Coordinated activation of the metabolic pathways induced by LED blue light in citrus fruit.

María T. Lafuente*, Paco Romero and Ana-Rosa Ballester

Instituto de Agroquímica y Tecnología de Alimentos, IATA-CSIC, Calle Catedrático Agustín Escardino Benlloch 7, 46980 Paterna, Valencia, Spain

* Corresponding author. Tel.: +34 900 022; fax: +34 963 636 301

E-mail address: mtlafuente@iata.csic.es, promero@iata.csic.es, ballesterar@iata.csic.es

Running title: LBL-induced responses in citrus fruit

Abstract

The biochemical changes induced by LED Blue Light (LBL) (450 nm) in Lane Late oranges were investigated. The selected quantum flux ($60 \mu\text{mol m}^{-2} \text{s}^{-1}$, 2 days) was associated with resistance against *Penicillium digitatum*, the main postharvest pathogen of citrus fruit. A holistic overview was obtained by a comparative transcriptome profile analysis, which revealed that LBL favored energy metabolism and redirected metabolic pathways toward the synthesis of diverse primary and secondary metabolism products. LBL favored reactive oxygen species homeostasis and metabolic activities involving lipid metabolism, specifically the synthesis of pigments and oxylipins, and the metabolism of carbohydrates, amino acids and indol- and alkaloid-derivatives. LBL also repressed limonene catabolism and triggered phenylpropanoid derivatives-related changes, which increased content in total flavonoids. Transferring fruit from LBL to darkness favored those processes involving amino acids, different phenylpropanoid, alkaloid and terpenoid classes, and ferrochelatase activity.

Keywords: disease, light, photooxidative stress, primary and secondary metabolism.

1. Introduction

Light-emitting diodes (LEDs) illumination is an emerging technology in agriculture to potentially improve the production, nutritional quality and commercial value of crops (Bantis, Smirnakou, Ouzounis, Koukounaras, Ntagkas, & Radoglou, 2018). Moreover, the antimicrobial properties of light may be very useful for controlling those pathogens affecting human health (Dai, Gupta, Murray, Vrahas, Tegos, & Hamblin, 2012), as well as food pathogens (Schmidt-Heydt, Rüfer, Raupp, Bruchmann, Perrone, & Geisen, 2011). For these reasons, and given the development of resistance to the standard methods followed to control pathogens, research on the antimicrobial properties of light, and the mechanisms underlying these actions, it is becoming increasingly interesting (Bantis, Smirnakou, Ouzounis, Koukounaras, Ntagkas, & Radoglou, 2018; Dai, Gupta, Huang, Yin, Murray, Vrahas et al., 2013; Ondrusch & Kreft, 2011).

LED blue light (LBL) may control harmful pathogens for consumers, such as *Listeria monocytogenes* (Ondrusch & Kreft, 2011), and other food-relevant fungi (Schmidt-Heydt, Rüfer, Raupp, Bruchmann, Perrone, & Geisen, 2011). Postharvest diseases result in major economic loss in the citrus industry. *Penicillium digitatum* (Pers.:Fr.) Sacc. is the major pathogen that causes fungal decay during citrus fruit storage, and an important problem for its control is the development of resistant strains to synthetic fungicides. Hence because of environment concerns and human health care, there is a growing interest in developing alternative methods to fungicides in fruit (Ballester, Lafuente, De Vos, Bovy, & González-Candelas, 2013; Palou, Smilanick, & Droby, 2008; Zhou, Ma, Xie, Deng, Yao, & Zeng, 2018). In recent years, it has been demonstrated that LBL is able to not only control the growth of this pathogen (Alferez, Liao, & Burns, 2012; Lafuente, Alferez, & González-

Candelas, 2018), but to also induce resistance against the fungus in citrus fruit (Ballester & Lafuente, 2017; Liao, Alferez, & Burns, 2013).

Limited research has been conducted to understand the mechanisms induced by LBL in fruit crops (Yuan, Deng, Yin, Yao, & Zeng, 2017; Zhang, Ma, Yamawaki, Ikoma, Matsumoto, Yoshioka et al., 2015). Likewise, the mechanistic effects by which LBL may reduce disease caused by pathogens are not well-known (Dai et al., 2013; Schmidt-Heydt, Rüfer, Raupp, Bruchmann, Perrone, & Geisen, 2011). Only three studies have investigated the mechanisms induced by LBL treatments in LBL-elicited resistance against *P. digitatum* in citrus fruit peel (Alferez, Liao, & Burns, 2012; Ballester & Lafuente, 2017; Liao, Alferez, & Burns, 2013). The use of profiling technologies, such as omics technologies, is very useful for providing global information about the biochemical features underlying biological cues. Transcriptomic studies on the effect of lights of different center wavelengths on processes of agronomic interest are scarce, and those related to the effect of LBL focus mainly on developmental processes. As far as we know, no study has included a transcriptomic approach to decipher the complex mechanisms induced by LBL in relevant fruit for food nutrition. Light-induced responses may vary with light quality and intensity, and also with fruit crops. This work focused on a LBL treatment that reduces disease caused by postharvest pathogens in citrus fruit (Ballester & Lafuente, 2017) and, hence, helps to reduce the use of chemical fungicides that may pose human health problems. Our goal was to obtain a comprehensive overview of the metabolic pathways affected by LBL in citrus fruit peel. This highlighted the potential of LBL for inducing oxidative stress-related processes, and modifications in the metabolism of lipids, cell wall polysaccharides and of very diverse natural products; i.e., amino acids, alkaloids, phenylpropanoids, carotenoids, and terpenoids.

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80 2. Material and Methods

81 2.1. Fruit material and blue light treatment

82 Mature Lane Late sweet oranges (*Citrus sinensis* (L.) Osbeck) were hand-picked from
83 six adult trees grown under standard conditions in a commercial orchard in Liria (Valencia,
84 Spain). Three hundred and sixty fruit were harvested on March 3 around the whole tree and
85 an equal number of fruit (60 fruit) were picked from each tree. Fruit were immediately
86 delivered to the laboratory. Those fruit void of damage and external defects, and of uniform
87 size and color, were selected, surface-sterilized with commercial bleach for 5 min, and
88 rinsed with water (Ballester & Lafuente, 2017). Fruit were dried at room temperature for 2
89 h and then assigned to two different groups. The fruit in the first group were left in the dark
90 (control fruit), while those in the second group were exposed to LBL treatment. Both
91 groups were kept at 20 °C and 90-95% relative humidity (RH).

92 LBL treatment was selected according to previous results demonstrating its ability
93 to elicit resistance against *P. digitatum* in citrus fruit (Ballester & Lafuente, 2017). Briefly,
94 non inoculated oranges were treated with LBL for 2 days at a quantum flux of 60 $\mu\text{mol m}^{-2}$
95 s^{-1} in Mammoth Pro dark growth tents (60 x 60 x 160 cm) (Mammoth Pro 60, Eltac
96 Hidrofarm, Zaragoza, Spain) inside a temperature-controlled room at 20 °C. To ensure a
97 uniform light quantum flux, tents were equipped with velcro-sealable ventilation panels and
98 tough fabric lined with reflective mylar (Lafuente, Alférez, & González-Candelas, 2018). A
99 LumiGrow Pro 650TM LED array (LumiGrow, Novato, CA, USA) was used for emitting
100 LBL at a center wavelength of 450 nm with a full width at the half-maximum of 20 nm.
101 The light quantum flux was adjusted using a spectroradiometer (GL Spectics, Stuttgart,
102 Germany) (Lafuente, Alférez, & González-Candelas, 2018). The flavedo (outer colored

peel part) samples employed for the analysis were always taken from the control and LBL-treated orange fruit that were not inoculated with the fungus. Samples were taken from freshly harvested (FH) fruit during the light treatment (at 12 h and 24 h) and immediately at the end of this treatment (at 48 h, denoted as 0 days post-treatment, 0 dpt), and 3 days after ending it (3 dpt) (Fig. S1, Supplementary Material). On these 3 days, both the control and LBL-elicited fruit were left in the dark at 20 °C and 90-95% RH. Fruit were infected only to estimate the efficacy of LBL treatment on reducing *P. digitatum* infection, and infection was performed after treating fruit with light (Fig. S1, Supplementary Material).

