Shedding light on the Methylerythritol phosphate (MEP)-pathway: long hypocothyl 5 (HY5)/ phytochrome-interacting factors (PIFs) transcription factors modulating key limiting steps.

Marel Chenge-Espinosa[1], Elizabeth Cordoba[1], Cynthia Romero-Guido[1], Gabriela Toledo-Ortiz [2]* and Patricia León[1]*

[1] Departamento de Biología Molecular de Plantas, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Av. Universidad # 2001, Col. Chamilpa, Cuernavaca, Morelos, México, C.P. 62210.

[2] Lancaster University, Lancaster Environment Centre, Lancaster LA1 4YQ, UK

*Corresponding authors:

Patricia León

Telephone +52 (55) 5622 7856 Fax: +52 (777) 313 9988

email: patricia@ibt.unam.mx

Gabriela Toledo-Ortiz

Telephone: +44 (0) 1524 595053

Email: g.toledo-ortiz@lancaster.ac.uk

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Significance statement

Light imposes a direct, rapid and potentially multi-faceted effect that leads to unique protein dynamics to the main flux-limiting steps of the MEP pathway, a key route essential for plants. Through differential direct transcriptional interaction, the key-master integrators of light signals HY5 and PIFs, target the main flux-limiting steps of the pathway. Our work illustrates how light signals can impose contrasting dynamics over a key pathway whose products multi-branch downstream to all chloroplastic isoprenoids.

Summary

The plastidial methylerythritol phosphate (MEP) pathway is an essential route for plants as the source of precursors for all plastidial isoprenoids, many of which are of medical and biotechnological importance. The MEP-pathway is highly sensitive to environmental cues as many of these compounds are linked to photosynthesis and growth and light is one of the main regulatory factors. However, the mechanisms coordinating the MEP-pathway with light cues are not fully understood.

Here we demonstrate that by a differential direct transcriptional modulation via the key master integrators of light signal transduction HY5 and PIFs that target the genes that encode the rate-controlling *DXS1*, *DXR* and *HDR* enzymes, light imposes a direct, rapid and potentially multi-faceted response that leads to unique protein dynamics of this pathway resulting in up to 10-fold difference in the protein levels. For *DXS1*, PIF1/HY5 act as a direct activation/suppression module. In contrast, *DXR* accumulation in response to light results from HY5 induction with minor contribution of de-repression by PIF1. Finally, *HDR* transcription increases in the light exclusively by suppression of the PIFs repression. This is an example of how light signaling components can differentially multi-target the initial steps of a pathway whose products branch downstream to all chloroplastic isoprenoids. These findings demonstrate the diversity and flexibility of light signaling components that optimize key biochemical pathways essential for plant growth.

Keywords:

Isoprenoids, MEP-pathway, Light responses, DXS1, DXR and HDR enzymes, Phytochrome interacting factors, Long Hypocotyl 5, Rate-limiting enzymes, *Arabidopsis thaliana*.

Introduction

Isoprenoids constitute a family of natural products synthesized in all organisms with diverse function (Chappell, 1995). Isoprenoids are essential for plant development, participating in several key processes such as photosynthesis, respiration and general plant growth (Bouvier *et al.*, 2005). All isoprenoids are produced from the condensation of two universal five-carbon precursors, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Condensation of these basic units gives rise to isoprenoid diversity.

Plants synthesize IPP and DMAPP by two non-related pathways present in different compartments. The cytoplasmic mevalonic pathway uses acetyl-CoA via mevalonic acid for the synthesis of IPP and DMAPP, and the methyl-D-erythritol 4-phosphate (MEP) pathway takes place in plastids (Croteau *et al.*, 2000, Hemmerlin *et al.*, 2012, Rodriguez-Concepcion and Boronat, 2015). The MEP pathway uses pyruvate and D-glyceraldehyde 3-phosphate (GAP) for IPP and DMAPP synthesis through the activity of seven consecutive enzymes (Phillips *et al.*, 2008). The first step of the MEP pathway is catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS) that produces 1-deoxy-D-xylulose 5-phosphate (DXP). DXP is rearranged into MEP by the action of the 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), this is the first committed step of the pathway. Subsequently MEP is converted to 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBPP) by four additional enzymatic steps and in the final step HMBPP is converted into a mixture of IPP and DMAPP via the HMBPP reductase (HDR) enzyme (Eisenreich *et al.*, 2004).

The MEP pathway is present in eubacteria, plastids and the apicoplast of apicomplexan but is absent in other eukaryotes, including humans (Lange *et al.*, 2000). Thus, the MEP pathway is considered an attractive target for development of new antibacterial and antiparasitic drugs, and herbicides (Rodríguez-Concepción, 2004, Rohdich *et al.*, 2005). In plants the MEP pathway is responsible

for the production of essential compounds, such as the precursors of photosynthetic pigments (carotenoids and the side chain of chlorophyll), tocopherols and plastoquinones, hormones including gibberellins, abscisic acid and strigolactone and a variety of monoterpenes, diterpenes and some sesquiterpenes (Bouvier *et al.*, 2005, Umehara *et al.*, 2008). Recent studies have reported that the MEP pathway is also essential for the production of stress-specific retrograde signaling molecules (de Souza *et al.*, 2017). Thus, in plants the MEP pathway is essential for plant development and also is an important target for biotechnological manipulation.

The enzymes in the MEP pathway are subject to modulation at different levels, according to the developmental status of the plant and the fluctuating environmental conditions (Guevara-García *et al.*, 2005, Rodriguez-Concepcion, 2006, Cordoba *et al.*, 2009, Banerjee and Sharkey, 2014). This multi-level dynamic is critical to ensure the supply of IPP and DMAPP precursors with the demand of downstream pathways, many of which are dependent of diurnal and light conditions, such as the biosynthesis of chlorophylls and carotenoids (Ruiz-Sola and Rodriguez-Concepcion, 2012). In plants, the plastid-localized enzymes of the MEP pathway come from nuclear-encoded genes. Experimental evidence has demonstrated that the transcript levels of all genes in the pathway are induced during plant development (Guevara-García *et al.*, 2005, Meier *et al.*, 2011), during tomato fruit ripening (Lois *et al.*, 2000) and in response to hormones (Oudin *et al.*, 2007). Also, biotic interactions (Walter *et al.*, 2000), circadian clock (Cordoba *et al.*, 2009, Vranova *et al.*, 2013) and light modulate the MEP transcripts levels in several plant species (Hsieh and Goodman, 2005, Cordoba *et al.*, 2009). Furthermore, post-transcriptional regulation of DXS, one of the rate-limiting steps of the pathway, leads to changes in its protein accumulation and enzymatic activity (Guevara-García *et al.*, 2005, Wright *et al.*, 2014).

