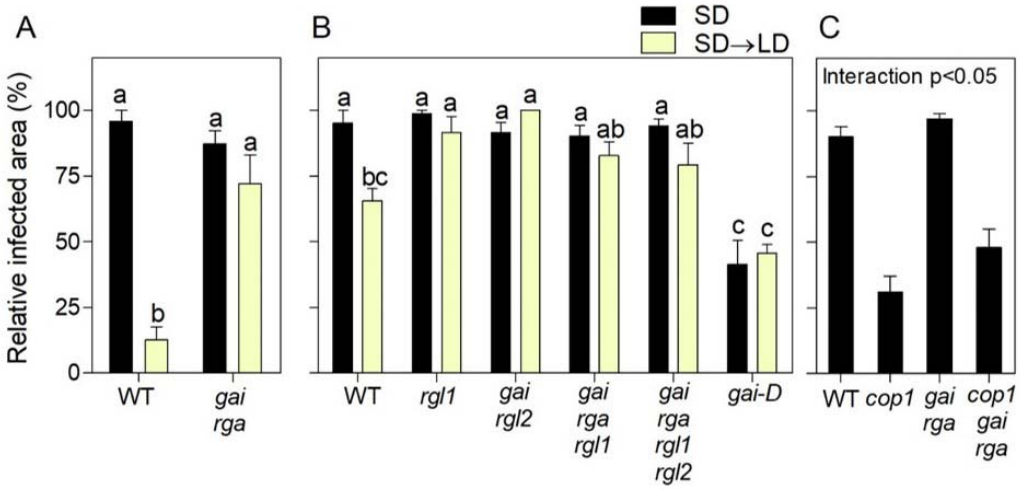

Figure 7. The effect of photoperiod on susceptibility to *B. cinerea* requires normal DELLA function. A and B, Resistance to *B. cinerea* infection represented by relative lesion size in mutants affected in DELLA genes in either Columbia (A) or Landsberg *erecta* (B) background. C, Resistance to *B. cinerea* infection under in WT and *cop1* mutant plants with or without compromised DELLA function conferred by *gai rga* double mutation. Data are means ± SE of 5 plant replicates. Different letters indicate significant differences (P <0.05) among means determined by Bonferroni post hoc tests. The significant interaction between *cop1* and *gai rga* determined by factorial ANOVA is shown in C.

its effects on DELLA proteins. The residual effect of COP1 may be mediated by remaining DELLA proteins or de-stabilisation of the MYC2 protein (Chico et al., 2014).

DISCUSSION

To investigate plant processes affected by photoperiod, we analysed transcriptome responses to SD→LD compared to that under SD, followed by the identification of overrepresented GO terms among responsive genes and a physiological screening. This procedure detected JA-dependent defense as one of the processes enhanced by LD compared to that under SD. We have identified a group of genes that increase their expression immediately in response to LD perceived by cry1 and cry2 (and in some cases also by phyA), and a group of genes that reduce their expression largely independently of these photoreceptors (Fig. 1A). This set of genes is robust (Supplemental Fig. S2, Supplemental Fig. S3, Supplemental Table S4), does not represent simply a transient response to the SD→LD shift (Supplemental Fig. S2, B and C), and does not normally respond to increased irradiance (Supplemental Fig. S4B). Highly overrepresented GO terms included light-harvesting complexes, phenylpropanoid metabolism, JA and ethylene-dependent systemic resistance (mainly response to JA stimulus), and oxygen and reactive oxygen species metabolism (Fig. 1B, Supplemental Table S3). The response of light-harvesting complex genes represents a shift of expression towards later hours of the daily cycle induced by SD→LD (Millar and Kay, 1996) without affecting the daily integral (Fig. 1B). No differences in chlorophyll or anthocyanin levels were observed after 3 LD (Supplemental Fig. S5), and the SD→LD transition caused at most modest oxidative stress (Bermúdez et al., 2010; Supplemental Fig. S6). However, compared to SD, LD significantly reduced the lesions caused by the necrotrophic pathogen *B. cinerea* (Fig. 2), which is consistent with the elevated JA-dependent defense predicted by transcriptome patterns.

