New Phytologist

Research

Phytochrome B regulates reactive oxygen signaling during abiotic and biotic stress in plants

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Received: *30 September 2022*
Accepted: *11 November 2022*

New Phytologist (2023) **237**: 1711–1727
doi: 10.1111/nph.18626

Key words: acclimation, phytochrome, reactive oxygen species, respiratory burst oxidase homolog, signal transduction, stress.

Summary

- Reactive oxygen species (ROS) and the photoreceptor protein phytochrome B (phyB) play a key role in plant acclimation to stress. However, how phyB that primarily functions in the nuclei impacts ROS signaling mediated by respiratory burst oxidase homolog (RBOH) proteins that reside on the plasma membrane, during stress, is unknown.
- *Arabidopsis thaliana* and *Oryza sativa* mutants, RNA-Seq, bioinformatics, biochemistry, molecular biology, and whole-plant ROS imaging were used to address this question.
- Here, we reveal that phyB and RBOHs function as part of a key regulatory module that controls apoplastic ROS production, stress-response transcript expression, and plant acclimation in response to excess light stress. We further show that phyB can regulate ROS production during stress even if it is restricted to the cytosol and that phyB, respiratory burst oxidase protein D (RBOHD), and respiratory burst oxidase protein F (RBOHF) coregulate thousands of transcripts in response to light stress. Surprisingly, we found that phyB is also required for ROS accumulation in response to heat, wounding, cold, and bacterial infection.
- Our findings reveal that phyB plays a canonical role in plant responses to biotic and abiotic stresses, regulating apoplastic ROS production, possibly while at the cytosol, and that phyB and RBOHD/RBOHF function in the same regulatory pathway.

Introduction

Light is indispensable for plants serving as an energy source, developmental signal, and regulator of many different responses to environmental cues and stresses. However, light can come in different intensities and qualities that can rapidly fluctuate. To hone their responses to light, and become as efficient as possible in harnessing it, plants utilize several different light receptors and signal transduction pathways that control multiple biochemical, physiological, metabolic, and molecular mechanisms, and allow them to successfully adapt to changes in light conditions (Quail, 2002, 2021; Legris *et al.*, 2019; Klose *et al.*, 2020; Cheng *et al.*, 2021; Gallé *et al.*, 2021; Kathare & Huq, 2021; Chen *et al.*, 2022; Li *et al.*, 2022).

While light energy is harvested by chloroplasts that can acclimate to changes in light conditions using different biochemical and retrograde mechanisms (Kovács *et al.*, 2006; Johnson *et al.*, 2011; Nath *et al.*, 2013; Kirchhoff, 2014; Wolf *et al.*, 2020; Mittler *et al.*, 2022), changes in light intensity and quality are sensed by light receptors, such as phytochromes (phys), cryptochromes (crys), UV-B resistance 8 (UVR8), and other proteins that regulate transcriptomic responses of plants (Liscum *et al.*, 2020; Cheng *et al.*, 2021). In addition to regulating photosynthesis and linking light cues with different developmental and environmental responses, some of the pathways described above evolved to prevent overloading of the photosynthetic apparatus during conditions of excess light stress, which could result in the unregulated production of potentially damaging reactive oxygen species (ROS; Exposito-Rodriguez *et al.*, 2017; Waszczak *et al.*, 2018; Smirnoff & Arnaud, 2019; Foyer & Hanke, 2022; Mittler *et al.*, 2022).

Most of the ROS accumulating in plant cells during excess light stress are thought to originate in chloroplasts and mitochondria, and in C_3 plants, in peroxisomes as well (Asada, 2006; Estavillo *et al.*, 2011; Kerchev *et al.*, 2016; Exposito-Rodriguez *et al.*, 2017; Mittler, 2017; Waszczak *et al.*, 2018; Shapiguzov *et al.*, 2019; Smirnoff & Arnaud, 2019; Foyer & Hanke, 2022). New findings in Arabidopsis (*Arabidopsis thaliana*) and rice

(*Oryza sativa*) revealed, however, that during excess light stress, some of the ROS that accumulate in leaf mesophyll and bundle sheath cells are produced at the apoplast by respiratory bust oxidase homologs (RBOHs; Devireddy *et al.*, 2020; Xiong *et al.*, 2021). This more recent work suggests that although $O_2^{\bullet-}$ and H_2O_2 are produced in the chloroplast, mitochondria, and peroxisomes during light stress, ROS, which could be important for stress signaling, is also produced in the apoplast by RBOHs. It was further proposed that H_2O_2 that is produced in the apoplast during light stress diffuses into the cytosol *via* aquaporins and alters the redox state of different proteins triggering signal transduction pathways (Mittler *et al.*, 2022). Alternatively, ROS produced in the apoplast could trigger signal transduction pathways in the cytosol *via* plasma membrane (PM)-localized apoplastic receptors such as HYDROGEN-PEROXIDE-INDUCED Ca^{2+} INCREASE 1 (HPCA1; Kimura *et al.*, 2020; Wu *et al.*, 2020; Fichman *et al.*, 2022). The highly regulated function of RBOHs, which generate $O_2^{\bullet-}$ (that subsequently dismutates to H_2O_2) at the apoplast in response to many different developmental-, abiotic-, mechanical-, and pathogen-derived signals (Fichman & Mittler, 2020; Lee *et al.*, 2020; Castro *et al.*, 2021; Mittler *et al.*, 2022), could therefore be utilized for ROS production and signaling during excess light stress. Moreover, this process was shown to occur during excess light stress even in etiolated plants that contain undeveloped chloroplasts that do not conduct photosynthesis (Xiong *et al.*, 2021). These tantalizing results highlight a new question of how is light sensed during excess light stress to trigger an apoplastic ROS production response?

A candidate regulator and/or receptor that could lead to ROS production by RBOHs during excess light stress is phyB. Phytochrome B is found in its inactive form in the cytosol and is mobilized to the nucleus upon sensing of red light. Once in the nuclei, phyB is involved in the transcriptional regulation of many light response genes through interactions with several different transcriptional regulators, such as PHYTOCHROME-INTERACTING TRANSCRIPTION FACTORS (PIFs; Kathare & Huq, 2021; Quail, 2021; Chen *et al.*, 2022; Li *et al.*, 2022). In Arabidopsis and tomato (*Solanum lycopersicum*), phyB was found to be required for systemic stomatal responses, systemic ROS accumulation, and systemic photosynthetic regulation, in response to a local treatment of high light stress (Guo *et al.*, 2016; Devireddy *et al.*, 2020). Here, we report that phyB and RBOHs function as part of a key regulatory module that controls apoplastic ROS production, transcript expression, and plant acclimation in response to excess light stress. We further show that phyB is required for apoplastic ROS production during excess light stress even if it is restricted to the cytosol and that phyB, RBOHD, and RBOHF coregulate 1000s of transcripts in response to excess light stress. Remarkably, we found that phyB is also required for ROS accumulation in response to heat, wounding, cold, and bacterial infection. Taken together, our findings suggest that phyB plays a pivotal role in plant responses to biotic and abiotic stresses, regulating ROS production, and that phyB and RBOHs likely function in the same regulatory pathway.

Materials and Methods

Plant material and growth conditions

Wild-type *A. thaliana* (L.) Heynh. (cv Columbia and Landsberg), *rbohD* (AT5G47910; Torres *et al.*, 2002), *rbohF* (AT1G64060; Torres *et al.*, 2002), *rbohD rbohF* (Kwak *et al.*, 2003), *phyB-5*, *phyB-9* (AT2G18790; Reed *et al.*, 1993), *phyB-5* YHB (*phyB*^{Y276H}; Fischer *et al.*, 2005; Su & Lagarias, 2007; Hu *et al.*, 2009), and *phyB-9* PHYB-GR1 (Huq *et al.*, 2003) were used (the *phyB* YHB mutant was backcrossed to eliminate the *phyA* mutation; Fischer *et al.*, 2005; Su & Lagarias, 2007; Hu *et al.*, 2009). Four-week-old plants were grown on peat pellets (Jiffy-7; Jiffy International, Kristiansand, Norway), at 21°C, under 10 h : 14 h, light : dark (50 µmol photons s^{-1} m^{-2}) conditions. To study green or etiolated seedlings, seeds from the above genotypes were germinated and grown on ½ Murashige & Skoog media (Caisson Labs, Smithfield, UT, USA) for 7 d, before light stress and ROS quantification. Plates were supplemented with 0.1 mM dexamethasone (DEX; Sigma-Aldrich, St Louis, MO, USA) for *phyB* PHYB-GR1 experiments. For acclimation experiments with and without DEX treatments, seedlings were grown in liquid cultures of ¼ Murashige & Skoog in six-well plates. Rice (*O. sativa* L.) plants studied included Nipponbare WT, *OsphyB*, *OsrbohA-1*, and *OsrbohB* (Xiong *et al.*, 2021). Rice plants were grown on peat pellet for 3 wk in 16 h : 8 h, light : dark (100 µmol photons s^{-1} m^{-2}) conditions.

Stress treatments

Whole plants were subjected to light stress for 10 or 50 min. White light stress (740 µmol photons s^{-1} m^{-2}; Supporting Information Fig. S1a) was applied using an LED array (Bestva, Commerce, CA, USA) and covered a spectrum of 300–800 nm, while red light stress (120 µmol photons s^{-1} m^{-2}; Fig. S1b) was applied using an LED array (Duostrip I033 light diodes array; LEDdynamics, Randolph, VT, USA) with a spectrum of 600–700 nm in a dark room. Paraquat (PQ; 10^{-6} M) was applied by fumigation of plants for 30 min. Plants were then subjected to a 10-min treatment of ambient (50 µmol photons s^{-1} m^{-2} white light), or white-, or red light stresses, as described above. For dark control, fumigation and 10-min incubation were conducted in a dark room. The effect of different red light intensities on ROS accumulation was measured following dark incubation of plants for 2 h and treatment with 8, 25, 50, or 120 µmol photons s^{-1} m^{-2} of red light using the LED array described above. Reactive oxygen species measurements of systemic stress signaling were performed as described previously (Fichman *et al.*, 2019; Zandalinas *et al.*, 2020a,b), following a 2-min local light stress treatment (1700 µmol photons s^{-1} m^{-2}) generated by a ColdVision fiber-optic LED light source (Schott, Mainz, Germany). Wounding was applied by simultaneously injuring a single leaf with 30 dresser-pines (Fichman *et al.*, 2019), cold stress was applied by placing an ice cube on a single leaf for 2 min (Jung *et al.*, 2016), heat stress was induced by placing a heat block 2 cm away from the treated leaf (Zandalinas *et al.*, 2020a), and

pathogen attack was applied by dipping a single leaf in a tube with *Pseudomonas syringae* DC3000 in phosphate buffer (for mock treatment, the leaf was dipped in a tube with the buffer without the bacteria; Fichman *et al.*, 2019).