The fruit from both the control and LBL-treated fruit groups were shorted into two subgroups. The first subgroup was used to take the flavedo samples for the subsequent analysis and contained three replicates of 5 fruit per incubation period. The second subgroup was employed to estimate the efficacy of light treatment in reducing infection. This subgroup included three replicates of 15 fruit to be inoculated with the fungus at 0 dpt, and three other replicates containing the same number of fruit to be inoculated at 3 dpt (Fig. S1, Supplementary Material).

2.2. Fruit infection, fungal material and disease severity

Fruit were inoculated with a 10 µL of a 10^5 conidia mL⁻¹ suspension of *P. digitatum* spores to check the effectiveness of the LBL elicitor treatment on reducing the infection caused by the pathogen at 0 and 3 dpt. To this end, each control and elicited fruit was pricked on the equatorial axis with a 2 mm (diameter) x 1 mm (deepness) sterilized needle immediately before applying the spore suspension. The needle was equipped with a stopper to ensure uniformity of wounds. One wound per fruit for each replicate and treatment was performed and the inoculated fruit were stored at 20 °C and 90–95% RH. *Penicillium*

digitatum (Pers.:Fr.) Sacc. isolate Pd1 (CECT 20795) (Lafuente, Alfrez, & Gonzlez-Candelas, 2018) was used to infect fruit. The conidia suspensions were prepared in sterile distilled water from the 7-day-old cultures grown on potato dextrose agar (PDA) (Thermo Scientific, Wilmington, DE, USA) at 24 C, and then adjusted to a concentration of 10⁵ conidia mL⁻¹ with a hemocytometer. Disease severity was estimated by measuring the diameter of the macerated tissue with a flexible ruler in two directions, and it was expressed as lesion area (cm²).

2.3. Total RNA isolation

Total RNA extraction from 1 g of the previously frozen and homogenized flavedo samples was performed as previously described (Ballester et al; 2011). A NanoDrop ND-1000 spectrophotometer (Thermo Scientific) was used to determine RNA concentration. For RNA integrity evaluations, 1 g RNA was migrated on agarose gel according to Ballester, et al. (2011). RNA quality was confirmed by a Model 2100 Total RNA BioAnalyzer (Agilent Technologies, Madrid, Spain) with the Eukaryote Total RNA Nano kit (Agilent Technologies).

2.4. cDNA labeling and microarray hybridization

The processing of RNA samples for the microarray hybridizations was done by following the manufacturer's two-color protocol (Two-Color Microarray-Based Gene Expression Analysis v. 6.5, Agilent Technologies). Dye incorporation was measured by a UV-VIS spectrophotometer (Nanodrop 1000, Agilent Technologies). Three biological replicate samples from the FH fruit, and from the samples of the control and LBL-treated

non inoculated fruit taken at 0 and 3 dpt, were analyzed with a Custom-made citrus cDNA microarray designed in the laboratory (Romero, Alferez, Establés-Ortiz, & Lafuente, 2020), generated using the Agilent Technologies platform. This microarray contains the 44000 unigenes from *Citrus sinensis* (L.) Osbec to, thus cover the whole genome from this species (<https://phytozome.jgi.doe.gov/Csinensis>).

2.5. Microarray data acquisition and analysis

After hybridization, microarrays were scanned by a DNA Microarray Scanner (Model G2505C, Agilent Technologies). Data acquisition and analyses were performed as previously described (Romero, Alferez, Establés-Ortiz, & Lafuente, 2020). A gene is considered differentially expressed if it displays a BH adjusted p-value below 0.05. The identification of the KEGG metabolic pathways, biological processes (BP) and molecular functions (MF) significantly under- or over-represented in a set of differentially expressed genes (DEGs), in relation to the reference sample, was performed by a hypergeometric analysis using the GOSTats package. Only the DEGs showing at least a 2-fold change in expression were considered in this analysis. A Fisher two-tailed test ($p < 0.05$) was independently performed for the gene ontology analysis of the induced and repressed genes. A Principal Component Analysis (PCA) was performed with the Bioconductor package, and the MapMan software package was used for incorporating DEGs into metabolic pathways.

2.6. Determination of lignin and total anthocyanin and flavonoid contents in flavedo

Lignin content was determined as described by Cajuste and Lafuente (2007). Briefly, freeze-ground flavedo was lyophilized and then washed with water, ethanol,

acetone and diethyl ether, in sequence, until the tissue became colorless. After drying the tissue at 70 °C for 1 h, 20 mg of the resulting powder were digested in a bath at 70 °C for 30 min by shaking with a solution of 25% (w/w) acetyl bromide in acetic acid (2.5 mL) and HClO₄ (70%, 0.1 mL). The digested samples were cooled on ice. Then 10 mL of 2 M NaOH and 12 mL of acetic acid were added. The absorbance of the resulting solution was measured at 280 nm after diluting it 2.5 times with acetic acid. The results of lignin content were expressed as absorbance at 280 nm g⁻¹ of fresh weight. Anthocyanins were determined by a pH differential spectrophotometry method (Rapisarda, Bellomo, Fabroni, & Russo, 2008) by extracting 250 µg of frozen flavedo with 1500 µL of a 80/20 (v/v) mixture of 95% ethanol and 37% HCl. The absorbance of the samples extract was measured at 510 and 530 nm using a Thermo Multiskan Spectrum spectrophotometer (Thermo Scientific). Total flavonoids were determined by following the method described by Lafuente, Alférez, and Romero (2014). Briefly, 250 µg of frozen flavedo were extracted with 1500 µL of ethanol in a Mini Beadbeater 8 Cell Disruptor (Biospec Products, Inc.). The extract was centrifuged at 13000 x g for 5 min at 4 °C and two supernatant aliquots were used to determine the total flavonoid content. Next 100 µL of each supernatant were diluted with 400 µL of water and 30 µL of 5% NaNO₂ were added. The mixture was incubated for 5 min at room temperature, 30 µL of 10% AlCl₃ were added and the reaction was stopped at 6 min with 200 µL of 1 N NaOH. The absorbance at 350 nm was measured and a standard curve was prepared with hesperidin to calculate the flavonoid content in flavedo. All the chemicals were purchased from Sigma-Aldrich (Madrid, Spain). The results are the means of three replicate samples±SEM.

2.7. Statistics

A mean comparison using the Tukey's test was performed to test whether the differences in disease severity, and in lignin and flavonoid contents in flavedo between the control and LBL-treated fruit, were significantly different at $p \leq 0.05$. The Statgraphics Plus 4.0 software (Manugistics, Inc.) was used to perform the analysis. The values represent the mean of three replicate samples $\pm$ SEM.