Both the transcriptional response of several genes involved in JA-dependent defense (Fig. 1B) and the resistance to *B. cinerea* infection (Fig. 2) were impaired in the *phyA cry1 cry2 triple* mutant, indicating that the effects of growth under LD are not

simply the result of sustained photosynthesis or oxidative stress (Supplemental Fig. S6; Rossi et al., 2017) driven by the extended daylength. The levels of JA (Goodspeed et al., 2012) and the abundance of MYC2 (Shin et al., 2012) are controlled by the circadian clock. The susceptibility to *B. cinerea* and the associated transcriptional signature are also clock controlled, causing responses that depend on the time of the day at which the plants are inoculated (Ingle et al., 2015). However, the effects of photoperiod reported here do not result from a light-induced shift in the circadian rhythm of sensitivity (gating) because all the plants were inoculated simultaneously before exposure to the different light conditions and gene expression responses occurred during the first day of light extension. LD increased the intensity of JA signalling but not absolute JA levels (Fig. 3), indicating that LD increase the sensitivity to JA by acting downstream the hormone itself.

cry2 (Zuo et al., 2011) and cry1 (Lian et al., 2011; Liu et al., 2011), activated by blue light, and phyA, activated by far-red light (Sheerin et al., 2014), interact with SPA1 and other SPA proteins reorganising the COP1/SPA complex. Here we show that, compared to that under SD, a single photoperiod of LD was enough to significantly reduce the nuclear abundance of COP1 measured at the end of the extended photoperiod (Fig. 5A). Reduced COP1 nuclear abundance is predicted to reduce its activity towards nuclear targets (Pacín et al., 2014). We therefore investigated if COP1 was involved in the defense response associated with LD. The *cop1* mutant showed elevated defense against *B. cinerea* under SD and no response to LD (Fig. 5B). The impact of the *cop1* mutation on the expression of genes involved in JA-dependent defense showed a negative correlation with the reported impact of the *myc2 myc3 myc4* mutation (Fig. 4), indicating that COP1 might act via these transcription factors. Among *MYC2*, *MYC3*, and *MYC4*, only *MYC2* was included among the genes that responded to LD (Supplemental Table S1), but this response was largely unaffected by the *cop1* mutation (Supplemental Table S4). Therefore, COP1 appears to control the activity of MYC transcription factors downstream of their gene expression levels.

COP1 has been reported to de-stabilise MYC2 in etiolated seedlings compared to that in young light-grown seedlings; however, MYC2 does not appear to be a direct target of COP1 (Chico et al., 2014). Here, we explored a different possibility involving DELLA proteins that are known to increase JA-dependent defense by binding JA ZIM-DOMAIN (JAZ) proteins, which are negative regulators of MYC2 (Wild et al., 2012). Loss- and gain-of-function mutations in DELLA genes eliminated the response to photoperiod concerning the area of the lesions induced by *B. cinerea* (Fig. 7), and even low-order mutants displayed clear increased susceptibility to the pathogen (Wild et al., 2012). We therefore investigated whether daylength affected DELLA stability. The levels of RGA increased under SD→LD compared to that under SD in a COP1-dependent manner (Fig. 6). In conclusion, the mechanisms that controls JA-dependent defense in response to daylength involve LD perception by cry1, cry2, and phyA, followed by a reduction of COP1 nuclear abundance and a subsequent increase in DELLA abundance. Whether the link between COP1 and DELLA is direct is currently under investigation. In addition, there is a COP1-independent action of daylength on the expression of *MYC2*.

There is a tight association between the light environment and plant defense (Ballaré, 2014), and light perceived by phyA or phyB increases the responses to JA. The *phyA* mutant shows reduced JA-induced inhibition of root growth and promotion of gene expression (Robson et al., 2010). Plants exposed to low red/far-red ratios that reduce phyB activity show compromised resistance to *B. cinerea* and impaired induction of gene expression by either JA or *B. cinerea* (Cerrudo et al., 2012; de Wit et al., 2013). Conversely, UV-B radiation perceived by UV RESISTANCE LOCUS 8 increases the resistance to *B. cinerea*, but this effect is likely mediated by increased production of sinapate and not by changes in JA signalling (Demkura and Ballaré, 2012). The reduced responses to JA in plants with low or null phyA or phyB activity are mediated by enhanced stability of JAZs (Robson et al., 2010; Leone et al., 2014). Therefore, the trade-off between growth and defense can be uncoupled in a sextuple