Whole-plant live ROS imaging, H_2O_2 and chlorophyll quantification, plant acclimation, and stomatal aperture measurements

Reactive oxygen species accumulation was measured as described previously (Fichman *et al.*, 2019). Plants were fumigated with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) solution (50 μM H_2DCFDA (Sigma-Aldrich), 0.001% Silwet L-77 (Sigma-Aldrich) in 50 mM phosphate buffer pH 7.4) for 30 min in a glass container placed in a chemical hood, using a medical nebulizer (Punasi Direct, Hong Kong, China; Fichman *et al.*, 2019). For PQ treatments, PQ (10^{-6} M) was included with the H_2DCFDA solution. Due to the toxicity of PQ, fumigation with PQ was also performed in a glass container placed in a chemical hood, and handling of plants was carried out with personal protective equipment. Internalized and oxidized DCF fluorescence (ex./em. 480 nm/520 nm) was measured using the IVIS Lumina S5 platform (PerkinElmer, Waltham, MA, USA). Accumulation of oxidized DCF was calculated using the Ivis Living Image 4.7.2 software (PerkinElmer). Hydrogen peroxide quantification was performed with Amplex-Red (10-acetyl-3,7-dihydroxyphenoxazine; ADHP; Thermo Fisher Scientific, Waltham, MA, USA) as described by Fichman *et al.* (2022). Chlorophyll content was measured as described previously (Balfagón *et al.*, 2019; Zandalinas *et al.*, 2020a). Acclimation was performed as described previously (Zandalinas *et al.*, 2020a; Fichman *et al.*, 2021). Acclimation of DEX-treated and untreated seedlings was performed with 1-wk-old seedlings grown in liquid ¼ Murashige & Skoog media (Caisson Labs). Seedlings were subjected to the same light treatments as described above, and conductivity was measured in the liquid growth media. Stomatal aperture measurements were taken as described previously (Devireddy *et al.*, 2020).

RNA extraction and transcript expression analysis

Sixty leaves were pooled from 15 different plants for each biological repeat (three biological repeats were used for each time point and genotype) of WT, *phyB-9*, *rbohD*, or *rbohF* subjected to 0 or 10-min white light or red light, and frozen in liquid nitrogen before grinding and isolation of RNA using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Isolated RNA was used as template for cDNA synthesis using PrimeScript RT Reagent Kit (TaKaRa Bio, Kusatsu, Japan). Transcript expression was quantified by qRT-PCR using iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA, USA). RNA sequencing was performed by Novogene (Sacramento, CA, USA) using NovaSeq 6000. Read quality control was performed using FastQC v.1.20.0 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), followed by alignment of reads onto the Arabidopsis reference genome (Berardini *et al.*, 2015; https://www.arabidopsis.org/) using Star aligner v.2.4.0.1 (https://github.com/alexdobin/STAR) and analysis of differential gene expression using DESeq2 v.1.20.0 (Love *et al.*, 2014; Zandalinas *et al.*, 2020a; https://bioconductor.org/packages/release/bioc/html/DESeq2.html). The genome index was built using TAIR 10. Differentially abundant transcripts were defined as those that have a log fold change with an adjusted $P<0.05$ (negative binomial Wald test followed by a Benjamini–Hochberg correction, both integral to the DESeq2 package). Venn diagram overlap was subjected to hypergeometric testing using phyper. Transcript overlap with different stress-, hormone-, and ROS-response transcripts was generated as described previously (Zandalinas *et al.*, 2019, 2020a; Fichman *et al.*, 2020). The overlap between gene lists was calculated with an in-house script available at https://github.com/sohamsg90/RNA-Seq-perl-scripts. Gene ontology (GO) annotation of transcripts was performed as described previously (Zandalinas *et al.*, 2019, 2020a; Fichman *et al.*, 2020). In addition, the GO application tool (http://bioinformatics.sdstate.edu/go/) was used to identify transcripts that can be assigned to specific categories with a false discovery rate (FDR) of $P<0.05$ (Ge *et al.*, 2020). RNA-Seq data files were deposited in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under the accession no. GSE188732.

Nuclear protein enrichment and immunoblotting

Seedlings (1 g) from WT, *phyB-9*, and *phyB-9* PHYB-GR1 without DEX or *phyB-9* PHYB-GR1 with DEX were collected and flash frozen in liquid nitrogen. Nuclei were isolated as described (Calikowski & Meier, 2006). Protein concentration was measured using the Pierce 660nm Protein Assay (Thermo Fisher Scientific), and 10 μg protein samples were denatured in Laemmli buffer and separated on 8.5% SDS-PAGE (Suzuki *et al.*, 2013). Proteins were blotted to PVDF membranes and incubated with phyB primary antibodies (PHY1733; PhytoAB, San Jose, CA, USA) or Histon H3 primary antibodies (PHY2460A; PhytoAB), followed by incubation with HRP-conjugated secondary antibodies goat antimouse (for phyB AB) or goat antirabbit (for Histon H3 AB; Jackson ImmunoResearch Laboratories, West Groove, PA, USA). Chemiluminescence was detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) in the IVIS Lumina S5 platform (PerkinElmer).

Transmission electron microscopy

Leaves of 4-wk-old *phyB-9* and WT plants were processed for transmission electron microscopy (TEM) as described (Fichman *et al.*, 2021). Sections were cut to a thickness of 80 nm using an ultramicrotome (Ultracut UCT; Leica Microsystems, Wetzlar, Germany). Images were acquired with a Jeol JEM 1400 TEM at 80 kV on a Gatan Ultrascan 1000 charge-coupled device camera (Gatan Inc., Plesanton, CA, USA). The numbers and size of chloroplasts in different mesophyll cells were quantified using ImageJ. Preparation of the samples and imaging were performed at the Electron Microscopy Core facility at the University of Missouri.

Statistical analysis

All experiments were repeated at least three times with at least three biological repeats. Box plots graphs are presented with mean as $X \pm$ SE; median is line in the box, and box borders are 25th and 75th percentiles; whiskers are the 1.5 interquartile range. ANOVA was followed by a Tukey's *post hoc* test. Different letters denote statistical significance at $P < 0.05$.

Results

PhyB, RBOHD, and RBOHF regulate ROS accumulation, physiological responses, and plant acclimation to excess light stress

To study the role of phyB in ROS production during excess light stress, we subjected WT plants and *phyB-9*, *rbohD*, *rbohF*, and *rbohD rbohF* mutants to an excess light stress treatment (740 μmol photons s^{-1} m^{-2}) for 10 min and measured ROS accumulation in whole plants grown in soil using our newly developed live ROS imaging method (Fichman *et al.*, 2019). This method uses the cell-permeant H_2DCFDA that detects a wide range of different ROS including H_2O_2 (Fichman *et al.*, 2019). In addition to white light, we also subjected plants to red light (120 μmol photons s^{-1} m^{-2}) using a light intensity that is proportional to the fraction of red light included within our white light treatment (Devireddy *et al.*, 2020). As shown in Fig. 1(a), treatment with excess white or red light resulted in ROS accumulation in WT plants, but not in *phyB-9*, *rbohD*, *rbohF*, or *rbohD rbohF* mutants. To study the impact of excess light stress on ROS production in plants that do not contain photosynthetically active chloroplasts, we used etiolated seedlings. As shown in Fig. 1(b), treatment of etiolated WT seedlings with white or red light resulted in ROS accumulation, while treatment of etiolated *phyB-9*, *rbohD*, *rbohF*, or *rbohD rbohF* seedlings did not. Interestingly, the amount of ROS produced in mature green plants treated with white light was higher than that of mature green plants treated with red light, while this difference was not observed in etiolated seedlings (Fig. 1a,b). This finding suggests that some ROS could be produced by photosynthetically active chloroplasts of mature green plants in response to white light. However, even in green plants, this amount of ROS production was completely abolished in the *rbohD rbohF* double mutant (Fig. 1a), suggesting that even this residual ROS production that could result from chloroplasts or differences in intracellular NADPH levels is under the control of RBOHs. Because DCF detects a broad range of different ROS, we also measured the levels of H_2O_2 that accumulate in green and etiolated WT, *phyB-9*, *rbohD*, and *rbohF* seedlings (Fig. 2). This analysis revealed that H_2O_2 accumulated in WT but not *phyB-9*, *rbohD*, and *rbohF* seedlings in response to light stress. In addition, because 120 μmol photons s^{-1} m^{-2} of red light could also cause ROS production in chloroplasts, we measured the production of ROS in WT, *phyB-9*, *rbohD*, and *rbohF* seedlings treated with 8, 25, 50, or 120 μmol photons s^{-1} m^{-2} of red light for 10 min (Fig. S2). This analysis revealed that even 8 μmol photons s^{-1} m^{-2} of red light was sufficient to trigger ROS production in WT seedlings (but not *phyB-9*, *rbohD*, and *rbohF* seedlings), supporting a role for PhyB in triggering ROS production during light stress.

To test the effect of phyB, RBOHD, and RBOHF on physiological responses of plants to light stress, we measured the stomatal aperture closure response of plants to excess light stress (Devireddy *et al.*, 2018; Balfagón *et al.*, 2019; Zandalinas *et al.*, 2020a). As shown in Fig. S3, treatment of mature green WT plants with excess white or red light for 10 min resulted in stomatal aperture closure. By contrast, treatment of *phyB-9*, *rbohD*, *rbohF*, or *rbohD rbohF* mutants did not. As the absence of ROS accumulation (Figs 1a, 2) and stomatal responses (Fig. S3) could lead to an impaired acclimation of plants to excess light stress (Suzuki *et al.*, 2013; Devireddy *et al.*, 2018; Zandalinas *et al.*, 2020a), we measured the acclimation (i.e. reduced tissue damage following exposure to light stress) of mature WT, *phyB-9*, *rbohD*, *rbohF*, and *rbohD rbohF* plants to a prolonged excess white light treatment following a short pretreatment with excess white or red light and an incubation period. As shown in Fig. 1(c), pretreatment of WT plants with 10 min of excess white or red light, followed by an incubation of 50 min under controlled growth conditions, protected plants from a subsequent exposure to 45-min excess white light (i.e. prevented leaf injury as measured by electrolyte leakage, compared with plants that were subjected to the 45-min excess light treatment without a 10-min pretreatment with excess white or red light). By contrast, pretreatment of *phyB-9*, *rbohD*, *rbohF*, or *rbohD rbohF* plants with excess white or red light failed to induce plant acclimation to a subsequent prolonged excess white light stress (Fig. 1c). The findings presented in Figs 1, 2, S2, and S3 demonstrate that phyB, RBOHD, and RBOHF are essential for ROS accumulation, physiological responses, and plant acclimation to excess light stress.