Light constitutes one of the most critical environmental signals for plant development. From the emergence of the germinating seedling, light changes the

developmental program from skotomorphogenesis to photomorphogenesis (Chen *et al.*, 2004). Upon light exposure etioplasts rapidly differentiate into chloroplasts upregulating many genes involved in photosynthesis and other plastid biosynthetic pathways (Jiao *et al.*, 2007). Complex photoreceptor systems allow plants to adjust diverse processes in response to variable light conditions (Franklin and Quail, 2010). The phytochrome photoreceptors fine-tune plant photomorphogenesis in response to Red and Far Red light. Mechanistically, light-activated nuclear-phytochromes bind directly to members of the bHLH family of phytochrome-interacting factors (PIFs), promoting their degradation. PIFs act as important repressors of photomorphogenic development in the dark and key signal integrators (Leivar and Quail, 2011, Leivar and Monte, 2014). In addition, active phytochromes prevent the degradation of activators of photomorphogenesis such as ELONGATED HYPOCOTHYL 5 (HY5) and its close homolog HYH. These bZIP transcription factors participate in the up-regulation of a variety of genes in response to light and display antagonistic functions to the PIFs in the expression of diverse genes (Tepperman *et al.*, 2001).

Given the strategic role that light has over isoprenoid production in the present study we investigate the mechanisms coordinating the MEP-pathway with light cues. We examined the role of light signaling components PIFs, HY5 and HYH in modulating the expression and imposing a differential and specific dynamic on *DXS1*, *DXR* and *HDR* genes encoding for flux-controlling enzymes of the MEP pathway (Estévez *et al.*, 2001, Mahmoud and Croteau, 2001, Botella-Pavía *et al.*, 2004, Carretero-Paulet *et al.*, 2006). Transcript analyses and ChIP assays confirmed that HY5 and PIFs are direct regulators of the light-modulated expression of these genes. We show that the function of these factors in tuning the MEP-pathway gene expression extends to different developmental stages potentially diversifying the synthesis of multiple compounds of isoprenoid origin, essential for plant growth and development at different stages of the plant life cycle. Furthermore, we demonstrate using protein accumulation studies that the transcriptional regulation mediated by PIF and HY5 impacts the accumulation of

DXS1 and HDR enzymes but not DXR. This analysis exemplifies the diverse mechanistic dynamics that the same master light regulators can impose to tune up essential metabolic pathways in response to light.

Results

Cis-acting elements in the DXS promoter are responsible for the regulation of the DXS1 gene.

Previous studies have shown that in developing seedlings, transcripts of the MEP pathway genes accumulate upon light exposure (Botella-Pavía *et al.*, 2004, Hsieh and Goodman, 2005, Cordoba *et al.*, 2009). To analyze if the light induction response is mediated at the transcriptional level, 3 day-old etiolated transgenic lines containing 1510 bp upstream from the *DXS1* gene ATG fused with the GUS gene (Estévez *et al.*, 2000) were exposed to light. GUS activity is detected in the cotyledons of dark grown seedlings (Figure 1a). However, after 6 h of light exposure this staining expands to the hypocotyl (Figure 1d). Quantitative determination of GUS activity confirmed an approximately 2-fold increase after light exposure in comparison to dark control seedlings (Figure 1g). This result confirms that in response to light, *DXS1* transcript levels are transcriptionally up-regulated and also demonstrates that the *cis*-acting elements important for this response are present within the 1.5 Kb upstream regulatory region of this gene.

To further delimit the region involved in the light response two additional transgenic lines containing 750 (Figure b and e) and 670 bp (Figure c and f) upstream from the *DXS1* ATG of were generated, and their expression in response to light was analyzed. We observed that the GUS expression in these lines is induced upon light exposure at similar levels to the 1.5 Kb original fragment (Figure 1g). These results support that the elements responsible for light response in the *DXS1* gene localize within the 670 bp region upstream from the ATG (360 from the transcription initiation site).

HY5 and HYH positively regulate transcription of MEP-pathway limiting *DXS1 and DXR* genes, but not of HDR.

The transcription factor HY5 plays a pivotal role as a positive regulator of photomorphogenesis and greening responses through direct binding to the promoters of diverse light-activated genes (Lee *et al.*, 2007, Zhang *et al.*, 2011). To determine whether HY5 contributes to the light induction of the *DXS1* gene, its transcript level was analyzed after illumination of dark-adapted wild-type and *hy5* mutant seedlings; since a clear accumulation by light for MEP genes was observed under these conditions (Cordoba *et al.*, 2009). Seedlings were grown for 3 days under 16 h light: 8h dark photoperiod and then transferred to darkness for 3 additional days (dark-adapted treatment) prior to illumination for 6 h. Northern blot analysis shows that the transcript of *DXS1* is significantly lower in the light *hy5* mutant compared to the Col-0 wild-type counterpart (Figure 2a). In the *hy5hyh* double mutant we observed further reduction of the *DXS1* transcript supporting the notion that while minor, HYH also plays a positive role in the expression of *DXS1* in response to light.

In addition to DXS, two other enzymes, DXR and HDR, limit the flux through the MEP-pathway (Mahmoud and Croteau, 2001, Botella-Pavía *et al.*, 2004, Carretero-Paulet *et al.*, 2006, Kim *et al.*, 2009). We also analyzed the light responsiveness of *DXR* and *HDR* transcripts in the *hy5* and *hy5hyh* mutants. As shown in Figure 2b the transcript of *DXR* accumulates upon light illumination in the Col-0 wild-type plants. This induction is substantially lower in the *hy5* mutant, demonstrating that HY5 also acts positively on the expression of the *DXR* gene in the light. Relative to *hy5*, the *hy5hyh* double mutant has no additive effect on the level of *DXR* transcript abundance, supporting the lack of involvement of HYH in the light induced up-regulation of this gene. In contrast, the transcript accumulation in response to light for *HDR* is marginal in the wild-type (Figure 2a, b) with no clear role of HY5 and HYH (Figure 2c).