3. Results and Discussion

The research field on the benefits of LBL for human health and food quality, and in reducing the food losses caused by pathogens, is being paid considerable attention. Nevertheless, information about the mechanistic effects of LBL is still scarce and fragmentary. In this study, we performed a high-throughput analysis coupled to biochemical determinations to obtain a holistic overview of the metabolic changes induced by LBL in citrus fruit peel, which is the main barrier against the stresses causing loss of external fruit quality and rot. LBL-derived consequences may depend on the light quantum flux and on treatment duration. Therefore, we focused on the effect of LBL treatment that helps to control postharvest pathogens (Ballester & Lafuente, 2017) and to, hence, reduce the amount of chemicals needed to avoid fruit loss deriving from pathogen infection in citrus fruit, and the putative health problems or environmental contaminations deriving from the abusive use of fungicides.

*3.1. LBL, applied to a quantum flux that increases fruit resistance to *P. digitatum*, affects multiple metabolic pathways in citrus fruit peel*

Treating Lane Late oranges with LBL at a quantum flux of $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 2 days was very effective in reducing the disease severity caused by *P. digitatum* (Fig. 1A).

Disease reduction in the LBL-elicited fruit was more marked when fruit were inoculated at 3 than at 0 dpt. This result highlights the relevance of the period between the end of light treatment and elicitation of resistance, and/or the mechanisms induced by the combination of light and darkness. Therefore, a comparative transcriptomic profile of the light-treated and untreated fruit was performed at 0 and 3 dpt. This experimental approach (Fig. 1, Supplementary Material) allowed us to know the LBL-induced responses, but also which responses persisted after transferring fruit from light to darkness, or which were produced by the combination of LBL and subsequent darkness. Samples were clustered according to their global gene expression profiles by performing a PCA (Fig. 1B). This analysis showed that the transcriptional profiles of the biological replicates of each sample were closely grouped together, which is indicative of the good repeatability of the microarray data across replications. Marked differences in the gene expression patterns between samples from the fruit exposed for 48 h to LBL treatment (denoted as 0 dpt L) and their respective control samples, left in the dark for the same period (denoted as 0 dpt D), were found. These differences sharply diminished when the LBL-treated fruit were transferred to darkness for 3 days (3 dpt L vs. 3 dpt D). The gene expression patterns of all these samples differed from that of the FH fruit.

The pair-wise comparison was also performed to determine the transcripts that showed significant changes in their expression in response to LBL at 0 and 3 dpt. Bioinformatics programs were used to group these DEGs into different processes and metabolic pathways and to, thus, decipher the most relevant biochemical changes that occurred at 0 and 3 dpt. The number of up- and down-regulated DEGs at $p \leq 0.05$ in the LBL-treated fruit and in their control fruit that were left in the dark in relation to the samples taken from the FH fruit was very high. Therefore, we restricted the analysis to the

DEGs that were induced or repressed by a factor of at least 2 ($1 \geq \log_2 \leq 1$) (Fig. 1C). The pair-wise comparison was also performed between the samples exposed to LBL treatment and their respective controls left in the dark. There were fewer DEGs in the last pair-wise comparison compared to the analyses performed for the FH fruit, and induction prevailed in relation to repression at both 0 and 3 dpt (Fig. 1C). The number of DEGs sharply dropped from 0 to 3 dpt, which indicates that most changes induced by LBL did not persist after transferring fruit to darkness. Many BP (Table 1), MF (Table S1, Supplementary Material), and metabolic pathways (Table 2) were altered by LBL compared to their control samples left in the dark (0 dpt). Most were not maintained after transferring fruit to darkness (3 dpt), while others were induced or repressed during this period. Given their complexity, they were visualized on an overview metabolic map (Fig. 2), were grouped and discussed in the following subsections, and summarized and highlighted in Fig. 3.

3.2. LBL affected light-reaction centers, pigments and iron homeostasis, and induced photo-oxidative-related mechanisms.

LBL treatment induced relevant changes related to photorespiration and light reactions centers to cover photosynthesis, photorespiration and photosynthetic electron transport (Table 1, Fig 2A). It also affected the metabolism of the xanthophylls and carotenoids pigments, which are necessary for absorbing light and converting light energy into chemical products. All this agrees with findings demonstrating that LBL increases total carotenoids, lutein, violaxanthin, β -cryptoxanthin and zeaxanthin during mandarin degreening (Yuan, Deng, Yin, Yao, & Zeng, 2017). Moreover, pigments protect the photosynthetic apparatus from oxidative damage and act as light screens to reduce excess light that leads to photo-oxidative damage in plants (Bantis, Smirnakou, Ouzounis,

[Koukounaras, Ntagkas, & Radoglou, 2018](#)). In line with this, LBL favored the ‘non photochemical quenching’ process (0 dpt), which involves carotenoids and helps to protect photosynthesis in environments where light energy absorption exceeds the light utilization capacity. Interestingly, this was the only LBL-induced process associated with light reaction centers that persisted after transferring fruit from light to darkness (3 dpt) ([Table 1](#)). Some BP over-represented at 0 dpt also included responses to different light types. Although we cannot rule out that some responses might frequently be induced by distinct light types, we should bear in mind that differences in plants’ sensitivity and responses to the light of distinct wavelengths can be influenced by the different sensitivity of light receptors ([Bantis, Smirnakou, Ouzounis, Koukounaras, Ntagkas, & Radoglou, 2018](#)).

The above results indicate that LBL may induce photo-oxidative-related mechanisms, which involves ROS, but also defense mechanisms against the photo-oxidative damage that derives from excess energy by preventing the effect of ROS ([Asada, 2006](#)). In line with this, the over-representation noted at 0 dpt of MF related to glycolate oxidase activity was remarkable because this enzyme is responsible for H₂O₂ accumulation in chloroplasts ([Table S1, Supplementary Material](#)). LBL also induced a set of responses for the purpose of detoxifying ROS. Thus LBL had a marked effect on not only the genes involved in tocopherol biosynthesis, which can quench ¹O₂ and suppress lipid peroxidation by trapping lipid radicals, but also on the ascorbate-glutathione cycle ([Fig. 2](#)), which also plays a key role in ROS homeostasis and detoxification ([Asada, 2006](#)). LBL also increased the expression of the genes encoding alternative oxidase, which prevents O₂ formation, ferredoxin and thioredoxin, whose expression increased by at least 10-fold. Further support for the link between ROS and the LBL-induced responses arises from the fact that the processes involving glutathione (GSH) peroxidase activity ([Table S1, Supplementary](#)

Material), metabolism of glutathione or glutathionylation of proteins were over-represented at 0 dpt (Table 1). Glutathionylation is an early adaptation process that allows cells to survive at sites with high oxidative stress, which arises from the formation of a reversible mixed disulfide between GSH and cysteine–SH groups of proteins.