Since *phyB-9* mutants could display alterations in chloroplast structure, function, and/or number, potentially impacting ROS production during light stress, we subjected WT and *phyB-9* plants to TEM analysis. As shown in Fig. S4, the structure and number of chloroplasts from the *phyB-9* mutant were similar to those of WT. To further test the capacity of chloroplasts from *phyB-9* mutants to generate ROS during light stress, we treated WT and *phyB-9* plants with the herbicide PQ that causes enhanced ROS production primarily in chloroplasts during light stress. As shown in Fig. S5, WT or *phyB-9* plants treated with PQ in the dark did not accumulate enhanced levels of ROS. By contrast, upon exposure to light (ambient, or excess red or white light), both PQ-treated WT and *phyB-9* plants accumulated enhanced levels of ROS (Fig. S5). The findings that *phyB* mutants subjected to excess light stress in the absence of PQ did not accumulate enhanced levels of ROS (Figs 1a,b, 2, S5), while in the presence of PQ they did (Fig. S5), demonstrated that *phyB* plants can produce ROS *via* their chloroplasts. This finding supports our working hypothesis that during light stress, some of the ROS accumulating in leaf cells of WT plants are produced by RBOHs at the apoplast (Figs 1a,b, 2, S5; Devireddy *et al.*, 2020; Xiong *et al.*, 2021).

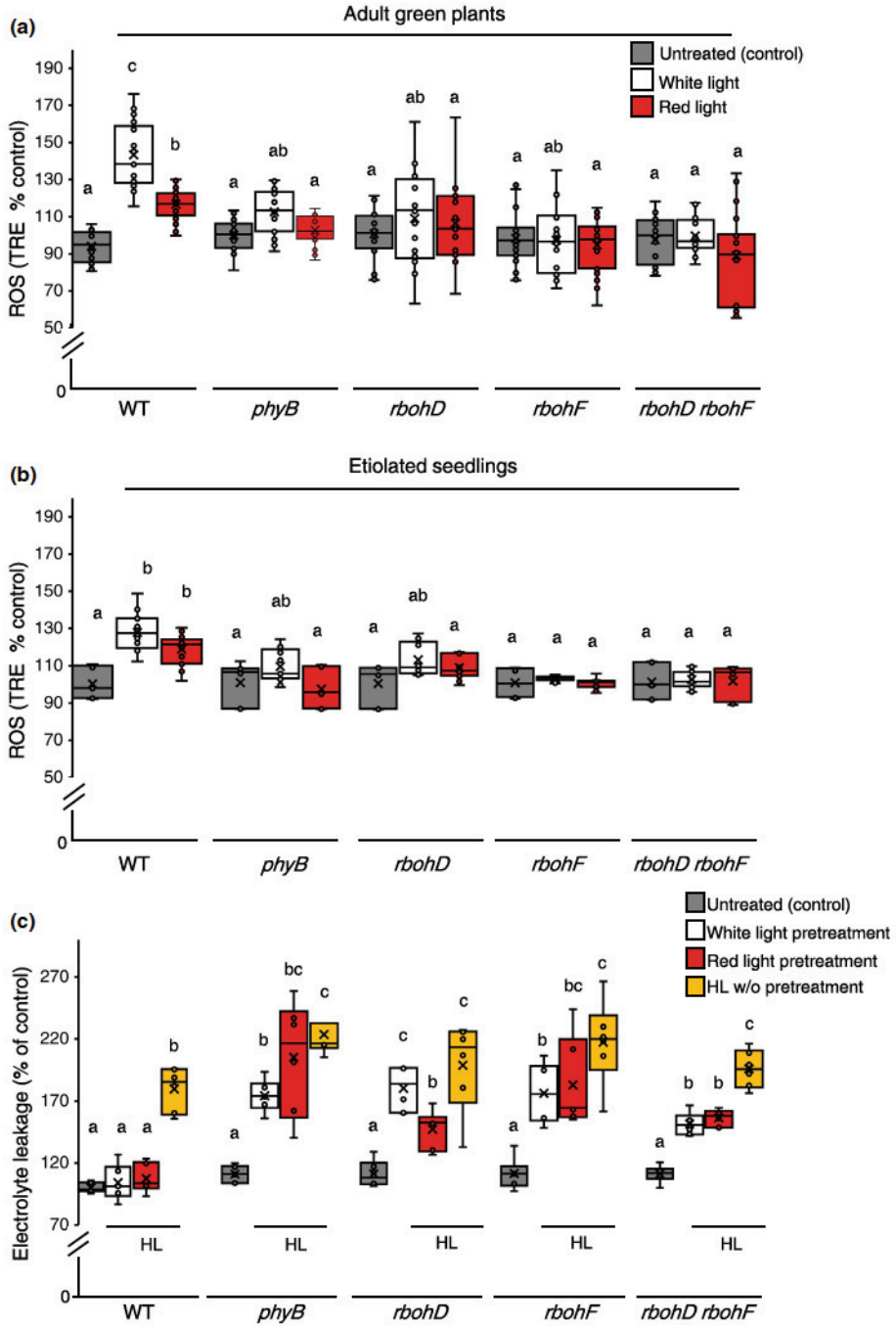

Fig. 1 PhyB, RBOHD and RBOHF regulate ROS accumulation, physiological responses, and plant acclimation to excess light stress. (a) ROS accumulation in WT, *phyB-9*, *rbohD*, *rbohF*, and *rbohD rbohF* 4-wk-old *Arabidopsis thaliana* plants subjected to 10-min excess white (white boxes) or red (red boxes) light. (b) Same as (a), but for 6-d-old etiolated seedlings grown on plates. (c) Acclimation of WT, *phyB-9*, *rbohD*, *rbohF*, and *rbohD rbohF A. thaliana* plants to excess light stress. Measurements are shown for untreated plants (control – gray boxes), plants that were pretreated with excess white or red light for 10 min, allowed to recover for 50 min, and then subjected to excess white light for 45 min (pretreated – white or red boxes), and plants that were treated with a 45-min excess white light without pretreatment (w/o pretreatment – yellow boxes). Data are represented as percentage of control (untreated plants; gray boxes) ± SE. All experiments were repeated at least three times with four plants per biological replicate. $n = 12$; different letters denote statistical significance at $P < 0.05$ (ANOVA followed by a Tukey's *post hoc* test). Results are shown as box-and-whisker plots with borders corresponding to the 25th and 75th percentiles of the data. Absolute values (in µS) for 100% leakage of untreated (control) are as follows: WT, 4.34; *phyB*, 5; *rbohD*, 3.97; *rbohF*, 3.57; *rbohD rbohF*, 4.2. Open circles represent data points and crosses the mean. DCF, 2′,7′-dichlorofluorescin; HL, highlight; phyB, phytochrome B; rbohD, NADPH/respiratory burst oxidase protein D; rbohF, NADPH/respiratory burst oxidase protein F; ROS, reactive oxygen species; TRE, total radiant efficiency; w/o, without; WT, wild-type

PhyB, RBOHD, and RBOHF regulate the expression of thousands of transcripts in response to excess light stress

To determine whether phyB, RBOHD, and RBOHF function in the same signaling pathway that regulates ROS accumulation, physiological responses, and plant acclimation to excess light stress (Figs 1, 2, S2, S3), we subjected mature WT, *phyB-9*, *rbohD*, and *rbohF* plants to a 10-min treatment with excess white (740 µmol photons s^{-1} m^{-2}) or red (120 µmol photons s^{-1} m^{-2}) light stress and studied their transcriptomic responses using RNA-Seq (Fig. 3; Datasets S1–S28). Quantitative RT-PCR (qPCR) analysis conducted on the different RNA samples obtained before the RNA-Seq analysis revealed a complex response of the different mutants to the different treatments (Fig. S6; Dataset S29). Some transcripts that were upregulated in WT but not *rbohD* or *rbohF* were still upregulated in *phyB* (e.g. *MYELOBLASTOSIS DOMAIN PROTEIN 30 (MYB30)*, *ZINC FINGER OF ARABIDOPSIS THALIANA 12 (Zat12)*, and *ASCORBATE PEROXIDASE 2 (APX2)*), while others, such as *ZINC FINGER HOMEODOMAIN* 5 (ZHD5), that were upregulated in WT were suppressed in all mutants (Fig. S6). Venn diagrams comparing the transcripts significantly altered in WT and *phyB* in response to the excess white light treatment reveal that 3091 transcripts altered in WT by this treatment were not altered