To further substantiate these results quantitative RT-PCR (qRT-PCR) on the same RNAs samples was performed, confirming the positive role of HY5 on the induction by light of *DXS1* and *DXR* transcripts (Figure 2d, e) but not on *HDR*. qRT-PCR data further corroborated that HYH does not appear to play a major role in the modulation by light of these genes. As monochromatic red light maximizes the light response we analyzed the expression of these genes in the dark-adapted seedlings illuminated with red light (Figure S1). This analysis corroborates an increase of the *HDR* transcript by light and that HY5 does not play a major role in this response. Red-light experiments also demonstrated that in the absence of these bZIP transcription factors there is still some light responsiveness, supporting the notion that additional unknown factors participate in this response.

PIFs are negative regulators of *DXS1, DXR and HDR* genes

For diverse light responses, PIF transcription factors act antagonistically to HY5 and HYH (Chen *et al.*, 2013). To test whether PIFs play an opposing role to HY5 in the light-mediated accumulation of the MEP pathway genes, the transcript level of the *DXS1, DXR* and *HDR* were analyzed by northern blots in *pif1*, *pif3, pif4* and *pif5* mutants compared to Col-0 wild-type seedlings, using dark-adapted treated seedlings (Leivar *et al.*, 2008). We observed that the transcript levels of the *DXS1* (Figure 3a), *DXR* (Figure 3b) and *HDR* (Figure 3c) genes are elevated in several of the *pif* single mutants in the dark and in the light, supporting a function of these transcription factors as transcriptional repressors of the expression of these rate-limiting MEP pathway genes.

To further analyze the contribution of the different PIFs we performed qRT-PCR on the same samples illuminated with white light (Figure S2) or with monochromatic red light to maximize response (Figure 3). In the case of *DXS1*, increased transcript accumulation is observed in the *pif1* and *pif3* mutants with a more moderate increment in *pif5,* supporting the repressing function of these factors (Figure 3d and S2a). For *DXR*, transcript levels are higher in the dark and in light-exposed *pif1, pif3* and *pif5* mutants demonstrating that these three PIFs

contribute to the low accumulation level of this gene in wild-type plants (Figure 3e). Finally, PIF1 appears to have the most significant effect on repressing *HDR* transcript levels, with some contribution of PIF3 (Figure 3f). Collectively our data demonstrate that the PIF factors in a partially redundant form are required to maintain low expression levels of three key genes of the MEP pathway.

The functional redundancy of PIFs in regulating *DXS1*, *DXR* and *HDR* genes is further illustrated with the quadruple *pifQ* mutant lacking PIF1, PIF3, PIF4 and PIF5 (Leivar *et al.*, 2009). Because *pifQ* could suffer from photoxidative damage caused by the overaccumulation of photochlorophyllide in the dark (Chen *et al.*, 2013), *pifQ* analysis was done in 3 day-old seedlings grown in(the reported?) photoperiod and dark-adapted for 36 h before exposure to 6 h of red light. We corroborated that in the *pifQ* mutant the expression level of *DXS1* (Figure 3g), *DXR* (Figure 3h) and *HDR* (Figure 3i) genes are significantly elevated in the dark and after light exposure compared to wild-type seedlings. Over all, our data demonstrates that PIFs are important negative regulators of the three rate-limiting MEP pathway genes.

HY5 and PIFs regulators modulate the expression of *DXS*, *DXR* and *HDR* genes during de-etiolation

It is known that PIFs display differential expression during distinct developmental responses (Jeong and Choi, 2013). One of the physiologically relevant responses to light is de-etiolation. Previous data demonstrated that during de-etiolation PIF1 represses *PSY* gene expression that plays a limiting role in carotenoid biosynthesis, but under the conditions used (1 h of induction) no regulation by PIF1 was detected for *DXS1* (Toledo-Ortiz *et al.*, 2010). Based on our observations that light induces *DXS*, *DXR* and *HDR* expression at later time points, we re-evaluated the contribution of PIFs and analyzed the role of HY5 by qRT-PCR in 3 day-old etiolated seedlings exposed to red light for 6 h. Similar to the dark-adapted seedlings, the transcription factor HY5 was shown to act as an activator of the *DXS1* and *DXR* genes expression (Figure 4a and b). After light exposure the

level of the *DXS1* and *DXR* transcripts is lower in *hy5* compared to the wild-type. There was no significant change in the *HDR* expression level in *hy5* (Figure 4c), indicating that, at this stage,HY5 does not play a major role in the light response of this gene .

On the other hand, removal of the PIFs results in a significant increase in the transcript levels of the *DXS1*, *DXR* and *HDR* genes at this developmental stage compared to the wild-type seedlings (Figure 4). This result supports and extends the partially redundant, repressive role of PIFs in down-regulating the expression of the *DXS1*, *DXR* and *HDR* genes during de-etiolation. Similar to the dark-adapted seedlings PIF1, PIF3 and PIF5 contribute most significantly to the regulation of these genes and we did not observe any major changes in the relative role of the individual PIFs at this developmental stage. In accordance the *pifQ* mutant accumulates at higher transcript levels than the three genes in the dark and in the light.

Commented [HG1]: Wasn't sure about this when looking at the plots. Certainly for DXS1 and DXR in the light this is true and also for DXS1 in the dark but pif1 looks to be greater than pifQ in DXR and for HDR pif3 in the dark and pif1 in the light. I could be missing something though.

PIF1 and HY5 interact with the promoters of the MEP pathway genes *in vivo*

To investigate if the changes in the gene expression result from a direct interaction of the PIFs and HY5 transcription factors with the promoters of the *DXS1*, *DXR* and *HDR* genes, we conducted chromatin immunoprecipitation (ChIP) experiments. We selected as a model PIF1, considering that this factor plays a major role in the regulation of the three MEP genes. ChIPs were carried out using seedlings that express PIF1 fused to a myc-tag in a *pif1* background (TAP-PIF1) (Moon *et al.*, 2008) or HY5 with an HA-tag in *hy5* background (HA-HY5) (Lee *et al.*, 2007). Lines with a TAP-GFP or HA-GUS in a wild-type background were included as negative controls for nonspecific binding of DNA to the tags used. Transgenic HA-HY5 and TAP-PIF1 are mild-overexpressors, with similar protein expression levels to endogenous HY5 and PIF1 and complement the mutant phenotypes in a wide range of tested-light responses (Lee *et al.*, 2007, Moon *et al.*, 2008).