The up-regulation of the genes encoding different glutathione peroxidases, glutathione S-transferase (GST), which may act as peroxidases or regenerate ascorbate, and of UDP-glucosyl transferases (UDPGT), which participate in both ROS detoxification and phenylpropanoids biosynthesis, was transitory as it occurred at 0 dpt, but not at 3 dpt (Table S2, Supplementary Material). This indicates that LBL-induced oxidative stress may be reversible, while antioxidant glutathione-related proteins and other protective oxidative-stress related responses may participate in the cross protection responses induced by LBL in citrus fruit against subsequent stress caused by *P. digitatum* infection. That is, LBL may induce ROS, which would be harmful to the pathogen, but also to fruit unless fruit develop an array of oxidative-stress protective responses to cope with this highly reactive toxic species. Hence it is remarkable to note that oxidative burst is an early response of plants to pathogen attack that mediates subsequent plant defense responses to cope with pathogen colonization (Hammond-Kosack & Jones, 1996; Tian, Torres, Ballester, Li, Vilanova, & González-Candelas, 2016); and that a LBL treatment that elicited resistance against *Botrytis cinerea* increased the antioxidant system's activity in tomato fruit (Kim, Kook, Jang, Lee, Kamala-Kannan, Chae et al., 2013). According to the results herein obtained, this appears to be the case for LBL-induced protection against *P. digitatum* in citrus fruit as LBL induced abundant antioxidant responses, but also responses associated with ROS generation, which could protect fruit from fungal colonization. These results agree with the fact that LBL may favor oxidative stress (Ballester & Lafuente, 2017), and that the

activation of the antioxidant enzymatic system is involved in elicited resistance against *P. digitatum* in citrus fruit (Ballester, Lafuente, & González-Candelas, 2006). Given the aforementioned LBL-induced responses, and that ascorbic acid (AsA) increases in response to LBL in juice sacs of citrus fruit (Zhang et al., 2015), we were surprised to find an infra-representation of the processes related to AsA metabolism, and also of the response to H₂O₂ at 0 dpt (Table 1). However, of the nine genes included in the ‘AsA metabolic’ process, seven encoded aldehyde dehydrogenase (ALDH) proteins (Table S3, Supplementary Material). This BP was over-represented at 3 dpt (Table 1). These data reflect the relevance of LBL-induced ROS homeostasis and redox balance for citrus fruit peel. Likewise, the response to H₂O₂ process was repressed because of the enrichment in the *FER1* genes encoding ferritin (Table S3, Supplementary Material), which may act as a buffer against iron deficiency and iron overload, and is down-regulated in the presence of an inducer of photo-oxidative stress (<https://www.ncbi.nlm.nih.gov/gene/831720>). Accordingly, LBL repressed the response to iron and cellular iron ion homeostasis BP.

It is remarkable that the oranges transferred from LBL to darkness (3dpt) displayed increased ferrochelatase activity (Table S1, Supplementary Material) and resistance against *P. digitatum* (Fig. 1A). This increase could be produced to cope with excess Fe²⁺, which may lead to the generation of the extremely destructive OH[•] radical from H₂O₂ by the Fenton reaction which can, in turn, initiate self-perpetuating lipid peroxidation (Hammond-Kosack & Jones, 1996). This highly reactive radical may cause major damage to DNA, lipids and proteins, and end in alterations to membranes, whereas photoreactive repair, which involves DNA repair, was affected by LBL treatment at 0 dpt (Table 1). Likewise, we should bear in mind that the availability of Fe²⁺ and other metals in the host is important for the disease development of some pathogens, including *P. digitatum* (Ballester, López-

Pérez, de la Fuente, & González-Candelas, 2019). Indeed some host defense strategies against infection consist of either metal starvation by sequestration or toxicity by the highly concentrated release of metals (Porcheron, Garénaux, Proulx, Sabri, & Dozois, 2013). LBL induced the ‘metal ion binding’ MF at 0 dpt (Table S1, Supplementary Material), which may indicate that metal availability is necessary for *P. digitatum* virulence.

3.3. LBL favors changes in the metabolism of the lipids associated with cell membranes and plant defenses

Besides inducing changes in carotenoids, LBL (0 dpt) affected the metabolism of the lipids associated with cell membranes or those participating in the synthesis of oxylipins. The KEGG analysis (Table 2) revealed that LBL repressed the metabolism of fatty acids (FA), glycerolipids and sphingolipids, as well as of glycosphingolipids (Table 2, in the ‘Glycan biosynthesis and metabolism’ pathway), and increased the expression of the genes encoding FA desaturase proteins (Fig. 2A). The pathways related to the metabolism of not only linoleic and arachidonic acids, but also of glycerolipids and glycerophospholipids, were activated after transferring the LBL-treated fruit to darkness (3 dpt) (Table 2). The above results coincide with previous findings indicating the participation of lipid metabolism in induced resistance against *P. digitatum* in sweet oranges (Ballester et al., 2011; Zhou, Ma, Xie, Deng, Yao, & Zeng, 2018), and also with LBL’s ability to increase phospholipase A₂ (PLA₂) activity, which catalyzes the formation of arachidonic acid and plays a role protecting citrus fruit against *P. digitatum* infection (Alferez, Liao, & Burns, 2012). Hence it is noteworthy that FA like linolenic, linoleic and arachidonic acids react with ROS to favor the production of reactive aldehydes (Hammond-Kosack & Jones, 1996), while LBL increased the expression levels of those genes

participating in the detoxification of the cytotoxic reactive aldehydes that belong to the aldo/keto reductase family protein. Likewise, LBL increased the expression of the genes encoding lipoxygenase at 0 dpt (Table S2, Supplementary Material) which, like PLA₂, occurs in the early steps of the synthesis of a vast diversity of lipid- and aldehyde-derived oxylipins that come from lipid oxidation (Mittler, Vanderauwera, Gollery, & Van Breusegem, 2004). LBL also activated the ‘regulation of fatty acid β-oxidation’ BP (Table 1), which may alter oxylipin profiling and other derivatives involved in plant defense against pathogens, other toxic volatiles and non volatile fatty acid-derived secondary metabolites that could directly attack pathogens (Hammond-Kosack & Jones, 1996). One of these oxylipin-derived volatiles, octanal, increases in the oil glands of Fallglo tangerine when exposed to intermittent LBL (blue/dark, 12 h/12 h 40 μmol m⁻² s⁻¹) (Liao, Alferez, & Burns, 2013).

3.4. Effect of LBL on metabolic events related to cell wall modification

The structural reinforcement of the plant cell wall by either cross-linking various hydroxyproline and proline-rich glycoproteins (HRGPs) to the polysaccharide matrix, or increasing lignification, makes the plant cell more resistant to microbial penetration and enzymatic degradation (Hammond-Kosack & Jones, 1996; Tian, Torres, Ballester, Li, Vilanova, & González-Candela, 2016). These results suggest that LBL favors H₂O₂ generation, up-regulates the genes encoding O-methyltransferases (OMTs) and over-represents the ‘lignin biosynthetic process’ (Table 1; Fig. 2 ‘lignin biosynthesis’). Both events may lead to higher lignin content (Hammond-Kosack & Jones, 1996). Our results (Fig. 4A) revealed that lignin content increased by 12 h in the fruit exposed to LBL. However, this increase is not likely relevant in the LBL-induced resistance against *P.*

digitatum as it was transitory, and no difference in lignin content between the control and the LBL-treated fruit was found when fruit were inoculated with the fungus at 0 or 3 dpt. So it is worth pointing out that plant OMTs constitute a large family of enzymes that methylate the oxygen atom of a variety of secondary metabolites, which leads to the synthesis of lignin, and also of different classes of alkaloids and phenylpropanoids (Buchanan, Grisse, & Jones, 2000; Dixon, Hawkins, Hussey, & Edwards, 2009), including isoflavonoids, flavonols, flavones and anthocyanins, and coumarins like scoparone, which is induced by LBL in citrus fruit peel (Ballester & Lafuente, 2017; Yamaga & Nakamura, 2018). In fact the results indicated the over-representation of the ‘biosynthesis of coumarins, flavonols and stilbene’ (Table 1). These genes also belong to lignan biosynthesis (Fig. 2A), which is closely related to non structural phenolic metabolites, and they play substantive roles in plant defense through their potent biocidal and antioxidant effects (Buchanan, Grisse, & Jones, 2000). Our results also revealed that the down-regulation of the genes encoding HRGPs or extensin prevailed over the up-regulation (Table S2, Supplementary Material) that took place in the LBL-treated fruit at 0 dpt, which were not induced at 3 dpt. Therefore, these results indicate that at the dose applied herein, LBL does not favor the structural reinforcement of the plant cell wall in citrus fruit by HRGPs, extensin proteins, or by increasing lignin content.