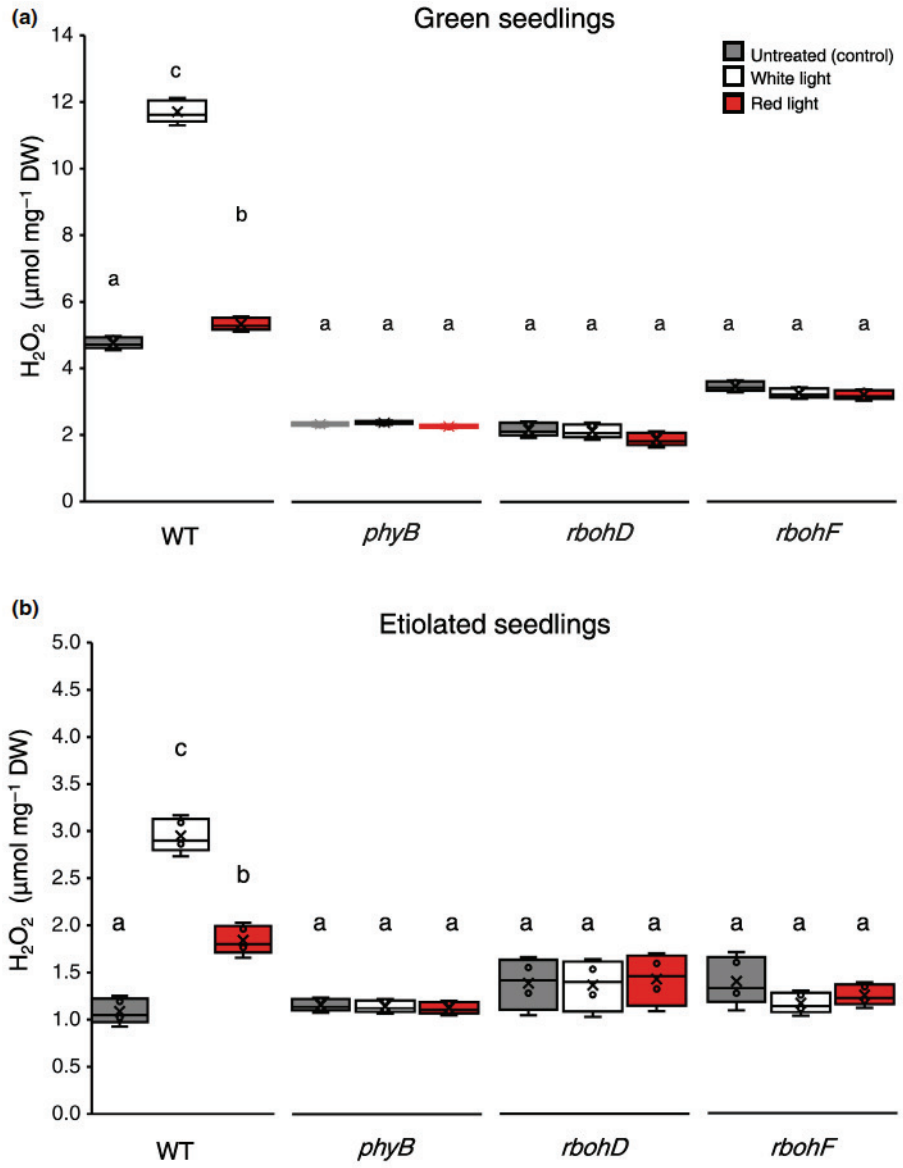

Fig. 2 H_2O_2 accumulation in WT, *phyB-9*, *rbohD*, and *rbohF Arabidopsis thaliana* seedlings in response to excess light stress. (a) H_2O_2 accumulation in 6-d-old WT, *phyB-9*, *rbohD*, and *rbohF* green seedlings grown on plates and subjected to 10-min excess white (white boxes) or red (red boxes) light. (b) Same as (a), but for 6-d-old etiolated seedlings grown on plates. All experiments were repeated at least three times with 10 seedlings per biological replicate. $n = 12$; different letters denote statistical significance at $P < 0.05$ (ANOVA followed by a Tukey's *post hoc* test). H_2O_2 was measured with Amplex-Red. Results are shown as box-and-whisker plots with borders corresponding to the 25th and 75th percentiles of the data. Open circles represent data points and crosses the mean. phyB, phytochrome B; rbohD, NADPH/respiratory burst oxidase protein D; rbohF, NADPH/respiratory burst oxidase protein F; WT, wild-type

in the *phyB-9* mutant (Fig. 3a; Dataset S17). A similar comparison of transcripts altered in WT and the *rbohD* mutant revealed that 3460 transcripts altered in WT were not altered in *rbohD* (Fig. 3a; Dataset S18). Interestingly, an overlap of 2674 transcripts was found between the 3091 transcripts putatively regulated by phyB during light stress and the 3460 transcripts putatively regulated by RBOHD during light stress, suggesting that phyB and RBOHD coregulate over 2500 transcripts during excess white light stress (Dataset S19). A similar comparison conducted between the response of *phyB* and *rbohF* revealed an overlap of 2446 transcripts (Fig. 3b; Datasets S20, S21). Interestingly, despite some differences in expression pattern between RBOHD and RBOHF in leaves (the latter being primarily expressed in vascular bundles; Morales *et al.*, 2016; Zandalinas *et al.*, 2020b), an overlap of 2217 transcripts was found between the transcripts common to phyB and RBOHD (2674) and phyB and RBOHF (2446), suggesting that phyB, RBOHD, and RBOHF regulate a large proportion of the plant response to excess light stress (Fig. 3b; Dataset S22). A similar analysis conducted for the transcriptomic response of WT, *phyB-9*, *rbohF*, and *rbohD* to excess red light (Fig. S7; Datasets S23–S28) revealed an overlap of 353 transcripts between the transcripts common to phyB and RBOHD (393) and phyB and RBOHF (490) in response to excess red light that should primarily activate phys (Fig. 3b; Dataset S28).

An analysis of stress, hormone, and ROS response transcripts found within these two groups of overlapping transcripts (2217 in response to white light and 393 in response to red light) revealed that both groups contained a large number of high light-, wounding-, drought-, heat-, and salt-response transcripts (Figs 3c, S8). In addition, they contained a high number of H_2O_2-, brassinosteroid-, and jasmonic acid-response transcripts (Fig. 3c). The identification of a large number of stress-response transcripts altered in both *phyB-9* and *rbohD*/*rbohF* during the

response to light stress could suggest that like RBOHs, phyB is involved in the response of plants to many different stress conditions.

The results presented in Figs 3, S7, and S8 reveal that phyB, RBOHD, and RBOHF coregulate thousands of transcripts during the response of plants to excess white light stress. Because RBOHD and RBOHF are thought to regulate plant responses *via* ROS production (Zandalinas & Mittler, 2018; Fichman & Mittler, 2020; Lee *et al.*, 2020), and in the absence of phyB, ROS do not accumulate in plants in response to excess light stress (red or white light; Figs 1, 2), it is likely that phyB functions upstream of RBOHD and RBOHF during plant responses to excess light stress.

Complementing *phyB-9* with a cytosolic-restricted phyB protein recovers ROS production during excess light stress

The findings that phyB is required for RBOH-mediated ROS production during excess light stress (Figs 1, 2) and that phyB, RBOHD, and RBOHF coregulate many transcripts during this response (Fig. 3) suggest that phyB regulates RBOH function. Although phyB was shown to interact with the PM and to exert some of its functions in the cytosol (Rosler *et al.*, 2010; Jaedicke *et al.*, 2012; Hughes, 2013), the majority of phyB functions are mediated following its nuclear localization and interactions with various transcriptional regulators (González *et al.*, 2012; Jung *et al.*, 2016; Jiang *et al.*, 2019, 2020; Kim *et al.*, 2020, 2021; Yan

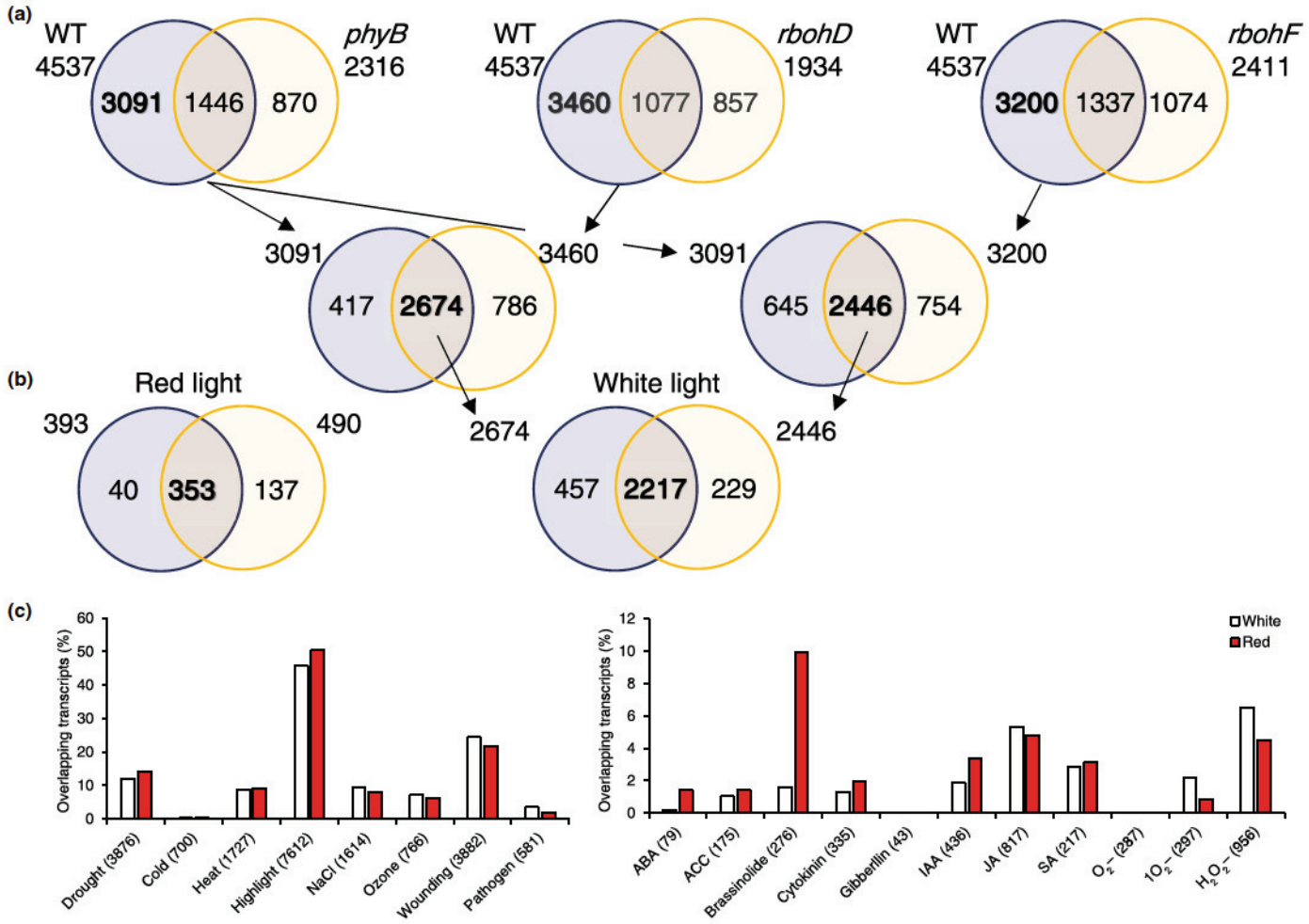

Fig. 3 PhyB, RBOHD and RBOHF regulate the expression of thousands of transcripts in response to excess light stress. (a) Venn diagrams showing the overlap between transcripts altered in their expression in WT, *phyB-9*, *rbohD*, and *rbohF Arabidopsis thaliana* plants in response to excess white light stress. (b) Venn diagram showing the overall between the transcripts altered in *phyB-9*, *rbohD*, and *rbohF A. thaliana* plants in response to excess red light stress. A complete analysis similar to (a), of the red light induced 353 transcripts that are coregulated by phyB, RBOHD, and RBOHF. Venn diagrams showing the overlap between transcripts altered in their expression in WT, *phyB-9*, *rbohD*, and *rbohF* plants in response to excess red light stress are shown in Supporting Information Fig. S7. Venn diagram overlap was calculated with hypergeometric testing and found to be significant ($P < 0.001$) for all diagrams. (c) Percentage representation of transcripts altered in response to different stresses, hormone treatments, or ROS in the white (2217) and red (353) light response transcripts common to *phyB-9*, *rbohD*, and *rbohF A. thaliana* plants (from (a) and (b); white columns for white light and red columns for red light). $^1O_2$, singlet oxygen; ABA, abscisic acid; ACC, 1-aminocyclopropane 1-carboxylic acid; H_2O_2, hydrogen peroxide; IAA, indole-3-acetic acid; JA, jasmonic acid; $O_2^{\bullet-}$, superoxide; phyB, phytochrome B; rbohD, NADPH/respiratory burst oxidase protein D; rbohF, NADPH/respiratory burst oxidase protein F; ROS, reactive oxygen species; SA, salicylic acid; WT, wild-type