To address if there is any difference in the interaction of these factors depending on the developmental stage, the ChIP was conducted in dark-adapted seedlings and etiolated seedlings, both exposed to 6 h of red light using myc or HA antibodies. qRT-PCRs were done using specific primers for selected promoter regions of each gene (Table S1, Figures S3, S5 and S6).

PIFs transcription factors bind to variants of the E boxes (CANNTG), including the G-box (CACGTG) and the PBE-box (CACATG/CATGTG), or to hexameric sequences G-box coupling elements (GCEs) containing the core "ACGT" elements (Toledo-Ortiz *et al.*, 2003, Zhang *et al.*, 2013, Kim *et al.*, 2016). The G-box, together with diverse GCE related motifs, such as the Z box (ATACTGTGT), CA (GACGTA) and CG hybrids (GACGTG), have also been identified as the interaction site of HY5 (Lee *et al.*, 2007, Toledo-Ortiz *et al.*, 2010, Zhang *et al.*, 2011). The analysis of the 1.5 kb *DXS1* promoter sequence using SOGO New PLACE software, showed no-presence of canonical G boxes. However, several E-box related elements, including a PBE-box (CACATG), a CG hybrid box (GACGTG), and a GCE element were found (Figure S3). Four specific oligonucleotide pairs were used to amplify the DNA enriched from the precipitated Protein-DNA complexes from the TAP-PIF1 and HA-HY5 transgenic lines maintained in the dark or exposed to 6 h red light. The qPCR using the oligonucleotide pairs 1 and 2 (Figure S3) showed no significant recovery in comparison to the negative TAP-GFP and HA-GUS controls, indicating no binding of PIF1 or HY5 to these promoter fragments. In contrast, enriched DNA sequences were amplified from the TAP-PIF1 and HA-HY5 immunoprecipitated fractions with the oligonucleotide pair P3 (F3/R3) (Figure S3). PIF1 binding was significant in the extracts from dark grown seedlings and a minor enrichment was seen in the light extracts (Figure 5a). For the HA-HY5 immunoprecipitate we only observed specific amplification in light-exposed seedlings (Figure 5b). For the P4 (F4/R4) primers pair (Figure S3) a minor amplification was detected from both the TAP-PIF1 and HA-HY5 extracts in comparison to the controls, indicating a very weak, and probably not-significant (just slightly above the in-specific GUS-HA background)

binding of both factors to this fragment (Figure 5c, d). The fragment that showed preferential binding to PIF1 and HY5 contains a PBE box, a GCE element and CG hybrid box in close proximity to each other (Figure S3). For PIF1, the PBE box (CACATG) is the most likely candidate binding-element and the GCE element (ACGT) for HY5. Very similar results were obtained from the ChIP experiments in de-etiolated seedlings demonstrating that the same sites are involved in the PIF1 and HY5 binding in these two light developmental stages (Figure S4).

We also analyzed the promoter sequences of *DXR* and *HDR* genes for potential PIF1 and HY5 binding sites. Within 1300 pb of the upstream sequence of *DXR* several G box-related sequences were found (Figure S5). Three pairs of primers covering the different elements from the DXR promoter were used in ChIP experiments (Table S1 and Figure S5). As shown in Fig. 5f the only significant enrichment detected was for HA-HY5 in the light with the primer pair 1 (F1 and R1) containing two GCE box-related sequences. These data demonstrate that *DXR* is directly upregulated by HY5. In contrast, binding of TAP-PIF1 in the dark was minor, although within statistical significance with the two primer pairs and close with the primer pair 3 (p 0.055) (Figure 5e, g and i). In these fragments, several putative G-box and E-box related sequences are present (Fig. S5). However, considering that the binding of PIF1 to the three sites is so close to the negative control in both photoperiodic and de-etiolation experiments (Figure S4e, g, i), this result probably indicates a non-preferential interaction of PIF1 with the promoter of *DXR*.

Finally, the *HDR* gene promoter includes 902 bp with only one PBE box and a GCE/ACE motif (Figure S6). Two pairs of primers were designed to cover the potential PIF1/HY5 binding sites and used to amplify the immunoprecipitates from the ChIPs (Table S1). In contrast to the *DXS1* and *DXR* genes, no enrichment was detected with HA-HY5 (Figure 5l), demonstrating that HY5 does not bind to the upstream sequences of the *HDR* gene. For TAP-PIF1 significant binding was observed in dark samples with the primer pair P2 that includes a PBE-box (Figure

5k). ChIPs results in de-etiolated seedlings (Figure S4k and l) tightly correlate with those of the photoperiodic-dark-adapted seedlings (Figure 5k, l). In conclusion, these data support the notion that modulation by light of *HDR* transcript levels results from its de-repression from the dark activity of PIF1/PIF3 and not from activation in light by HY5. Whether there is another light regulated activation factor, remains to be investigated. Over all, our ChIP studies likely reflect light imposed changes in the PIFs/HY5 regulators dynamic behavior and differential promoter-binding capacity in response to the light environment.

Impact of transcriptional imposed regulation over light modulated levels of MEP proteins

The MEP-pathway provides with the intermediaries for the production of multiple compounds essential for photomorphogenic development, including multiple hormones (GA, Cytokinin, ABA, Strigolactones) and photopigments (carotenoids and chlorophylls).

A previous study demonstrated that carotenoids and chlorophylls accumulate upon light exposure and this accumulation is affected in the *pifQ* and *hy5* mutants (Toledo-Ortiz *et al.*, 2014). Since the synthesis of these metabolites depends directly on the MEP pathway, the transcriptional regulation of the MEP-transcripts by light can impact the accumulation of the final pathway products. To evaluate the importance that light imposed transcriptional regulation of the *DXS1*, *DXR* and *HDR* transcripts has over the pathway, the levels of the corresponding proteins were analyzed in wild-type, *pif*s and *hy5* mutants. Total protein extracts from dark-adapted seedlings during 3 d (Wt, *pif1*, *pif3*, *pif5* and *hy5*) or 36 h (*pifQ*) were obtained and the level of the DXS1, DXR and HDR proteins compared to dark controls.