Some other results also revealed that LBL has a strong effect on cell wall polysaccharides (Table 1, Fig. 2A). At 0 dpt, the LBL infra-represented processes were involved in the catabolism of glycosaminoglycan and in the biosynthesis of cellulose, which can mediate plant defense responses and the pathogen triggering integrity (Bacete, Mérida, Miedes, & Molina, 2018). These processes are also related to homogalacturonan biosynthesis (Table 1 and Fig. 2A, ‘Cellulose synthesis’), and to the response to chitin

(Table 1). The MAPMAN analysis data also indicated that LBL repressed the genes involved in cell wall degradation, which include different hydrolases that act on O-glycosyl compounds and pectate lyase and polygalacturonase (Fig. 2A, ‘Cell wall degradation’), and up-regulated genes encoding pectinesterases (Fig. 2A, ‘Pectin esterases’). In the context of the present work, it is noteworthy that pectins were considered a source of signaling molecules that play a role in plant defense against biotic and abiotic stresses (Hématy, Cherk, & Somerville, 2009). Moreover, LBL repressed abundant genes encoding xyloglucan endotransglycosidase or expansin (Fig. 2A, ‘Cell wall modification’). Therefore, these results indicate that fruit exposure to LBL (0 dpt) induced abundant changes associated with cell wall modification, some of which could contribute to elicited resistance in citrus fruit peel against *P. digitatum* as they trigger defense responses against pathogens. At 3 dpt, it was remarkable that the processes ‘response to chitin’ and ‘chitin metabolism’ were over-represented (Table 1). In this regard, the marked induction of a gene encoding a basic chitinase after fruit transfer from light to darkness (Table S2, Supplementary Material) deserves our attention because chitinases are pathogenesis-related proteins (PRs) related to the elicitation of resistance against *P. digitatum* in citrus fruit (Ballester et al., 2011).

3.5. LBL affects cell energy, and carbohydrate and amino acid metabolism, leading to the synthesis of secondary metabolites

LBL strongly influenced cell energy, carbohydrate metabolism and the metabolism of several amino acids (Table 1 and 2 and Fig. 2 and Table S1, Supplementary Material), which may lead to the synthesis of diverse secondary metabolites (Buchanan, Grisse, & Jones, 2000). Thus LBL exposure (0 dpt) activated energy-related processes, which

involved AMP or pyruvate metabolic processes, as well as gluconeogenesis (Table 1 and Fig. 2), which can allow plant lipids to be converted into sucrose, and ATP synthesis (Buchanan, Gruissem, & Jones, 2000), while repressed genes involved in fermentation (Fig. 2). The major increases in transcript levels corresponded to the genes encoding glyceraldehyde 3-phosphate dehydrogenase ($\approx$ 10-fold increase) (Table S2, Supplementary Material), which is involved in glycolysis and, thus, serves to break down glucose to produce energy and carbon scaffolds. Our results also highlight LBL's ability to repress the processes related to carbohydrate transport and metabolism, and to the metabolism of starch and sucrose (Tables 1 and 2), which may help citrus fruit to cope with carbon starvation after detachment. Such repression is attenuated in detached fruit by adding ATP, which induces defense responses against pathogens (Romero, Alferez, Establés-Ortiz, & Lafuente, 2020).

LBL activated the tricarboxylic acid cycle (TCA). This cycle participates in cell energetics, the generation of carbon skeletons and the incorporation of inorganic nitrogen into assimilatory amino acid glutamate (Table 1). This occurs by the concerted action of the glutamine synthase/glutamate 2-oxoglutarate aminotransferase (GS/GOGAT) cycle, which was activated by LBL in flavedo (Fig. 2A). In contrast, the biosynthesis of other amino acids like tryptophan, and of subsequent indole glucosinolate- and indole phytoalexin-derivatives, was repressed while fruit were exposed to LBL (Table 1, 0 dpt), and tryptophan metabolism was over-represented after transferring fruit from light to darkness (Table 2, 3 dpt). These results, plus others revealing many changes in the metabolism/catabolism of diverse amino acids, including 'rare amino acids' (Table 2) such as cyanoamino and taurine and hypotaurine metabolisms (0 dpt), or changes in drug or xenobiotic metabolism (3 dpt), point out that complex regulatory networks involving diverse amino acids play a role in

LBL-induced elicitation against *P. digitatum* in citrus fruit. Some may possess antimicrobial properties, whereas others would participate in the synthesis of the subsequent secondary metabolites involved in fruit resistance.

3.6. LBL induces changes in natural products, involving alkaloids, phenylpropanoids and terpenoids

LBL had a marked impact on the three major groups of natural products: terpenoids, phenylpropanoids and alkaloids (Tables 1 and 2, Fig. 2B). Alteration to these metabolic pathways persisted after transferring fruit from light to darkness or were induced by the combined effect of light and darkness at 3 dpt (Table 1 and 2), when a major elicitation of resistance was achieved (Fig. 1A).

In line with the effect of LBL on amino acids, this physical treatment had a marked effect on the metabolism of alkaloids, which mostly derive from an amino acid as a precursor. At 0 dpt, LBL favored the infra-representation of tryptophan metabolism and the drop in the biosynthesis of acridone alkaloids, formed from this aromatic amino acid (Table 2). This alkaloids class, found solely in Rutacea genera, comprises a relatively small group with over 100 known structures isolated from citrus plants (Wink, 2010). At 3 dpt when elicitation of resistance against *P. digitatum* was greater, LBL triggered metabolic pathways, which led to betalains that are structurally related to alkaloids, and include betacyanins and betaxantins (Wink, 2010), and three different alkaloid classes (Table 2; Fig. 2B). One of these alkaloid classes involves the metabolism of caffeine, a purine alkaloid deriving from xanthosine, which has been related to *P. digitatum* infection in citrus fruit (Gonzalez-Candelas, Alamar, Sanchez-Torres, Zacarias, & Marcos, 2010). The second alkaloid subclass, which was over-represented at 3 dpt, has been related to isoquinoline

alkaloid biosynthesis deriving from tyrosine (Wink, 2010). A third one is related to indole alkaloid biosynthesis, which derives from tryptophan (Wink, 2010), whose metabolism was activated at 3 dpt. In this group, alkaloid strictosidine merits special attention because the expression of several genes encoding strictosidine synthase-like proteins increased by at least two-fold after LBL exposure (0 dpt) in flavedo, while the repression of the strictosidine synthase family has been associated with the ability of the yeast *Rhodosporidium paludigenum* to infect citrus fruit (Lu, Wang, Zhu, Lu, Zheng, & Yu, 2015).