422 mutant lacking both phyB and the five JAZs, which shows constitutively high JA
423 responses and no growth reductions (Campos et al., 2016). Low red/far-red ratios also
424 reduce the stability of MYC2 (Chico et al., 2014) and DELLA (Leone et al., 2014) and
425 PHYTOCHROME INTERACTING FACTOR4 has recently been described as a
426 negative regulator of defense (Gangappa et al., 2017). Therefore, although here we
427 have focused on the COP1-DELLA pathway, other aspects of the plant defense
428 network could also be affected by photoperiod.
429 A priori, there are several reasons why enhanced defense under LD might be
430 advantageous for the plant. These include the potentially higher availability of products
431 of photosynthesis to be invested in defense under LD, and the protection of
432 reproductive development initiated under LD. However, it is intriguing that *B. cinerea*
433 forms conidia in the light (air-borne macroconidia are a major source of infection;
434 Canessa et al., 2013) and the concentration of airborne inoculum is significantly higher
435 during day periods than at night (Blanco et al., 2006; Leyronas and Nicot, 2012).
436 Compared to that under SD, LD mainly extends the high expression of genes involved
437 in JA-dependent defense during the period of additional light exposure (i.e. LD does
438 not enhance expression compared to that under SD during the period where both are
439 exposed to light; Fig. 1B). Therefore, the plant response might be an adaptation to the
440 light response of the pathogen under LD.
441
442 **MATERIALS AND METHODS**
443
444 **Plant material and growth conditions**
445
446 Plants of Arabidopsis were grown at 20°C under SD (8 h light, 16 h darkness) for 3
447 weeks and then either transferred to LD (16 h light, 8 h darkness, same lighting) or left
448 as SD controls. White light (300 µmol m^{-2} s^{-1} between 400 and 700 nm) was provided
449 by 400 W Philips SON lamps, except in microarray Experiment 2 (160 µmol m^{-2} s^{-1}),

where 36 W Philips tubes were used to test the selected gene list under different conditions. The *phyA*, *cry1*, and *cry2* single and multiple mutants (Mazzella et al., 2001) and the *rgl1-1*, *gai-t6 rgl2-1*, *gai-t6 rga-t2 rgl1-1* or *gai-t6 rga-t2 rgl1-1 rgl2-1* (Lee et al., 2002; Achard et al., 2006) and the gain-of-function *gai-1* mutant (Koorneef et al., 1985) in Landsberg *erecta* and the *myc2-3* (Yadav et al., 2005), *phyA cry1 cry2* (Buchovsky et al., 2008), *cop1-4*, *cop1-6* (McNellis et al., 1994a), and *gai-td1 rga-29* (Plackett et al., 2014; Park et al., 2017) mutants in Columbia were compared to their respective WTs. For *COP1* overexpression, the *p35S:COP1* transgenic line in Nossen (McNellis et al., 1994b) was compared to its Nossen WT. The lines *p35S:YFP-COP1* in Columbia (Oravecz et al., 2006) and *pRGA:GFP-RGA* in Landsberg *erecta* (Silverstone, 2001) were used for confocal microscopy.

Microarray Experiments

Total RNA was extracted from SD→LD and SD plants in two different experiments (Supplemental Fig. S1) by using the RNEASY Plant mini kit (Qiagen). cDNA and cRNA synthesis and hybridization to 22 K (ATH1) Affymetrix Gene Chips were performed according to Affymetrix instructions. The scaling tab of the Affymetrix microarray suite in the mode ‘‘all probe sets’’ was used to standardize the trimmed mean signal of each array to the ‘‘target signal’’ according to the manufacturer’s instructions.

Analysis of microarray data

Two different experiments were conducted. In Experiment 1 (Supplemental Fig. S1), plants of the WT and *phyA*, *cry1*, *cry2*, *phyA cry1*, *phyA cry2*, *cry1 cry2*, and *phyA cry1 cry2* mutants were harvested at the end of the first LD (SD→LD) or simultaneously, 8 h after the end of the SD as controls. Expression data for each microarray was first normalised by dividing the expression of each gene by the ratio between the average