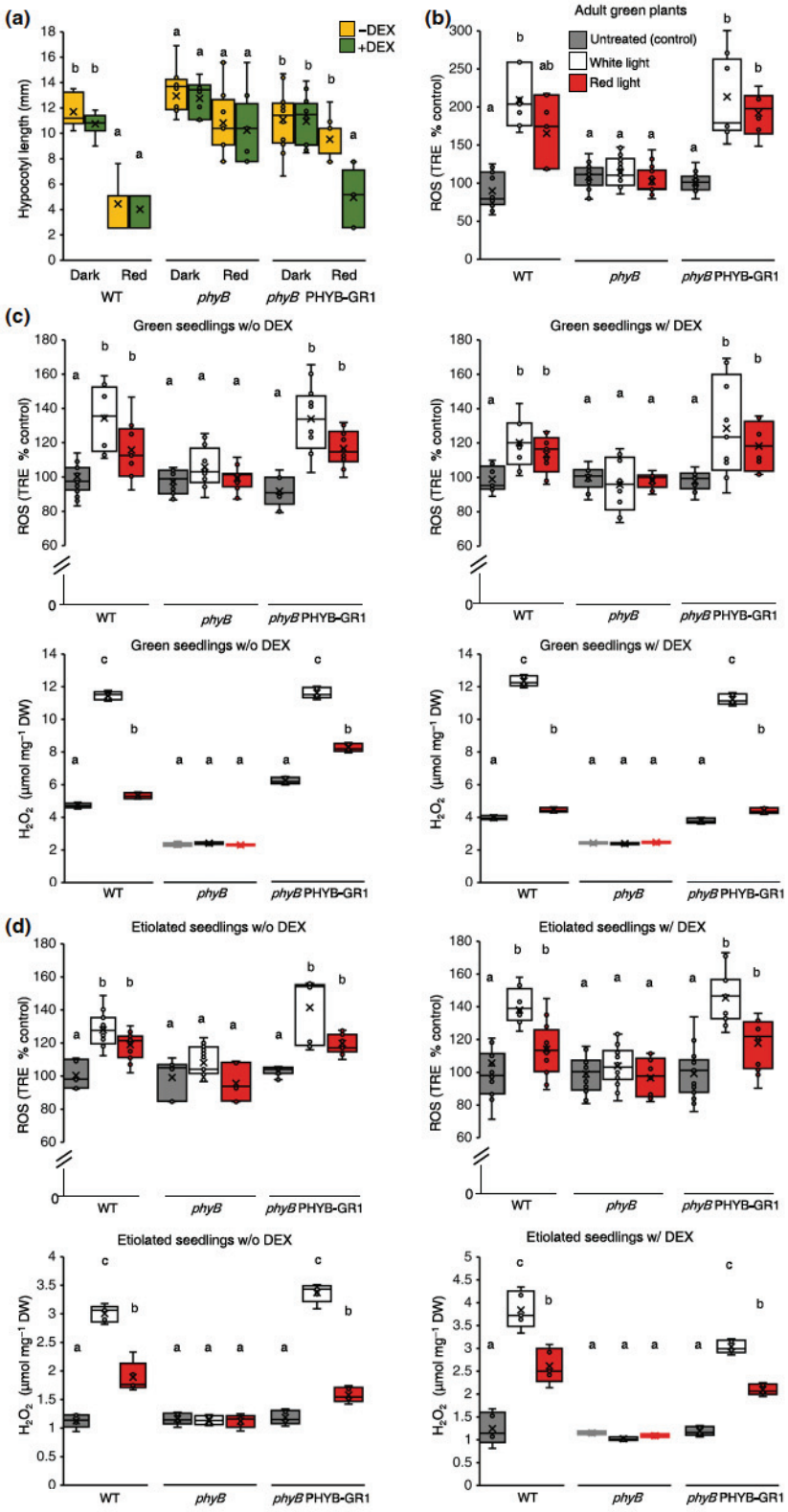

Fig. 4 Complementing *phyB-9* with a cytosolic-restricted phyB protein recovers ROS production during excess light stress. (a) Hypocotyl length of WT, *phyB-9*, and *phyB-9* PHYB-GR1 *Arabidopsis thaliana* seedlings grown without DEX (yellow) or with DEX (green) in the dark or under constant red light. (b) ROS accumulation in WT, *phyB-9*, and *phyB-9* PHYB-GR1 adult green *A. thaliana* plants subjected to 10-min excess white (white boxes) or red (red boxes) light stress. (c) ROS (upper) and H_2O_2 (lower) accumulation in *A. thaliana* green seedlings of WT, *phyB-9*, and *phyB-9* PHYB-GR1 subjected to 10-min excess white (white boxes) or red (red boxes) light stress in the presence or absence of DEX. (d) Same as (c), but for etiolated *A. thaliana* seedlings grown on plates with or without DEX. H_2O_2 was measured with Amplex-Red. Data are represented as percentage of control (untreated plants; gray boxes) ± SE. Different letters denote statistical significance at $P < 0.05$ (ANOVA followed by a Tukey's *post hoc* test). Results are shown as box-and-whisker plots with borders corresponding to the 25th and 75th percentiles of the data. Open circles represent data points and crosses the mean. DEX, dexamethasone; GR1, glucocorticoid receptor; HL, highlight; phyB, phytochrome B; ROS, reactive oxygen species; TRE, total radiant efficiency; w/o, without; WT, wild-type

et al., 2020). To test whether phyB functions in the cytosol or nuclei to regulate RBOH function, we used a transgenic line that expresses phyB fused in frame to glucocorticoid receptor 1 (GR1) in the *phyB-9* mutant background (*phyB-9* PHYB-GR1). Previous studies showed that the phyB-GR1 hybrid protein is retained in the cytosol unless DEX is applied (Samach *et al.*, 2000; Huq *et al.*, 2003; Figs S9, S10). To validate the function of the phyB-GR1 system, we germinated WT, *phyB-9*, and *phyB-9* PHYB-GR1 on plates in the presence or absence of DEX in the dark or under red light for 3 d. As shown in Figs 4(a) and S10, WT seedlings

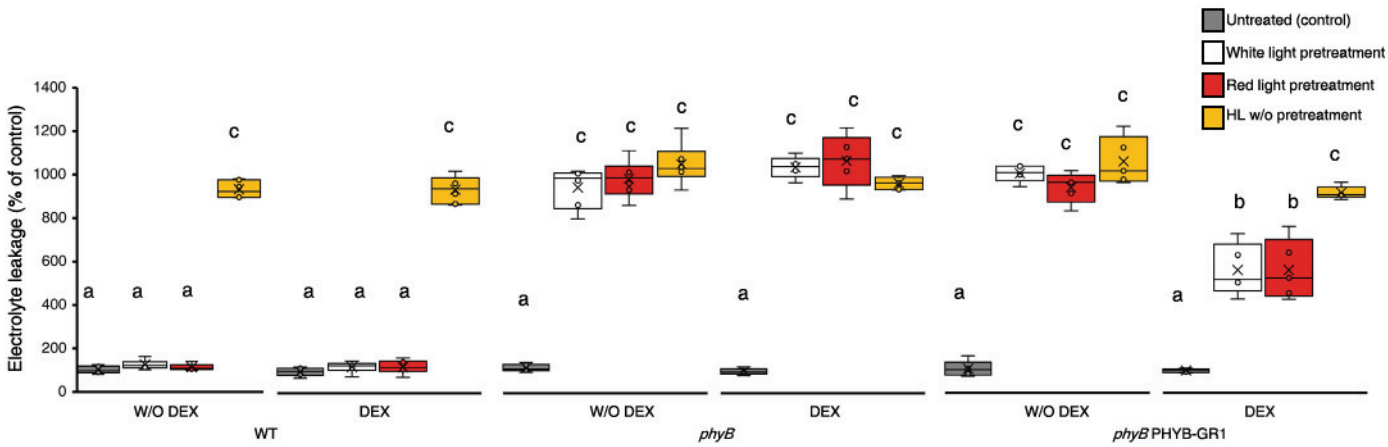

Fig. 5 Plant acclimation to excess light stress requires nuclear localization of phyB. Acclimation of WT, *phyB-9*, and *phyB-9* PHYB-GR1 *Arabidopsis thaliana* seedlings grown in liquid in the presence or absence of DEX to excess white light stress. Measurements are shown for untreated seedlings (control – gray boxes), seedlings that were pretreated with excess white or red light for 10 min, allowed to recover for 50 min and then subjected to excess white light for 45 min (pretreated – white or red boxes), and seedlings that were treated with a 45 min excess white light without pretreatment (w/o pretreatment – yellow boxes). Data are represented as percentage of control (untreated plants; gray boxes) ± SE. All experiments were repeated at least three times with 45 seedlings per biological replicate. Different letters denote statistical significance at $P < 0.05$ (ANOVA followed by a Tukey's *post hoc* test). Results are shown as box-and-whisker plots with borders corresponding to the 25th and 75th percentiles of the data. Absolute values (in μS) for 100% leakage of untreated (control) are as follows: WT, 3.9; *phyB*, 4.44; *phyB* PHYB-GR1 (w/o DEX), 4.9; (w/ DEX), 3.63. Open circles indicate data points and crosses the mean. DEX, dexamethasone; GR1, glucocorticoid receptor; HL, highlight; phyB, phytochrome B; ROS, reactive oxygen species; TRE, total radiant efficiency; w/o, without; WT, wild-type

grown in darkness had an elongated hypocotyl. In response to red light, however, their hypocotyl was short. In contrast to WT, *phyB-9* seedlings did not respond to red light and had an elongated hypocotyl under dark or red light conditions. Compared with WT or *phyB-9*, which were not responsive to the DEX treatment, the *phyB-9* PHYB-GR1 line grown in the absence of DEX behaved like the *phyB-9* line, while the *phyB-9* PHYB-GR1 grown in the presence of DEX behaved like the WT (as evident by hypocotyl length and chlorophyll content; Figs 4a, S10). These results suggest that in the absence of DEX, the phyB-GR1 protein is retained in the cytosol, while in the presence of DEX, it is mobilized to the nuclei (Samach *et al.*, 2000; Huq *et al.*, 2003).