In natural products, we identified major changes in the expression of genes and on the processes involved in the synthesis of different classes of phenylpropanoid derivatives, including coumarins, lignin/lignans, stilbene and flavonols, which may share some key genes. Some, like that encoding chalcone and stilbene synthase (CHS) proteins, were highly induced (≈ 23 fold) by LBL (Table S2, Supplementary Material) and may participate in the synthesis of other secondary metabolites like alkaloids. Phenylpropanoids play a relevant role as antioxidants and light screens, and also in the elicitation of resistance against *P. digitatum* in citrus fruit (Ballester et al., 2011; Zhou, Ma, Xie, Deng, Yao, & Zeng, 2018). At 0 dpt, we observed marked increases (> 6 fold) in the expression of two transcription factors, HY5 and MYB111, whose combinatorial action triggered anthocyanin biosynthesis and the over-representation of MF ‘anthocyanidin 3-O-glucosyltransferase activity’ (Table S1, Supplementary Material). We also found marked increases in the expression of the genes coding for different proteins (CHS, 4-coumarate:CoA ligase 3 (4CL3), ferulic acid 5-hydroxylase (FAH), flavanone 3-hydroxylase (F3H), UDPGT and GST) (Table S2, Supplementary Material) involved in the accumulation of this photo-protective pigment (Carmona, Alquézar, Marques, & Peña, 2017). Based on this result, and

510 although anthocyanin levels are negligible in blond oranges (Rapisarda, Bellomo, Fabroni,
511 & Russo, 2008), we studied whether LBL would be able to increase these pigments in the
512 blond orange Lane Late when exposed to LBL. However, no anthocyanins were detected at
513 either 0 or 3 dpt (data not shown), which could be related to the lack of expression of the
514 other key genes in its synthesis like those encoding anthocyanidin synthase (Carmona,
515 Alquézar, Marques, & Peña, 2017). Some of them, along with OMTs and FAH induced by
516 LBL in flavedo, also participate in the synthesis of lignin precursors, such as sinapic acid.
517 However as previously mentioned, changes in lignin were transient and did not parallel the
518 elicitation of resistance against *P. digitatum* (Fig. 4A). These results might be explained by
519 the fact that hydroxycinnamic acids can be easily esterified to the arabinose and galactose
520 units of some pectic polysaccharides, and end in the formation of complex lignans, which
521 can vary from dimers to higher oligomers (Buchanan, Grisse, & Jones, 2000). This idea
522 is plausible as lignans may be induced in response to LBL (Fig. 2), and LBL strongly
523 impacted cell wall modifications, which may release pectic polysaccharides. The genes
524 encoding CHS, 4CL3 and F3H are also important for the synthesis of flavonols. In line with
525 this, given the marked LBL-induced rise (≈ 6) in the accumulation of flavonol synthase
526 (FLS) transcripts, and the fact that LBL did not induce any relevant changes in common
527 flavonoids in citrus fruit flavedo (Ballester & Lafuente, 2017), we cannot rule out that LBL
528 may induce metabolic shifts that lead to the synthesis of flavonols in citrus fruit. LBL did
529 not increase the levels of quercetin and its glycoside rutin (Ballester & Lafuente, 2017),
530 which are common flavonols in citrus fruit flavedo. However, we cannot rule out the
531 synthesis of other less common flavonols and/or their glycosides, or other flavonoid-related
532 compounds like flavanones given the marked LBL induction of a gene coding for chalcone-
533 flavanone isomerase (CFSI). In fact LBL increased total flavonoid content in flavedo (Fig.

4B). LBL also up-regulated the expression of the genes encoding the OMTs required for the synthesis of some coumarins with antifungal activity against *P. digitatum* (Angioni, Cabras, D'Hallewin, Pirisi, Reniero, & Schirra, 1998). Although this situation is not simple, we should consider that thousands of natural products in the phenylpropanoids pathway have been described in nature. Our overall results point out that LBL, if applied at an intensity that elicits resistance against *P. digitatum*, is able to induce complex phenylpropanoid molecules in citrus fruit.

LBL also brought about major changes in the metabolism of terpenoids, which are the most abundant, and structurally the most versatile group of, natural products in plants with about 30000 described compounds (Wink, 2010). Therefore, it was not surprising to find both repressions and inductions in the metabolic pathways of these compounds in response to LBL. Besides favoring the changes involved in the synthesis of carotenoid-related pigments and on tocopherol, LBL induced C5-branched dibasic acid metabolism (Table 2) and other metabolic pathways related to C-21-steroid hormones metabolism. Likewise, LBL repressed thailanol metabolism (Table 1), which has been related to metabolic pathways leading to the degradation of monoterpenes limonene and pinene (Table 2). Therefore, LBL may favor the accumulation of these essential oils, which may be relevant for the elicitation of resistance during fruit exposure to light as both limonene and pinene reduce the spore germination of *P. digitatum* and, hence, the infection caused by this pathogen (Droby, Eick, Macarisin, Cohen, Rafael, Stange et al., 2008; Rodríguez, San Andrés, Cervera, Redondo, Alquézar, Shimada et al., 2011; Tao, Chen, Wu, Wang, Li, & Zhu, 2019). The findings of this work also highlight that LBL is a potent inductor of the terpenoid cyclases/protein prenyltransferases superfamily protein, and of terpene synthase (TP21) (Table S2, Supplementary Material), responsible for the synthesis of caryophyllene,

and that this induction persists after transferring fruit to darkness. This volatile has only a mild effect on the control of *P. digitatum*, but is relevant in agriculture because it controls the spread of bacterial disease citrus Huanglongbing (HLB), which is associated with *Candidatus Liberibacter* spp. This pest severely threatens the production of fruit crops worldwide, for which no cure is yet available. The results indicated that LBL induced complex changes in the metabolism of different terpenoid classes, and some may serve to control the distinct pests that cause major citrus fruit loss.

In the context of this work, it is worth mentioning that some LBL-induced changes may mimic others induced upon pathogen challenge in citrus fruit to cope with infection (Gonzalez-Candelas, Alamar, Sanchez-Torres, Zacarias, & Marcos, 2010; Tang, Chen, Hu, Deng, Chen, & Li, 2018), or when treating fruit with other elicitors of resistance against *P. digitatum* (Ballester et al., 2011). Of these, changes in lipid metabolism, alkaloids, complex phenylpropanoids and terpenoids have been identified and should, therefore, act as good candidates to be involved in the LBL-induced protection of citrus fruit against *P. digitatum*. Therefore, and given the vast diversity and complexity of these natural products, future studies should prioritize focusing on their identification and putative antifungal activity.

4. Conclusions

The overall results indicate that LBL induces complex responses in citrus fruit peel that involve very diverse metabolic pathways. LBL has a key effect on ROS homeostasis, which may participate in the elicitation of resistance against *P. digitatum* in citrus fruit. LBL also favors cell energy and redirects metabolic pathways toward the synthesis of very diverse natural products in primary and secondary metabolisms. These include amino acids, alkaloids, and complex phenylpropanoid- and isoprenoid-derivatives, which can be grouped

as carotenoids and terpenoids. The identification of these complex/rare metabolites, as well as their putative antifungal activity, deserve further research in the near future to identify natural products to help reduce the use of chemical fungicides. As iron homeostasis is also relevant in the elicited resistance, further attention should be paid to the role of iron on the virulence of the pathogen when seeking healthy alternatives to today's fungicides.

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Appendix. Supplementary Material

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Table 1. Gene ontology (GO) analysis (GOSTats, $P < 0.05$) of the biological processes (BP) over- (↑) or infra-represented (↓) in the fruit treated with LBL for 48 h (0 dpt), and from the fruit treated for the same period with light and then transferred for 3 days to darkness (3 dpt) in relation to their controls left in the dark. BP were grouped according to their biochemical function. Three biological replicates per condition were used.