478 expression of all the genes in that microarray and the average of all microarray
479 averages. The factor used for normalisation ranged between and 0.84 and 1.14,
480 indicating that there were no large differences among microarrays. To investigate the
481 genes that respond to LD and the role played by phyA, cry1, and cry2 in their
482 response, we first used ANOVA and calculated P and *q* values (Storey and Tibshirani,
483 2003). Since the experiment was focused on the response to LD and not on the
484 differences among genotypes that could already be present in the SD controls, we
485 pooled the data corresponding to the different genotypes under SD. This procedure
486 offered an objective criterion to eliminate those genes where the differences were
487 mainly present already under SD because these genes showed high error estimates
488 compared to the response to daylength. Therefore, the ANOVA included 9 treatments:
489 8 corresponding to each genotype under SD→LD (two biological replicates for each
490 genotype) and one corresponding to the SD control (8 pooled data corresponding to
491 one microarray per genotype). We identified 1124 genes with P values <0.005 and *q*
492 values <0.1. We restricted the list to 984 genes by using a WT SD→LD/SD gene
493 expression ratio >1.2 or <0.8 as a cut off. By using DChip (Li and Wong, 2003), 805 of
494 the 984 genes were grouped into three major clusters. The clustering step is
495 conservative and reduces the chances that a gene becomes incorporated into the list if
496 it does not share the major patterns of response. For instance, the list does not include
497 *FT*, which is known to respond to LD because, although this gene showed significant
498 effects of treatment (P <0.003, q <0.08, normalised expression: WT, SD→LD= 1.1,
499 SD= 0.3, phyA cry1 cry2, SD→LD= 0.2, SD= 0.3), it was not included in clusters 1 or 2.
500 Each cluster was further restricted by testing for each gene the statistical
501 significance of the features of each cluster. For cluster 1, we used multiple regression
502 $y= a + b\ x_1 + c\ x_2$, where b represents the additive effects of *CRY1* and *CRY2* WT
503 alleles under SD→LD, x_1 is 2 for the WT SD→LD, 1 for the *cry1* or *cry2* backgrounds
504 under SD→LD and 0 for the *cry1 cry2* background under SD→LD and all genotypes

505 under SD, c represents the effect of the *phyA* mutant allele in the *cry1* and/or *cry2*
506 mutant background under SD→LD, and x_2 is 1 for the *phyA cry1* and *phyA cry2*
507 mutants under SD→LD, 2 for the *phyA cry1 cry2* mutant under SD→LD, and 0 for all
508 other conditions. For cluster 2 we used simple regression y= a + b x, where b
509 represents the redundant effect of *PHYA*, *CRY1* and *CRY2* WT alleles under SD→LD,
510 and x assumes 1 for the WT and the single and double mutants under SD→LD and 0
511 for the *phyA cry1 cry2* triple mutant under SD→LD and all the genotypes under SD. For
512 cluster 3 we used simple regression y= a + b x, where b represents the effect of
513 SD→LD compared to SD and x is 1 for all genotypes under SD→LD and o for all
514 genotypes under SD. Limitation of the clusters by this procedure ensured the
515 homogeneous composition of the clusters by statistical criteria. Therefore, 749 genes
516 were grouped among cluster 1 (163 genes), cluster 2 (265 genes), and cluster 3 (321
517 genes).
518 Overrepresented functions were investigated for each cluster and for the
519 combination of the two cluster that included genes with expression promoted in
520 SD→LD compared to SD by using ATCOECIS (Vandepoele et al., 2009).
521 In Experiment 2, SD→LD and SD control plants of the WT were harvested at the end of
522 the first LD and at the end of the 6th day. Two biological replicates were included in
523 each case. Expression data were normalised as described for Experiment 1 and used
524 here to test the robustness of the gene list and the persistence of the effects several
525 days after transition.
526
527 **Bioassays of *B. cinerea* resistance**
528
529 Plants were grown for 3 weeks under SD. Seven hours after the beginning of day 21, a
530 single droplet of 5 µL of *B. cinerea* spore suspension (2–3 × 10^5 spores mL^{-1}) was
531 placed on the adaxial surface of each one of four mature leaves (Muckenschnabel,
532 2002). Pots were enclosed in individual clear polyester chambers to prevent