To test whether phyB functions in the cytosol or nuclei to regulate ROS accumulation during excess light stress, we subjected mature WT, *phyB-9*, and *phyB-9* PHYB-GR1 plants to excess white or red light stress in the absence of DEX (hybrid protein is restricted to the cytosol; Fig. S9; Samach *et al.*, 2000; Huq *et al.*, 2003) and measured ROS accumulation. As shown in Fig. 4 (b), excess white or red light caused enhanced ROS accumulation in WT or *phyB-9* PHYB-GR1 plants, suggesting that the phyB-GR1 protein present in the cytosol is sufficient to trigger ROS production under conditions of excess light stress. To test the effect of DEX on ROS production in WT, *phyB-9*, and *phyB-9* PHYB-GR1 plants, we grew seedlings of these lines in the presence or absence of DEX and treated them with excess white or red light. As shown in Fig. 4(c), the presence of DEX did not prevent the accumulation of ROS or H_2O_2 in WT or *phyB-9* PHYB-GR1 plants, suggesting that not all the PHYB-GR1 protein localized to the nuclei in *phyB-9* PHYB-GR1 plants following DEX application or that the PHYB-GR1 protein can also induce ROS formation upon localization to the nuclei. Similar results were found with etiolated seedlings of the different lines grown in the presence or absence of DEX and subjected to excess white or red light (Fig. 4d). Although we cannot rule out the possibility that residual PHYB-GR1 is present in the nuclei in the absence of DEX, the results presented in Figs 4, S9, and S10 strongly suggest that phyB does not need to localize into the nuclei to trigger ROS production in response to excess light stress.

The findings that phyB can regulate ROS production during excess light stress without entering the nuclei (Figs 4, S9, S10) prompted us to test whether it can also trigger plant acclimation to excess light stress while being restricted to the cytosol. For this purpose, we measured the acclimation of WT, *phyB-9*, and *phyB-9* PHYB-GR1 seedlings, growing in liquid media in the presence or absence of DEX, to excess light stress. As shown in Fig. 5, WT seedlings could acclimate (as evident by reduced tissue damage following exposure to light stress) to a prolonged excess white light stress treatment following a pretreatment with a short excess white or red light stress followed by incubation, regardless of the presence or absence of DEX. By contrast, and regardless of the presence or absence of DEX, *phyB-9* seedlings were unable to acclimate to the excess light stress treatment (Fig. 5). Interestingly, while *phyB-9* PHYB-GR1 seedlings were unable to acclimate to the excess white light treatment in the absence of DEX, they were able to partially acclimate to it in the presence of DEX. This finding suggests that nuclear localization of phyB could be required for plant acclimation to excess light stress.

Complementing *phyB-5* with a constitutively active phyB protein that is localized to the nuclei fails to recover ROS production during excess light stress

To complement our study with the PHYB-GR1 fusion protein that restricts phyB to the cytosol in the absence of DEX (Figs 4,

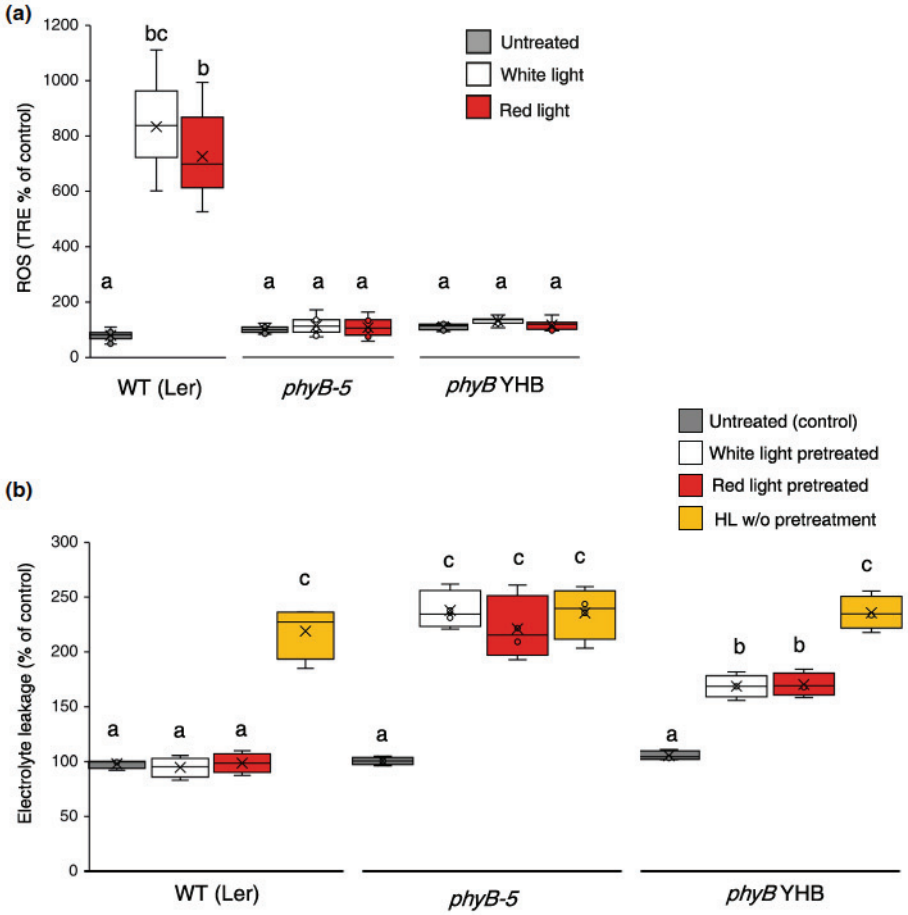

Fig. 6 ROS production and acclimation in response to light stress in the *phyB-5* YHB mutant that is localized to protein bodies in the nuclei and is constitutively active. (a) ROS accumulation in WT, *phyB-5*, and *phyB-5* YHB adult green *Arabidopsis thaliana* plants subjected to 10-min excess white (white boxes) or red (red boxes) light stress. (b) Acclimation of WT, *phyB-5*, and *phyB-5* YHB *A. thaliana* plants to excess light stress. Measurements are shown for untreated plants (control – gray boxes), plants that were pretreated with excess white or red light for 10 min, allowed to recover for 50 min and then subjected to excess white light for 45 min (pretreated – white or red boxes), and plants that were treated with a 45 min excess white light without pretreatment (w/o pretreatment – yellow boxes). Data are represented as percentage of control (untreated plants; gray boxes) ± SE ($n = 64$ in (a) and 12 in (b)). Different letters denote statistical significance at $P < 0.05$ (ANOVA followed by a Tukey's *post hoc* test). Results are shown as box-and-whisker plots with borders corresponding to the 25th and 75th percentiles of the data. Absolute values (in μS) for 100% leakage of untreated (control) are as follows: WT, 5.04; *phyB-5*, 3.02; *phyB* YHB, 3.26. Open circles indicate data points and crosses the mean. HL, highlight; Ler, Landsberg ecotype; phyB, phytochrome B; ROS, reactive oxygen species; TRE, total radiant efficiency; w/o, without; WT, wild-type; YHB, *phyB*^{Y276H}

5, S9, S10), we studied white and red light stress-induced ROS production in a different mutant of phyB (i.e. *phyB-5* YHB; *phyB*^{Y276H}; Fischer *et al.*, 2005; Su & Lagarias, 2007; Hu *et al.*, 2009). In this mutant, the phyB protein is constitutively active for responses to white light- or red light-dependent hypocotyl growth inhibition and localized to protein bodies in the nuclei (Fischer *et al.*, 2005; Su & Lagarias, 2007; Hu *et al.*, 2009; Fig. S11). As shown in Fig. 6, when the *phyB-5* YHB mutant was subjected to white or red light stress, it did not accumulate ROS (Fig. 6a). By contrast, the *phyB-5* YHB mutant was able to partially acclimate to white or red light stress (Fig. 6b; similar to the DEX-treated and nuclear-localized PHYB-GR1; Fig. 5). These findings support our results obtained with *phyB-9* PHYB-GR1 plants (Figs 4, 5, S9, S10) and demonstrate that nuclear localization of phyB is important for plant acclimation, while cytosolic localization of phyB is important for regulating ROS production during stress (Figs 4–6, S9–S11).

PhyB is required for ROS production in response to wounding, heat, cold, and pathogen infection

We previously reported that RBOHD is required for rapid ROS production and systemic signaling in *Arabidopsis* in response to many different abiotic and biotic stimuli (Miller *et al.*, 2009; Fichman *et al.*, 2019; Zandalinas *et al.*, 2020a). The findings that phyB is required for rapid ROS production in response to excess light stress (Figs 1, 2), that phyB and RBOHs are required for the expression of the same set of transcripts during light stress (Fig. 3), and that phyB can regulate rapid ROS production even if it is restricted to the cytosol (Figs 4–6) prompted us to test whether phyB is required for rapid ROS production and systemic signaling during the response of *Arabidopsis* to other stresses. Remarkably, as shown in Fig. 7(a–d), we found that phyB is required for rapid local and systemic ROS accumulation in response to excess light, cold, heat, or pathogen infection. By contrast, we found that phyB is required for systemic, but not local, ROS accumulation during wounding (Fig. 7e). The findings presented in Fig. 7 suggest that phyB is required for the rapid ROS accumulation response of plants to several different abiotic and biotic stresses, potentially functioning as part of a signaling module with RBOHD and/or RBOHF.