Go Term	Description	0 dpt	3dpt	Go Term	Description	0 dpt	3dpt	Go Term	Description	0 dpt	3dpt
Light reactions				Amino Acids				Hormones			
GO:0009773	photosynthetic electron transport in photosystem I	↑		GO:0006542	glutamine biosynthetic process	↑		GO:0080167	response to karrikin	↑↓	↑
GO:0010196	nonphotochemical quenching	↑	↑	GO:0006563	L-serine metabolic process	↑		GO:0009751	response to salicylic acid stimulus	↓	
GO:0018298	protein-chromophore linkage	↑		GO:0006544	glycine metabolic process	↑		GO:0009753	response to jasmonic acid stimulus	↓	
GO:0010218	response to far red light	↑		GO:0006547	histidine metabolic process	↓	↑	GO:0009690	cytokinin metabolic process	↓	
GO:0010114	response to red light	↑		GO:0006528	asparagine metabolic process	↑		GO:0009696	salicylic acid metabolic process	↓	
GO:0019253	reductive pentose-phosphate cycle	↑		GO:0019482	beta-alanine metabolic process	↓	↑	GO:0010540	basipetal auxin transport	↓	
GO:0080093	regulation of photorespiration	↑		GO:0009065	glutamine family amino acid catabolic process	↑		GO:0009733	response to auxin stimulus	↓	
GO:0010207	photosystem II assembly	↑		GO:0006554	lysine catabolic process	↑		mRNA-related processes			
GO:0010205	photoinhibition	↑		GO:0006550	isoleucine catabolic process	↑		GO:0031124	mRNA 3'-end processing		↑
GO:0010206	photosystem II repair	↑		GO:0006574	valine catabolic process	↑		GO:000956	nuclear-transcribed mRNA catabolic process		↑
GO:0009768	photosynthesis, light harvesting in photosystem I	↑		GO:0006552	leucine catabolic process	↑		Transport and cation response and homeostasis			
GO:0009854	oxidative photosynthetic carbon pathway	↑		GO:0006560	proline metabolic process	↓	↑	GO:0006817	phosphate ion transport		↑
GO:0071483	cellular response to blue light	↑		GO:0006525	arginine metabolic process	↓	↑	GO:0055069	zinc ion homeostasis	↓	
GO:0010304	PSII associated light-harvesting complex II catabolic process	↑		GO:0000162	tryptophan biosynthetic process	↓		GO:0010043	response to zinc ion	↓	
GO:0009903	chloroplast avoidance movement	↑		GO:0009086	methionine biosynthetic process	↓		GO:0006814	sodium ion transport	↓	
GO:0009904	chloroplast accumulation movement	↑		GO:0006534	cysteine metabolic process	↓		GO:0071577	zinc ion transmembrane transport	↓	
GO:0010224	response to UV-B	↑		GO:0009068	aspartate family amino acid catabolic process	↓		GO:0006855	drug transmembrane transport	↓	
GO:0009772	photosynthetic electron transport in photosystem II	↑		Indoles				GO:0006879	cellular iron ion homeostasis	↓	
GO:0015994	chlorophyll metabolic process	↑		GO:0042432	indole biosynthetic process	↑		GO:0010039	response to iron ion	↓	
GO:0010027	thylakoid membrane organization	↑		GO:0009759	indole glucosinolate biosynthetic process	↓		GO:0006857	oligopeptide transport	↓	
GO:0048564	photosystem I assembly	↑		GO:0009684	indoleacetic acid biosynthetic process	↓		Others			
GO:0010362	negative regulation of anion channel activity by blue light	↑		GO:0009700	indole phytoalexin biosynthetic process	↓		GO:0010951	negative regulation of endopeptidase activity		↑
GO:0048579	negative regulation of long-day photoperiodism, flowering	↑		Phenylpropanoids				GO:0019260	1,2-dichloroethane catabolic process	↓	↑
GO:0032544	plastid translation	↑		GO:0009805	coumarin biosynthetic process	↑	↑	GO:0006516	glycoprotein catabolic process		↑
Lipids				GO:0009809	lignin biosynthetic process	↑	↑	GO:0032259	methylation		↑
GO:0031998	regulation of fatty acid beta-oxidation	↑		GO:0051555	flavonol biosynthetic process	↑		GO:0006476	protein deacetylation	↑	
GO:0046486	glycerolipid metabolic process	↓		GO:0009811	stilbene biosynthetic process	↑	↑	GO:0007623	circadian rhythm	↑	
Cuticle and cell wall components				Isoprenoid/triterpenoid derivatives				GO:0000719	photoreactive repair	↑	
GO:0010200	response to chitin	↓	↑	GO:0042572	retinol metabolic process	↑↓	↑	GO:0009704	de-etiolation	↑	
GO:0006032	chitin catabolic process		↑	GO:0008210	estrogen metabolic process	↑		GO:0006352	DNA-dependent transcription, initiation	↑	
GO:0030244	cellulose biosynthetic process	↓		GO:0008209	androgen metabolic process	↑		GO:0035999	tetrahydrofolate interconversion	↑	
GO:0042335	cuticle development	↓		GO:0008207	C21-steroid hormone metabolic process	↑		GO:0009399	nitrogen fixation	↑	
GO:0052544	defense response by callose deposition in cell wall	↓		GO:0006699	bile acid biosynthetic process	↑↓	↑	GO:0048441	petal development	↓	
GO:0010289	homogalacturonan biosynthetic process	↓		GO:0016117	carotenoid biosynthetic process	↑		GO:0010214	seed coat development	↓	
GO:0006027	glycosaminoglycan catabolic process	↓		GO:0016122	xanthophyll metabolic process	↑		GO:0009835	fruit ripening	↓	
GO:0043447	alkane biosynthetic process	↓		GO:0016132	brassinosteroid biosynthetic process	↓		GO:0001887	selenium compound metabolic process	↓	
Energy				GO:0080003	thalianol metabolic process	↓		GO:0009641	shade avoidance	↓	
GO:0006108	malate metabolic process	↑		GO:0010263	tricyclic triterpenoid biosynthetic process	↓		GO:0048443	stamen development	↓	
GO:0006090	pyruvate metabolic process	↑		GO:0010686	tetracyclic triterpenoid biosynthetic process	↓		GO:0072529	pyrimidine-containing compound catabolic process	↓	
GO:0006097	glyoxylate cycle	↑		GO:0019745	pentacyclic triterpenoid biosynthetic process	↓		GO:2000037	regulation of stomatal complex patterning	↓	
GO:0006096	glycolysis	↑		GO:0046251	limonene catabolic process	↓	↑	GO:2000038	regulation of stomatal complex development	↓	
GO:0006167	AMP biosynthetic process	↑		Response to biotic and abiotic stress				GO:0006120	mitochondrial electron transport, NADH to ubiquinone	↓	
GO:0019643	reductive tricarboxylic acid cycle	↑		GO:0042742	defense response to bacterium	↑		GO:0060147	regulation of posttranscriptional gene silencing	↓	
GO:0006094	gluconeogenesis	↑		GO:0009627	systemic acquired resistance	↓		GO:0008361	regulation of cell size	↓	
Carbohydrate metabolism, transport and response				GO:0009611	response to wounding	↓	↑	GO:0048366	leaf development	↓	
GO:0006000	fructose metabolic process	↑		GO:0009682	induced systemic resistance	↓		GO:0000187	activation of MAPK activity	↓	
GO:0006013	mannose metabolic process	↑		GO:0009624	response to nematode	↓					
GO:0015976	carbon utilization	↑		GO:0050832	defense response to fungus	↓					
GO:0009744	response to sucrose stimulus	↑		GO:0009409	response to cold	↑↓					
GO:0009915	phloem sucrose loading	↑		GO:2000070	regulation of response to water deprivation	↓					
GO:0005982	starch metabolic process	↓		Oxidative stress							
GO:0005985	sucrose metabolic process	↓		GO:0010731	protein glutathionylation	↑					
GO:0015798	myo-inositol transport	↓		GO:0006749	glutathione metabolic process	↑					
GO:0006011	UDP-glucose metabolic process	↓		GO:0019852	L-ascorbic acid metabolic process	↓	↑				
GO:0008643	carbohydrate transport	↓		GO:0042542	response to hydrogen peroxide	↓					

Table 2. Metabolic pathways identified as over- (↑) or infra-represented (↓) by the KEGG analysis in the flavedo of the fruit treated with LBL (60 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 48 h (0 dpt), and from those treated for the same period in the light and then transferred for 3 days to darkness (3 dpt) in relation to their controls left continuously in the dark. Three biological replicates from each condition were used.