533 desiccation of the droplets. Forty eight hours after inoculation, the leaves were
534 harvested and photographed to measure the area of the lesion with the aid of Adobe
535 PhotoShop CS3.
536
537 **Confocal microscopy**
538
539 Confocal fluorescence images were taken with an LSM5 Pascal (Zeiss,
540 http://www.zeiss.com) laser scanning microscope with a
541 water-immersion objective lens (C–Apochromat 40 X/1.2; Zeiss). For chloroplast
542 visualization, probes were excited with a He-Ne laser (543nm) and fluorescence was
543 detected using an LP560 filter. For COP1-YFP and RGA-GFP fusion proteins
544 visualization, probes were excited with an Argon laser (488nm) and fluorescence was
545 detected using a BP 505–530 filter. Fluorescent nuclei were defined as regions of
546 interest (ROIs) and fluorescence intensity was measured using IMAGEJ from the
547 National Institutes of Health (Abràmoff et al., 2004). A transmission image was also
548 included to count cells in each image. Representative cells of the leaf parenchyma (first
549 layers beneath the epidermis) were documented by photography during the first 15 min
550 of microscopy analysis.
551
552 **Reverse Transcription Quantitative PCR**
553
554 Seedlings were harvested in liquid nitrogen, then total RNA was extracted with the
555 RNEasy Plant Mini Kit (Qiagen) and subjected to a DNAse treatment with RQ1 RNase-
556 Free DNase (Promega, http://www.promega.com). cDNA derived from this RNA was
557 synthesized using Invitrogen SuperScript III and an oligo-dT primer. The synthesized
558 cDNAs were amplified with FastStart Universal SYBR Green Master (Roche) using the
559 7500 Real Time PCR System (Applied Biosystems, http://www.appliedbiosystems.com)
560 cycler. The *UBIQUITIN-CONJUGATING ENZYME 2* (*UBC2*) gene was used as

561 normalisation control (Czechowski et al., 2005). The primers are listed in Supplemental
562 Table S5.
563
564 **Extraction, purification, and estimation of JA content**
565
566 JA was extracted from Arabidopsis dry shoots by using a modified version of the
567 protocol of Durgbanshi et al. (2005). Plant material was homogenized and dissolved in
568 5 mL ultra-pure water. Fifty nanograms of [2H6]-JA (OlChemIm Ltd, Olomouc, Czech
569 Republic) was added as internal standard. Extracts were transferred to 50-mL tubes,
570 centrifuged at 1500 *g* for 15 min. The supernatant was collected, adjusted to pH 2.8
571 with 15% (v/v) acetic acid and extracted twice with an equal volume of diethyl ether.
572 The aqueous phase was discarded and the organic fraction was evaporated under
573 vacuum. Dried extracts were dissolved in 1 mL methanol. Samples were filtered
574 through a syringe filter tip on a vacuum manifold at flow rate less than 1 mL min-1, and
575 the eluate was evaporated at 35°C under vacuum in a SpeedVac SC110 (Savant
576 Instruments, Inc., New York, USA). Mass spectrometry analysis for JA quantification
577 was performed on a quadruple tandem mass spectrometer (MS–MS, Quattro Ultima;
578 Micromass, Manchester, UK) outfitted with an electrospray ion source (ESI). A mixture
579 containing unlabelled compound and internal standard was separated by reversed-
580 phase high performance liquid chromatography (HPLC) and analysed by tandem mass
581 spectrometry with multiple reaction monitoring (MRM) for JA retention time
582 determination. This compound was monitored at m/z transitions of 209/59–15/59 with
583 retention time of 13.5 min. The collision energy used was 20 eV (electron volts). The
584 cone voltage was 35V.
585
586 **ACCESSION NUMBERS**
587 Sequence data from this article can be found in the GenBank/EMBL data libraries
588 under accession numbers AT1G09570 (PHYA), AT4G08920 (CRY1), AT1G04400

589 (CRY2), AT1G32640 (MYC2), AT5G46760 (MYC3), AT4G17880 (MYC4), AT2G32950
590 (COP1), AT2G01570 (RGA1), AT1G14920 (GAI), AT1G66350 (RGL1) and
591 AT3G03450 (RGL2).
592
593 **SUPPLEMENTAL DATA**
594
595 The following supplemental materials are available
596 **Supplemental Figure S1.** Experimental protocols.
597 **Supplemental Figure S2.** Transcriptome responses to the initial period of LD are
598 robust and persistent.
599 **Supplemental Figure S3.** Transcriptional network involving genes present in clusters
600 1, 2, and 3.
601 **Supplemental Figure S4.** Specific signature of gene expression responses to
602 daylength.
603 **Supplemental Figure S5.** Chlorophyll and anthocyanin contents do not exhibit rapid
604 responses to daylength.
605 **Supplemental Figure S6.** Negligible effects of daylength on oxidative stress markers.
606 **Supplemental Table S1.** List of genes corresponding to clusters 1, 2 and 3..
607 **Supplemental Table S2.** Expression of genes corresponding to clusters 1, 2 and 3 in
608 experiment 2..
609 **Supplemental Table S3.** GO term enrichment.
610 **Supplemental Table S4.** Expression of JA and ethylene-dependent systemic
611 resistance genes in WT and *cop1* mutants.
612 **Supplemental Table S5.** Sequence of primers used for RT-qPCR
613
614 **ACKNOWLEDGMENTS**
615