We found that the transcriptional regulation mediated by the HY5/PIF1 module results in significant changes in the DXS1 enzyme level. Compared to the wild-type, the *hy5* mutant has lower accumulation of DXS1 protein whereas *pif* single mutants and *pifQ* contain up to >10 times higher protein content than wild-

type in the dark (Figure 6B). It is worth noticing that although the DXS1 level in the light is higher in the *pifQ* mutant, it does not maintain the same difference observed in the dark (Figure 6b). We hypothesize that this might be the result of post-transciptional regulation that keeps this protein within certain levels (Flores-Perez *et al.*, 2008). This response correlates well with the transcript trends observed in *pif*s. The accumulation of DXS resembles the model of de-repression by PIFs in the dark /activation by HY5 in the light.

We also observed accumulation of HDR protein in *pif3* and *pifQ* with a contributed additive effect of primarily PIF3 and PIF1. We did not detect protein differences in *hy5* vs wild-type. The role of PIFs in de-repressing from the dark leads to high protein levels in the light, but no further light-induced upregulation was detected at the time point assessed.

No major differences were detected in the level of DXR protein in the different mutants analyzed (Figure 6), in agreement with the more moderate transcript differences observed for the *pif*s and *hy5*.

Discussion

The MEP pathway is a key biosynthetic route responsible for the synthesis of essential compounds and signals that modulate developmental and stress responses (Bouvier *et al.*, 2005, Umehara *et al.*, 2008, Hemmerlin *et al.*, 2012, Walley *et al.*, 2015, Benn *et al.*, 2016). Also, several of the MEP pathway products have importance for human health and nutrition. Addressing how such a central metabolic pathway is modulated by external and internal cues has big implications for future efforts to regulate its outputs including many compounds of biotechnological or pharmaceutical interest

Light is one of the most relevant signals that affect plant metabolism, including the production of essential photopigments, growth regulators and stress

hormones derived from the MEP-pathway (von Lintig *et al.*, 1997, Rodríguez-Concepción *et al.*, 2004). Previous work on photopigment production demonstrated that light-responsive transcriptional factors HY5 and PIF1 control central carotenoid and chlorophyll biosynthetic genes (Huq *et al.*, 2004, Toledo-Ortiz *et al.*, 2010). In the case of phytoene synthase PIF1 and HY5 act as a module that antagonistically balance the expression of this key carotenogenic gene (Toledo-Ortiz *et al.*, 2014). Our data demonstrate that via the same master-modulators, an additional higher-order layer arises via the light-induced tune up of the MEP-pathway for the coordinated production of the precursors used for multiple compounds involved in photomorphogenesis. Such is the case of carotenoids and the phytol side chain of the chlorophylls.

The gene products of the MEP pathway accumulate upon light-exposure (Botella-Pavía *et al.*, 2004, Hsieh and Goodman, 2005, Cordoba *et al.*, 2009), however the molecular mechanisms for this upregulation and its impact on the pathway are still not fully understood. The coordinated tuning by light of the MEP pathway genes leads to the possibility that common mechanisms regulate the expression of these genes in response to light. Here we show that the master regulators of light signals transduction HY5 and PIFs directly interact with the up-steam elements of the flux-controlling *DXS1*, *DXR* and *HDR* genes (Estévez *et al.*, 2001, Botella-Pavía *et al.*, 2004, Carretero-Paulet *et al.*, 2006) and fine-tune their expression levels in response to light. This regulation involves the interplay and differential contribution of each factor for each gene, leading to unique transcriptional dynamics (Figure 7) that lead to changes in protein accumulation. Considering the flux-controlling capacity of DXS, DXR and HDR (Estévez *et al.*, 2001, Botella-Pavía *et al.*, 2004, Enfissi *et al.*, 2005, Carretero-Paulet *et al.*, 2006, Banerjee *et al.*, 2013, Ghirardo *et al.*, 2014), the dynamics observed could importantly modify the flux through the pathway in response to the prevailing light environment.

Our findings on the role of PIFs contrast to previous reports that concluded that *DXS1* expression was not regulated by PIF1 (Toledo-Ortiz *et al.*, 2010). This

discrepancy probably results from differences in the quantification of transcript levels through a lower-sensitivity microarray analysis compared to qRT-PCR and the kinetics of light induction at earlier or later time points, as all these genes present light/dark oscillatory patterns (Cordoba *et al.*, 2009). In the case of PIF5, using overexpressing PIF5 cell lines it was concluded that this factor was the main positive regulator of all the MEP pathway genes without major participation for PIF1 or PIF3 (Mannen *et al.*, 2014). However, in our study we did not observe major differences of PIF1, PIF3 and PIF5 contribution as negative regulators of the MEP pathway genes during de-etiolation and dark-adaptation. It is possible that these differences result from a dominant negative effect of PIF5-over-expression. Also, we cannot exclude that PIF5 activity could change in other developmental or environmental conditions as a result of interaction with other elements (Mannen *et al.*, 2014).

Our studies demonstrate that the same key master integrators of light signal signals have the capacity to coordinate the core of the MEP pathway and its multiple outputs through particular mechanisms for each gene **(**Figure 7). *DXS1* expression is repressed in the dark by the direct binding of PIF1, probably through the PBE box (CACATG), whereas HY5 induces the expression of this gene in the light through direct binding to the nearby GCE element (ACGT). Despite the use of mild-over-expressors of PIF1 or HY5 for ChIP assays, the plant material likely resembles the behavior of the endogenous proteins, as these transgenic lines express comparable protein levels to the endogenous proteins, complement the *pif1* and *hy5* mutant phenotypes and maintain the dark/light dynamics imposed on these proteins by phytochromes and COP1, among others. Since PIFs/HY5 targeted *cis*-acting elements are located very close to one another, it is likely that the binding of one regulator results in allosteric interference of binding the second regulator, resulting in an antagonistic mechanism. This regulation resembles the one described for the phytoene synthase (*PSY*) gene (Toledo-Ortiz *et al.*, 2010, Toledo-Ortiz *et al.*, 2014).

In contrast, the accumulation of *HDR* in the light results exclusively from the degradation of the PIF repressors upon light exposure, without contribution from HY5. PIF1 directly interacts with *cis*-elements in the regulatory region of *HDR* (Figure 7). The strongest candidate as binding-element is a PBE motif present 156 bp upstream of the ATG.