PhyB is required for ROS accumulation during excess light stress in rice

We previously reported that RBOHA is required for ROS production during excess light stress in rice (Xiong *et al.*, 2021). To test whether phyB is also involved in the response of rice to excess

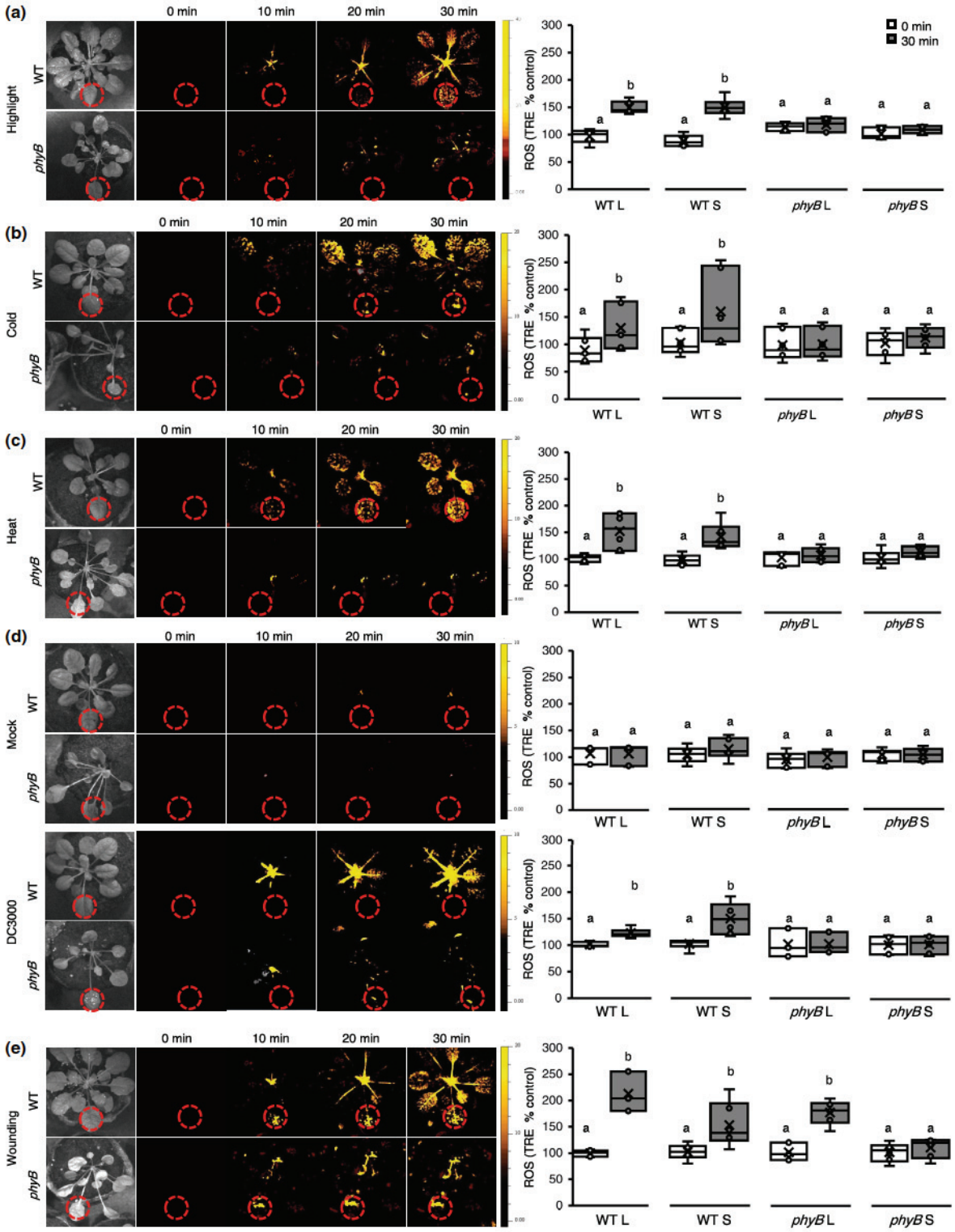

Fig. 7 PhyB is required for ROS production in response to wounding, heat, cold, and pathogen infection. (a) Representative time lapse images (left) and quantitative bar graphs (right) of ROS accumulation in local (L) and systemic (S) leaves (local leaf is marked with red broken line circle) of WT and *phyB-9* *Arabidopsis thaliana* plants subjected to an excess light stress treatment applied to the local leaf. (b) Same as (a), but for cold stress applied to the L leaf. (c) Same as (a), but for heat stress applied to the L leaf. (d) Same as (a), but for mock or *Pseudomonas syringae* DC3000 infection applied to the L leaf. (e) Same as (a), but for mechanical wounding applied to the L leaf. Data are represented as percentage of control (0 min; white boxes) ± SE. Different letters denote statistical significance at $P < 0.05$ (ANOVA followed by a Tukey's *post hoc* test). Results are shown as box-and-whisker plots with borders corresponding to the 25th and 75th percentiles of the data. Open circles indicate data points and crosses the mean. L, local; phyB, phytochrome B; ROS, reactive oxygen species; S, systemic; TRE, total radiant efficiency; WT, wild-type

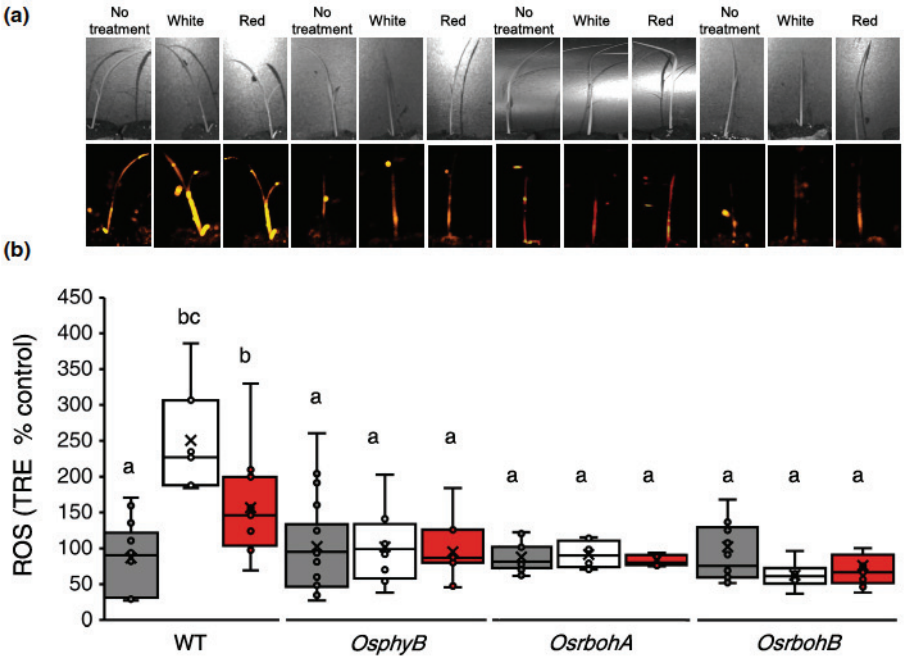

Fig. 8 PhyB is required for ROS accumulation during excess light stress in rice. (a) Representative images of whole-plant ROS accumulation in WT, *OsphyB*, *OsrbohA*, and *OsrbohB* rice plants subjected to 10-min excess white or red light. (b) Quantitative analysis of ROS accumulation in WT, *OsphyB*, *OsrbohA*, and *OsrbohB* rice plants subjected to 10-min excess white (white boxes) or red (red boxes) light. All experiments were repeated at least three times with 10 plants per biological replicate. Data are represented as percentage of control (untreated plants; gray boxes) ± SE. Different letters denote statistical significance at $P < 0.05$ (ANOVA followed by a Tukey's *post hoc* test). Results are shown as box-and-whisker plots with borders corresponding to the 25th and 75th percentiles of the data. Open circles indicate data points and crosses the mean. DCF, 2′,7′-dichlorofluorescin; phyB, phytochrome B; rbohD, NADPH/respiratory burst oxidase protein D; rbohF, NADPH/respiratory burst oxidase protein F; ROS, reactive oxygen species; TRE, total radiant efficiency; WT, wild-type

light stress, we subjected WT, *phyB*, *rbohA*, and *rbohB* rice plants to excess white or red light stress and measured ROS accumulation using our whole-plant live ROS imaging method (Fichman *et al.*, 2019). As shown in Fig. 8, WT rice plants subjected to excess light stress accumulated ROS. By contrast, rice *phyB*, *rbohA*, and *rbohB* mutants did not. These findings demonstrate that phyB could play an important role in ROS production during excess light stress in other plants.