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LEGENDS OF THE FIGURES

Figure 1. Robust responses of the transcriptome to initial LD exposure. A, Three major clusters grouped 749 genes showing statistically significant responses to photoperiod. Plants of Arabidopsis accession Landsberg *erecta* were grown under SD for three weeks, then transferred to LD and harvested after the end of the first LD photoperiod (Experiment 1, Supplemental Fig. S1A). The expression of each gene was normalised to the average for that gene across the genotypes and treatments and then the cluster average and SE was calculated for each genotype and condition. B, For each overrepresented GO term and cluster, average normalised expression and SE for each genotype and condition is shown in boxes corresponding to (left to right): Experiment 1, Experiment 2 (Supplemental Fig. S1A, independent experiment with accession Columbia following the same protocol as experiment 1 but followed up for 6 d), and publicly available data (Supplemental Fig. S1B, time course corresponding to plants of accession Landsberg *erecta* grown continuously under either SD or LD (Michael et al., 2008)).

Figure 2. LD enhances resistance to *B. cinerea*. Plants of Arabidopsis accession Columbia were grown under SD for three weeks and inoculated at 7 h of day 21. One group was transferred to LD while the other remained under SD, and leaves were harvested 48 h after inoculation. Data are means and SE of at least 11 plants. Different letters indicate significant differences ($P < 0.05$) among means determined using Bonferroni post hoc tests. Leaves were photographed individually and a composite image was produced with representative cases.

Figure 3. LD enhances JA signalling but not JA levels. A, Expression of a set of 100 genes whose expression is promoted by JA (Goda et al., 2008) was used as a proxy for JA signalling. Left: Experiment 1. Middle: Experiment 2. Right: published data

(Michael et al., 2008). B, JA levels in plants exposed to SD→LD. Data are averages ± SE. Different letters indicate significant differences (P <0.05) among means determined using Bonferroni post hoc tests and the significant effect of photoperiod in factorial ANOVA is shown in B.

Figure 4. Negative correlation between the impact of the *myc2 myc3 myc4* (Fernández-Calvo et al., 2011) and *cop1* mutations (Data in Supplemental Table S4) compared to the WT. Regression: P <0.01.

Figure 5. COP1 increases the lesions inflicted by *B. cinerea* under SD, whereas LD reduces COP1 nuclear abundance. A, Nuclear abundance of YFP-COP1 at the end of the first photoperiod under LD and in SD controls. Data are means ± SE of 8–9 plant replicates and representative images (arrows point to nuclei with detectable YFP-COP1, size bar= 20 μm). B, Resistance to *B. cinerea* in *cop1* mutants and the COP1 overexpressor (*COP1-OX*) under SD and LD represented by relative lesion size. Data are means ± SE of 13 plant replicates. Different letters indicate significant differences (P <0.05) determined using Student's *t*-test (A) or Bonferroni post hoc tests (B).

Figure 6. LD increases RGA abundance in a COP1-dependent manner. Data are means ± SE of 6 plant replicates and representative images are shown (size bar= 20 μm). Different letters indicate significant differences (P <0.05) among means determined using Bonferroni post hoc tests.

Figure 7. The effect of photoperiod on susceptibility to *B. cinerea* requires normal DELLA function. A and B, Resistance to *B. cinerea* infection represented by relative lesion size in mutants affected in DELLA genes in either Columbia (A) or Landsberg erecta (B) background. C, Resistance to *B. cinerea* infection under in WT and *cop1* mutant plants with or without compromised DELLA function conferred by *gai rga* double

mutation. Data are means ± SE of 5 plant replicates. Different letters indicate significant differences ($P < 0.05$) among means determined by Bonferroni post hoc tests. The significant interaction between *cop1* and *gai rga* determined by factorial ANOVA is shown in C.

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