Finally, the expression of *DXR* accumulates in the light as a consequence of the direct interaction of HY5 to elements located around 1.2 Kb upstream of the ATG of this gene (Figure 7). In contrast, PIF1 has a weak interaction in more than one region of the HDR promoter (Figure 7). The weak interaction observed with PIF1 could reflect that its binding capacity might depend on other PIFs. In agreement with this possibility our analyses demonstrate that in addition to PIF1, PIF3 and PIF5 also affect the expression level of the *DXS1, DXR* and *HDR* genes. Differential affinity of various PIFs for the promoters of MEP-pathway genes could result in modified kinetics of light-responsiveness and modulation by other environmental cues such as photoperiodism and the circadian clock.

Other studies have demonstrated high affinity of PIF1 and HY5 for G-box elements *in vitro* and *in vivo* (Toledo-Ortiz *et al.*, 2003, Huq *et al.*, 2004, Oh *et al.*, 2009, Zhang *et al.*, 2013). None of the MEP pathway genes analyzed contain canonical G-boxes and our data support that for MEP-genes, the PBE box (CACATG) and the GCE element (ACGT) are the strongest candidates to bind PIF1 for HY5 respectively. This differs and extends from the signal integration module established by G-boxes in the case of photopigment related genes. Our analysis from the DXS1::GUS transgenic lines carrying different promoter regions of the *DXS1* also supports our ChIP data, positioning the important *cis*-acting elements within 300 bp from the transcription initiation site. It is worth to mention that with the DXS1::GUS transgenic constructs although we observed the same response trend, only a 2-fold increase between dark and light conditions was detected with high GUS activity in the dark. This is in contrast to the low *DXS1* transcript levels found in the dark and its increase in response to light detected in our quantification

analyzes. This apparent discrepancy probably results from the accumulation of the GUS protein in the dark in the transgenic lines, as the stability of this protein is known to extend for more than 3 days (Kavita and Burma, 2008). Thus, in the case of *DXS1,* GUS is a good marker to identify regulatory *cis* acting elements but not for quantitative analyzes of its expression.

Hence together, our results indicate that the transcriptional regulation of MEP-pathway genes by PIFs-HY5 results in a unique dynamic behavior for each gene, providing additional flexibility to integrate inputs perceived by these master regulators, such as time keeping or temperature signals. Interestingly, the HY5 and PIF binding motives in *DXS1* gene localize proximal to a potential CCA1 binding site (AAAATCT). CCA1 encodes a MYB-related protein that binds to *Lhcb1*3* and that participates in the phytochrome regulation of this gene (Wang *et al.*, 1997). This factor is also an important component of circadian regulation in coordination of HY5 (Nagel *et al.*, 2015). Since *DXS1,* as well as other genes of the MEP pathway are regulated by the circadian clock (Cordoba *et al.*, 2009), it is possible that the enhanced regulatory dynamic imposed by a PIF/HY5 co-acting module would also bring unique capacity for the light and circadian regulation of *DXS1* via interaction with circadian components such as CCA1.

Importantly, we provide evidence that the differences in the transcript de-repression/activation by light mediated by PIFs/HY5 reflects on changes at the protein level leading to unique protein dynamics. DXS1 follows a good correspondence with the transcriptional fluctuations including low protein level in the dark and accumulation in the light in a HY5/PIFs dependent manner, resulting in a significant difference in protein levels. These rapid changes in the protein accumulation in response to light supports the possibility of a rapid turnover for this protein and are consistent to its central role as a major flux controller of the pathway in diverse environmental and developmental conditions (Estévez *et al.*, 2001, Enfissi *et al.*, 2005, Banerjee *et al.*, 2013, Ghirardo *et al.*, 2014, Wright *et al.*, 2014). However, we observed some discrepancies between the levels of the

transcript and their corresponding protein such as in the *pifQ* mutant, where only a 1.5-fold increase after light exposure is detected. This result probably reflects post-translational regulatory events over the DXS1 that adjust the level of this protein in response to the product demand, as has been previously reported (Pulido *et al.*, 2013, Pokhilko *et al.*, 2015, Pulido *et al.*, 2016). Thus, while transcription regulation of *DXS1* plays an important role in control the levels of this enzyme post-translational regulatory events also act as an additional layer of regulation that feedbacks metabolic requirements and impact upon the overall accumulation of this protein.

On the other hand, HDR protein levels also reflect on the transcriptional regulation mediated by light, following a different dynamic that results from the de-repression of transcript levels in the dark with no further changes associated with the light. The accumulation of HDR in response to light might be important to fulfill the synthesis requirement of photopigments in coordination to DXS1. This result is consistent with the co-limiting role previously observed for HDR during carotenoid synthesis in dark/light transition and during fruit ripening (Botella-Pavía *et al.*, 2004, Kim *et al.*, 2009). This type or regulation could be particularly important in conditions where different levels of PIFs accumulate, such as photoperiodic conditions, and at the same time could limit the acute responses to the light signal. Finally, changes in the DXR protein accumulation in response to light was reflected in constant protein levels in the light within the time frame analyzed. This result is intriguing and it may reflect on particularities of the half-life of this protein or post-transcriptional events that control protein abundance. Future analyses in this respect are important to address these possibilities.

Overall, our data support a model where a differential contribution of the master light regulators PIFs and HY5 to light-modulated transcriptional effects reflects in protein changes in MEP-pathway flux-controlling enzymes DXS1 and HDR. The significance of this differential regulation may impact on multiple downstream pathways such as chlorophyll and carotenoid biosynthesis as well as hormone and secondary metabolite synthesis, maintaining a very sensitive

responsiveness to the prevailing external conditions. Our studies exemplify how differential multi-targeting of the initial steps of a pathway whose products multi-branch downstream could impose a fine and unique modulation of all chloroplastic isoprenoids.

Methods

Plant Material and Growth Conditions.

The *Arabidopsis thaliana* lines used in this work are in Columbia (Col-0) background. Seeds from *pif1-2*, *pif3-3*, *pif4-2*, *pif5-2*, and *pifQ* were kindly provided by P. Quail (University of California Berkeley). Seeds were grown on 1X Murashige and Skoog (MS) media with Gamborg vitamins (Phytotechology Laboratories, Shawnee Mission, KS) supplemented with 1% (w/v) sucrose and 0.8% (w/v) phytoagar and stratified at 4°C for 4 days. For the light gene expression analysis two treatments were used. For the dark-adapted treatment, seedlings were grown for 3 days in a 16 h light: 8h dark photoperiod at 120 µmol m^2 sec^{-1}, followed by 3 day dark adaptation for the *hy5, hy5hy5* and *pif* single mutants or 36 h for the *pifQ*. Light treatment was done using 6 h with (100 µmol m^2 sec^{-1}) cool white (Philips F25T8/TL841) or (40 µmol m^2 sec^{-1}) of red (Phillips LED module HF Deep Red 177354) lights. For de-etiolation experiments seedlings were exposed to 3 h of white light, transferred to darkness for 3 days and exposed to 6 h of red light (40 µmol m^2 sec^{-1}). Control seedlings were maintained in darkness. Growth temperature was maintained between 21- 22°C in all cases.