KEGG Pathway Map	Metabolic pathway	0 dpt	3 dpt
Carbohydrate metabolism			
map00660	C5-Branched dibasic acid metabolism	↑	
map00500	Starch and sucrose metabolism	↓	
map00040	Pentose and glucuronate interconversions	↓	↑
map00640	Propanoate metabolism	↓	
map00053	Ascorbate and aldarate metabolism	↓	
Metabolism of other amino acid			
map00460	Cyanoamino acid metabolism	↑↓	
map00430	Taurine and hypotaurine metabolism	↑	
map00473	D-Alanine metabolism	↑	
map00472	D-Arginine and D-ornithine metabolism	↑	
map00410	beta-Alanine metabolism	↓	
Glycan biosynthesis and metabolism			
map00511	Other glycan degradation	↑↓	
map00604	Glycosphingolipid biosynthesis - ganglio series	↓	
map00531	Glycosaminoglycan degradation	↓	
Metabolism of terpenoids and polyketides			
map00900	Terpenoid backbone biosynthesis	↑	
map04660	T cell receptor signaling pathway	↑	
map00903	Limonene and pinene degradation	↓	
Lipid metabolism			
map01212	Fatty acid metabolism	↓	
map00561	Glycerolipid metabolism	↓	↑
map00600	Sphingolipid metabolism	↓	
map00121	Secondary bile acid biosynthesis	↓	
map00591	Linoleic acid metabolism		↑
map00564	Glycerophospholipid metabolism		↑
map00590	Arachidonic acid metabolism		↑
map00140	Steroid hormone biosynthesis		↑
Amino acid metabolism			
map00340	Histidine metabolism	↓	↑
map00380	Tryptophan metabolism	↓	↑
map00310	Lysine degradation	↓	
Metabolism of cofactors and vitamins			
map00830	Retinol metabolism	↓	↑
Biosynthesis of other secondary metabolites			
map00940	Phenylpropanoid biosynthesis	↓	
map01058	Acridone alkaloid biosynthesis	↓	
map00965	Betalain biosynthesis		↑
map00901	Indole alkaloid biosynthesis		↑
map00232	Caffeine metabolism		↑
map00950	Isoquinoline alkaloid biosynthesis		↑
Xenobiotics biodegradation and metabolism			
map00983	Drug metabolism - other enzymes	↓	
map00625	Chloroalkane and chloroalkene degradation	↓	
Metabolism of other amino acid			
map00982	Drug metabolism - cytochrome P450		↑
map00980	Metabolism of xenobiotics by cytochrome P450		↑

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Figure Captions

Figure 1. Macerated area (cm^2) of infected fruit (A), Principal Component Analysis (PCA) (B), and number of DEGs ($p < 0.05$) (C) in the flavedo of Lane Late oranges treated for 48 h with $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ LBL (0 dpt) before being transferred to darkness for 3 days (3dpt) in relation to their untreated control samples maintained for the same period in darkness. Macerated area values at 4 and 7 d post-inoculation are the means of three replicates $\pm$ SEM. Different letters in Fig. 1A denote significant differences ($p \leq 0.05$) between the light-treated and the control fruit for the same analysis day; black and gray bars correspond to both the control fruit left in the dark and the LBL-treated fruit, respectively. PCA was based on the large-scale transcriptional profiles of the DEGs satisfying an ANOVA test ($p < 0.05$) for all the represented conditions: FH: Freshly harvested fruit; 0 dpt L: LBL-treated fruit at 0 dpt; 0 dpt D: control fruit left in the dark at 0 dpt; 3 dpt L: LBL-treated fruit transferred to darkness for 3 days; 3 dpt D: control fruit left in the dark of the 3 dpt L samples. To visualize Fig. 1B's color, readers are referred to the web version of this article. Only the DEGs showing at least a 2-fold change in expression are included.

Figure 2. Metabolic overview (A) and secondary metabolism (B) using MapMan when comparing transcript accumulation in the flavedo of the fruit treated for 48 h with $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ LBL (0 dpt) in relation to their untreated control samples. The control fruit were continuously left in the dark. Red and blue squares represent the genes with decreasing and increasing transcript levels in the treated fruit, respectively, vs. the control samples. The color scale is indicated in the figure and is expressed as logFC (fold change in expression shown as $\log_2\text{FC}$). To visualize these colors, readers are referred to the web version of this article.

Figure 3. Schematic integration of the metabolic changes induced by LBL (0 dpt) and the combination of light+darkness (3 dpt). Blue arrows and pink lines indicate induction and repression, respectively. To visualize these colors, readers are referred to the web version of this article.

765 **Figure 4.** Changes in the total lignin (A) and flavonoids (B) content in the flavedo of the
766 Lane Late oranges treated for up to 48 h with $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ LBL (0 dpt) and then
767 transferred to darkness for 3 days ($\circ$). The control fruit ($\bullet$) were left continuously in the
768 dark. The values are the means of three replicates $\pm$ SEM. Asterisks indicate significant
769 differences ($p \leq 0.05$) between the control fruit and those treated with LBL for the same
770 analysis day.

Fig. 1

Fig. 2

Figure 3

Fig. 4

Table S3, Supplementary Material. DEGs included in the ‘Response to hydrogen peroxide’ and in the ‘L-ascorbic acid metabolism’ processes repressed by the LBL treatment at 0 dpt.

Response to hydrogen peroxide			
SystematicName	TAIR10symbol	TAIR10define	Fold-repression
orange1.1g017954m	BT1	BTB and TAZ domain protein 1	-2.025840069
orange1.1g021905m	BT1	BTB and TAZ domain protein 1	-2.027044708
orange1.1g023956m	ATFER1,FER1	ferretin 1	-2.290461759
orange1.1g024844m	ATFER1,FER1	ferretin 1	-2.202934596
orange1.1g028161m	ATFER1,FER1	ferretin 1	-2.224068747
orange1.1g031791m	CML23	calmodulin like 23	-2.426285198
orange1.1g035557m		UDP-Glycosyltransferase superfamily protein	-2.564415237
orange1.1g037646m	ATBT4,BT4	BTB and TAZ domain protein 4	-2.87675834
L-ascorbic acid metabolism			
SystematicName	TAIR10symbol	TAIR10define	Fold-repression
orange1.1g008366m	LAC7	laccase 7	-2.01789574
orange1.1g009035m	ALDH2B,ALDH2B7	aldehyde dehydrogenase 2B7	-2.6742336
orange1.1g009054m	sks5	SKU5 similar 5	-14.70717497
orange1.1g012658m	ALDH2,ALDH2A,ALDH2B4	aldehyde dehydrogenase 2B4	-4.390416868
orange1.1g012802m	ALDH2,ALDH2A,ALDH2B4	aldehyde dehydrogenase 2B4	-3.822593839
orange1.1g014515m	ALDH2B,ALDH2B7	aldehyde dehydrogenase 2B7	-4.18501009
orange1.1g016240m	ALDH2,ALDH2A,ALDH2B4	aldehyde dehydrogenase 2B4	-4.045970191
orange1.1g017060m	ALDH2B,ALDH2B7	aldehyde dehydrogenase 2B7	-4.058872009
orange1.1g020284m	ALDH2B,ALDH2B7	aldehyde dehydrogenase 2B7	-4.172269179