Expression analysis

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. For northern-blot analysis, 5 µg of total RNA was fractionated and transferred onto a Hybond-N^+ nylon membrane (GE, Buckinghamshire, UK). Hybridizations and washes were performed under stringent conditions. Probes were ^{32}P-radiolabeled using the Megaprime DNA labeling system (GE, Buckinghamshire, UK). All probes were

obtained by PCR amplification as previously reported (Guevara-García *et al.*, 2005). For qRT-PCR experiments seedlings were harvested in the dark for the dark samples and RNA extraction conducted using the RNeasy Plant Mini Kit (Qiagen) or TRIzol. Complementary DNA (cDNA) was obtained from DNase-treated RNA with M-MLV Reverse Transcriptase kit (Invitrogen, Carlsbad, CA). The qRT-PCR experiments were performed using FastStart DNA Master^{PLUS} SYBR Green I (Roche) on an Agilent Technologies Stratagene MX3005P or a Light Cycler 480 Roche. Analyses were done with three independent experiments and technical duplicates were included in each case (n=2). The reference gene used in the qPCR analyses is *ACT7* since the expression of this reference gene has been shown to not have major fluctuations in the conditions analyzed.

DXS1 promoter analysis.

From the transcriptional *DXS1::*GUS clone (Estévez *et al.*, 2000), two additional clones were generated containing 750 bp and 660 bp deletions from the ATG of *DXS1.* The fragments were subcloned into the pBin19 binary vector and used to generate transgenic lines through *Agrobacterium tumefaciens*-mediated transformation into the Col-0 ecotype (Clough and Bent, 1998). At least three independent homozygous lines were selected for each construct and analyzed.

GUS histochemical and fluorimetric analyses

Three day-old etiolated seedlings exposed to light or dark for 6 h were stained using the GUS histochemical assay (Jefferson *et al.*, 1987). Plants were clarified as reported (Malamy and Benfey, 1997) and visualized using a stereoscopic microscope (Nikon SMZ1500). For the fluorometric analysis the seedlings were homogenized in GUS extraction buffer (50 mM $NaHPO_4$, pH 7.0; 10 mM Na_2EDTA; 1% Triton X-100; 0.1% N-lauroyl sarcosine and 10 mM β-mercaptoetanol). The enzymatic reaction was done using 5 µl of the extracts. Fluorometric quantification was done with TKO 100 fluorimeter (Hoeffer). Specific activity was determined as nmol of methyl-umbelliferone per µg protein^{-1} per min^{-1}.

Protein gel blot analysis

Total protein was obtained from seedlings and 20 µg of the samples was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto nitrocellulose membrane (Amersham Protan Premium 0.45 µm NC GE Healthcare Life Science). To verify equal loading, a parallel gel was run and stained with Coomasie blue. Immunodetection was performed as previously reported (Guevara-García *et al.*, 2005). Detection was done using the Luminata Crescendo Western HRP Substrate (Millipore, USA). Bands from three independent experiments were quantified by densitometric analysis using ImageJ software (Schneider *et al.*, 2012).

Chromatin Immunoprecipitation assays

ChIP assays were conducted following the protocol reported previously (Moon *et al.*, 2008) except that in our assays, 2 week old- seedlings were used. Plants were grown as described in (Toledo-Ortiz et al., 2014), and dark adapted for 72h before light treatments and sample collection. Samples were collected for dark time points (0h, before lights on at the end of dark adaptation) or after 6 h illumination with red light (40 µmol m^{-2} s^{-1}). Plant material used (35S::HA-HY5 in *hy5-215* and 35S::TAP-PIF1 in *pif1-2*) was previously described (Lee *et al.*, 2007, Moon *et al.*, 2008) and kindly provided by the Deng Lab (Yale) and Huq Lab (UT Austin). Both lines are mild-over expressors that complement the mutant phenotypes and show comparative levels and light responses to native *PIF1* and *HY5.* . qRT-PCR was conducted on a Roche 480 Light cyler according to standard protocol by the manufacturer. The oligonucleotides sequences used to amplify upstream promoter regions of individual genes are shown in Table S1. Upstream sequences of the *DXS1, DXR* and *HDR* genes were analyzed for possible light responsive elements using SOGO New PLACE software (https://sogo.dna.affrc.go.jp/cgibin/sogo.cgi?sid=&lang=en&pj=640&action=page&page=newplace) and are shown in Figures S2, S3 and S4 (Higo *et al.*, 1999).

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Supporting information

Supplemental Figure 1. Expression analysis of the *DXS1*, *DXR* and *HDR* genes in *hy5* mutants in Red light.

Supplemental Figure 2. Expression analysis of the *DXS1*, *DXR* and *HDR* genes in the *pif* single mutants in Red light.

Supplemental Figure 3. *DXS1* upstream regulatory region.

Supplemental Figure 4. Chromatin immunoprecitation assays for 35S::TAP-PIF1 and 35S::HA-HY5 in de-etiolated seedlings**.**

Supplemental Figure 5. *DXR* upstream regulatory region.

Supplemental Figure 6. *HDR* upstream regulatory region.

Supplemental Table I. Sequence of the oligonucleotides used in the work.

Figure Legends

Figure 1. **Transcriptional regulation of *DXS1* by light**. Histochemical expression of 3 day-old transgenic representative lines grown in the dark (a-c) or exposed to 6 h of light (d-f) expressing the GUS marker from 1510 bp (a and d), 753 bp (b and e) and 660 bp (c and f) upstream sequences from the ATG of *DXS1*. (g) GUS specific activities from the dark (dark gray) or exposed to light (gray) transgenic lines. The induction level is reported relative to the mean of the specific activity (nmol of methyl-umbelliferone per µg of protein^{-1} min^{-1}) of the corresponding dark sample. Each bar is the mean of three independent experiments and error bars represent ±SD. The numbers above the bars indicate the *P* values according to a Student´s t test.

Figure 2. **Expression analysis of the *DXS1*, *DXR* and *HDR* genes in *hy5* and *hy5hyh* mutants.** Representative northern blots of *DXS1* (a), *DXR* (b) and *HDR* (c), each lane contains 5 µg of RNA from 3 day-old dark-adapted seedlings maintained in the dark (D) or exposed to light for 6 h (L) from wild-type (Wt), *hy5* and *hy5hyh* mutants and hybridized with probes for the *DXS1* (a), *DXR* (b) and *HDR* (c) genes. The 28S rRNA is shown as loading control. The membrane is representative of three independent biological experiments. qRT-PCR analysis of *DXS1* (d), *DXR* (e) and *HDR* (f) transcript levels from Col-0, *hy5* and *hy5hyh* dark-adapted seedlings maintain in the dark (gray column) or exposed to 6 h of light (white column). Expression is normalized to the Col-0 dark samples and adjusted to *Actin 7* (*ACT7*). Bars are means ±SE of triplicate biological experiments (each with n=2 technical replicas). The numbers bars indicate P values $p<0.05$ and the * marks statistical difference ($p<0.05$) between the light samples from the *hy5* and *hy5hyh* mutants compared to the induction in the Wt according to a Student´s t test.

Figure 3. **Expression analysis of the *DXS1*, *DXR* and *HDR* genes in the *pif* single mutants**. Representative RNA blots of *DXS1* (a), *DXR* (b) and *HDR* (c)

from 3 day-old dark-adapted wild-type Col*0* (Wt), *pif1*, *pif3*, *pif4* and *pif5* maintained in darkness (D) or exposed to 6 h light (L). The 28S rRNA is shown as a loading control (28S). Membranes are representative of three independent biological experiments. Analysis by qRT-PCR of *DXS1* (d,g), *DXR* (e,h) and *HDR* (f,i) transcripts from Col-0, *pif1*, *pif3*, *pif4* and *pif5* (d-f) dark-adapted for 3 days (gray column) or 36 h for *pifQ* (g-i) and exposed 6 h of red light (white column). Expression is reported relative to the dark Col-0 sample and adjusted relative to *Actin 7* (*ACT7*). Bars are means ±SE of triplicate biological experiments (each with n=2 technical replicas). The letter above the bars indicate P values $p<0.05$ between dark (a) or light (b) of Wt compared to the mutants (Student´s t test).

Figure 4. **Expression analysis of the *DXS1*, *DXR* and *HDR* genes in the *hy5, pif* single mutants and *pifQ* during de-etiolation**. qRT-PCR analysis of *DXS1* (a), *DXR* (b) and *HDR* (c) genes from etiolated seedlings of Col-0, *hy5*, *pif1*, *pif3*, *pif4, pif5* and *pifQ* maintained in the dark (gray columns) or exposed to 6 h of red light (white columns) relative to the Col-0 dark samples and adjusted relative to *Actin 7* (*ACT7*). Bars are means ±SE of triplicate biological experiments (each with n=2 technical replicas). The letter above the bars indicate values ($p<0.05$) decrease (a and b) or increase (c and d) between the dark (a and c) or light (b and d) values between the Col*0* and the mutants (Student´s t test).

Figure 5. **Chromatin immunoprecitation assays for 35S::TAP-PIF1 and 35S::HA-HY5 in dark-adapted seedlings**. Diagrams of the upstream regions of *DXS1*, *DXR* and *HDR* genes. Primers used for the analyses (arrows) and the potential PIFs (black) and HY5 (gray) binding elements (rectangles). ChIP of three days dark-adapted TAP-PIF and HA-HY5 transgenic seedlings (Lee *et al.*, 2007, Moon *et al.*, 2008) maintained in the dark (grey zone) or exposed to 6h red-light (clear zone). ChIP was conducted using specific antibodies against MYC for PIF1 the HA for HY5. 35S::GFP-TAP or 35S::GUS-HA lines were used as controls for unespecific binding. The ChIP/qPCR was done using specific primer pairs (F, forward primer and R, reverse primer) covering the regions containing putative

binding elements. ChIP-enriched DNA regions of the TAP-PIF *DXS1* (a and c), *DXR* (e,g and i) and *HDR* (k) or for HA-HY5 *DXS1* (b and d) *DXR* (f,h and j) and *HDR* (l) samples. The bars are the mean ± SE of triplicate independent experiments (each with technical duplicates n=2). The asterisk indicate the values with significance ($p<0.05$) between the negative control according to a Student´s t test.

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Figure 6. Protein accumulation of the DXS1, DXR and HDR in mutants of the HY5 and PIFs. (a) Immunoblots with 20 µg of protein extracts from seedlings dark-adapted for 3 days (Col-0, *hy5*, *pif1*, *pif3*, *pif4* and *pif5*) or 36 h (*pifQ*) and maintained in the dark (D) or exposed to 6 h light (L) using specific antibodies for DXS1, DXR or HDR proteins. A Coomassie blue–stained gel (Coo) is shown as a loading control. A representative gel from three independent biological experiments is shown. (b) Densitometric analyses of the DXS1, DXR and HDR protein levels from Col-0, *hy5*, *pif1*, *pif3*, *pif5* and *pifQ* immunoblots from the dark (grey bars) or exposed to 6 h light (white bars) samples. The expression level is reported relative to the Col-0 light samples and adjusted to the corresponding loading control. The bars correspond to the average of three independent biological experiments ± SD of biological triplicates.

Figure 7. Model of the differential light regulation of *DXS1*, *DXR* and *HDR* gene expression to modulate the MEP pathway. Light via phytochrome (Pfr) results in the degradation of PIFs and in the accumulation of the HY5. For the *DXS1*, Pfr accumulation inhibits the direct repression of PIF1 (solid arrow), PIF3 and PIF5 (dashed arrows) and the activation of HY5 (solid arrow). For *DXR,* Pfr impairs the weak repression of PIF1 in multiple sites (solid arrows), PIF3 and PIF5 (dashed arrows). Light via HY5 activate *DXR* (solid line). Finally, Pfr accumulation induces *HDR* expression through the degradation of the PIFs. The dashed arrows mean that a direct interaction was not experimentally demonstrated. The thickness of the arrows reflects the RNA levels and the enrichment detected in the ChIP

analyses. The orange arrow reflects the dynamic of the light activation observed for each gene.