Discussion

The photoreceptor protein phyB plays a key role in the sensing of light and heat leading to the activation and/or modulation of many different developmental programs and environmental responses (Kathare & Huq, 2021; Quail, 2021; Chen *et al.*, 2022; Li *et al.*, 2022). Recent studies revealed that in addition to light and heat, phyB is involved in responses of plants to chilling, salt, drought, pathogen, and cold stresses (Faigón-Soverna *et al.*, 2006; González *et al.*, 2012; Ha *et al.*, 2018; Kwon *et al.*, 2018; Arico *et al.*, 2019; Jiang *et al.*, 2020). To regulate plant responses to environmental stimuli, phyB interacts with, or affects the function of, multiple proteins including transcriptional regulators such as PIFs, binds to different promoters, and/or associates with large multiprotein complexes called photobodies (Quail, 2002, 2021; González *et al.*, 2012; Jung *et al.*, 2016; Jiang *et al.*, 2019, 2020; Legris *et al.*, 2019; Kim *et al.*, 2020, 2021; Klose *et al.*, 2020; Yan *et al.*, 2020; Cheng *et al.*, 2021; Kathare & Huq, 2021). Phytochrome B primarily functions therefore in the nuclei to alter gene expression. Here, we reveal that in addition to its nuclear functions, phyB may act in the cytosol to regulate ROS production *via* RBOHs during excess light stress (Figs 4, 6). Moreover, we show that phyB is essential for ROS production during excess light stress in both the dicot *Arabidopsis* (Figs 1, 2) and the monocot rice (Fig. 8), suggesting that its function upstream of RBOHs is conserved and widespread in plants. We further show that phyB is required for local and/or systemic ROS accumulation in plants in response to pathogen infection, cold, heat, or wounding (Fig. 7). These findings shed new light on phyB function in plants, that is, the regulation of ROS production at the apoplast in response to multiple stimuli. This new role is highly important for our understanding of plant biology since ROS signaling is central to plant responses to many different environmental conditions and stresses (Choudhury *et al.*, 2017; Mittler, 2017; Waszczak *et al.*, 2018; Kollist *et al.*, 2019; Smirnoff & Arnaud, 2019; Foyer & Hanke, 2022). Phytochrome B could therefore function in both the nucleus and the cytosol to coordinate apoplastic ROS production during stress with different transcriptional responses (Figs 3, 9). Supporting this hypothesis are our findings that although the cytosolic localization of phyB was sufficient to activate ROS production (Fig. 4), it was not sufficient to induce plant acclimation (Fig. 5). Instead, nuclear functions of phyB appear necessary to induce plant acclimation to excess light stress (Figs 5, 6). As phyB is required for a broad spectrum of stresses that may or may not

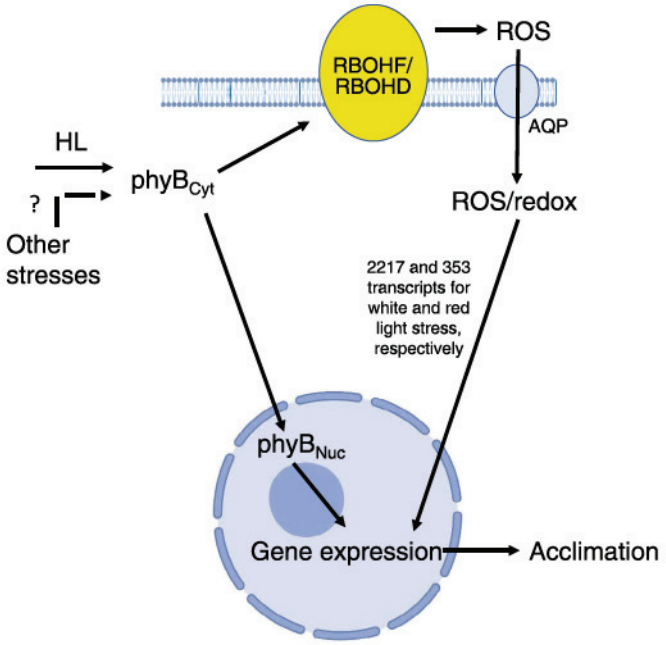

Fig. 9 Model for phyB-RBOH signaling during the response of plant to stress. PhyB is shown to integrate different environmental stresses and to activate ROS production by RBOHD and RBOHF at the apoplast. Phytochrome B can mediate this function even if it is restricted to the cytosol. To induce plant acclimation to stress, phyB needs, however, to localize to the nucleus. In response to excess white light stress, pyhB, RBOHD, and RBOHF are shown to regulate the expression of thousands of transcripts that depend on ROS production at the apoplast. ROS at the apoplast are thought to enter into the cytosol *via* aquaporins. Question mark indicates the possible involvment of other abiotic or biotic stresses in triggering ROS signaling through phyB. AQP, aquaporin; Cyt, cytosolic; HL, highlight; Nuc, nuclear; phyB, phytochrome B; PM, plasma membrane; rbohD, NADPH/respiratory burst oxidase protein D; rbohF, NADPH/respiratory burst oxidase protein F; ROS, reactive oxygen species

involve changes in light intensity and/or quality (Fig. 7), it is possible that phyB functions in a light-independent manner, perhaps as a scaffold or regulatory protein, linking stress responses to ROS production.

Our findings that in the absence of phyB, ROS do not accumulate in plant cells in response to excess light stress (Figs 1, 2, 8), that phyB can regulate ROS production without localizing to the nucleus (Figs 4, 6), and that phyB function overlaps with that of RBOHs in regulating transcriptomic responses during excess light stress (Figs 3, S7) strongly suggest that phyB functions upstream of RBOHs (Fig. 9). Taking into consideration the broad signaling function of RBOHs in mediating ROS production during biotic and abiotic stresses (Zandalinas & Mittler, 2018; Fichman & Mittler, 2020; Lee *et al.*, 2020), and the importance of phyB for many of these responses (González *et al.*, 2012; Jung *et al.*, 2016; Jiang *et al.*, 2019, 2020; Kim *et al.*, 2020, 2021; Yan *et al.*, 2020), it is likely that a phyB-RBOH regulatory module is involved in ROS production during plant responses to many different stresses (Fig. 7). Several mechanisms could link phyB function in the cytosol with the PM-localized RBOHs. Phytochrome B was shown, for example, to associate with the plant PM forming a complex with phototropin (Jaedicke *et al.*, 2012) and could potentially bind to or interact with RBOHs as well. In addition, phyB contains a Histidine kinase domain, and RBOHs are regulated by phosphorylation (Quail, 2002; Rosler *et al.*, 2010; Hughes, 2013; Zandalinas & Mittler, 2018; Legris *et al.*, 2019; Fichman & Mittler, 2020; Klose *et al.*, 2020; Lee *et al.*, 2020; Cheng *et al.*, 2021; Gallé *et al.*, 2021; albeit, Li *et al.*, 2022). Phytochrome B was also found to interact with different known activators of RBOHs (https://thebiogrid.org/; Drerup *et al.*, 2013; Kollist *et al.*, 2019) and could therefore function as a regulatory and/or scaffold protein involved in RBOH regulation during stress.

The possible broad function of phyB in regulating ROS production during plant responses to different stresses is supported by previous studies showing the involvement of phyB in plant responses to different abiotic stresses (González *et al.*, 2012; Jiang *et al.*, 2020), by our findings of a large overlap in transcripts regulated by phyB and RBOHs (Figs 3a, S7), and by the nature of the transcripts regulated by phyB and RBOHs (Figs 3c, S8). These include a high proportion of excess light-, drought-, heat-, salinity-, pathogen-, wounding-, and ozone-response transcripts. In addition, they contain H_2O_2-, jasmonic acid-, salicylic acid-, and auxin-response transcripts (Fig. 3c). Taken together, these data suggest that the phyB–RBOH signaling module could play a central role in regulating ROS levels and plant acclimation to many different abiotic and biotic stresses in plants (Figs 7, 9). While phyB is involved in ROS production in response to stress in leaves (Figs 1, 8), it was also shown to trigger a shoot-to-root systemic signal that suppresses ROS production in roots *via* the activation of a peroxidase (Ha *et al.*, 2018). The outcome of stress sensing by the phyB–RBOH module could therefore depend on the tissue type and stress involved.

Our findings that some of the ROS accumulating in plant cells during excess light stress are dependent on RBOH function are intriguing (Figs 1, 2, 8; Devireddy *et al.*, 2020; Xiong *et al.*, 2021). In particular, since ROS were also found to be produced by chloroplasts, peroxisomes, and/or mitochondria during light stress and to have an important regulatory function (Exposito-Rodriguez *et al.*, 2017; Waszczak *et al.*, 2018; Smirnoff & Arnaud, 2019; Foyer & Hanke, 2022). One possible explanation for these findings is that ROS produced in different organelles during excess light stress trigger retrograde signaling pathways that affect nuclear responses but are simultaneously scavenged within these organelles and do not accumulate to high levels. It is also possible that low levels of ROS produced in these organelles diffuse into the cytosol to regulate redox and signaling pathways. Because ROS can accumulate in the apoplast to high levels without having a toxic effect (Miller *et al.*, 2009; Fichman *et al.*, 2019; Devireddy *et al.*, 2020; Zandalinas *et al.*, 2020a), it is possible that apoplastic ROS production by RBOHs could serve as a transient stress memory mechanism, as previously shown for light stress in *Arabidopsis* that triggered an RBOHD-dependent 3–6 h high apoplastic ROS production state (Devireddy *et al.*, 2020). During stress, ROS could therefore be produced in organelles to trigger different retrograde signaling pathways, as well as in the apoplast to serve as a ROS reservoir (much like calcium is stored in the apoplast and other compartments; Luan & Wang, 2021), and a delicate interplay between retrograde

mechanisms and low ROS levels that enter the cytosol from different organelles or the apoplast could control different signaling pathways and lead to acclimation (Mittler *et al.*, 2022). In this respect, it should be mentioned that at least one retrograde pathway was recently shown to regulate phyB function (Jiang *et al.*, 2019). Further studies are needed to determine how different ROS and retrograde signals generated in different compartments are coordinated to trigger plant acclimation to excess light or other stresses.

Acknowledgements

We thank Dr Enamul Huq for the *phyB-9* PHYB-GR1 seeds and Dr Clark Lagarias for the *phyB-5* YHB seeds. This work was supported by funding from the National Science Foundation (IOS-2110017; IOS-1353886; MCB-1936590; IOS-1932639) and the Interdisciplinary Plant Group, University of Missouri.

Competing interests

None declared.

Author contributions

YF, HX, JMH, EL and RM designed the research. YF, JM and HL performed the research. YF, JM, SS, RKA, EL and RM analyzed the data. YF, EL, JMH and RM wrote the paper with contributions from all authors.

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Data availability

Data supporting the findings of this work are provided in the main paper and Supporting Information. Raw and processed RNA-Seq data files were deposited in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under the accession no. GSE188732.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Wavelength spectrum of LED light sources used in this study.

Fig. S2 Reactive oxygen species accumulation in wild-type, *phyB-9*, respiratory burst oxidase protein D, and respiratory burst oxidase protein F plants in response to different intensities of red light.

Fig. S3 Stomatal aperture of wild-type, *phyB-9*, respiratory burst oxidase protein D (*rbohD*), respiratory burst oxidase protein F (*rbohF*), and *rbohD rbohF* plants subjected to a 10-min excess white or red light.

Fig. S4 Chloroplast structure, size, and number in mesophyll cells from 4-wk-old wild-type and *phyB-9* plants.

Fig. S5 Reactive oxygen species accumulation in wild-type and *phyB-9* plants in response to paraquat treatment under dark, ambient 50 μmol photons m^{-2} s^{-1} or excess white (740 μmol photons m^{-2} s^{-1}) or red light (120 μmol photons m^{-2} s^{-1}) conditions.

Fig. S6 Transcript expression (qPCR) analysis following excess white or red light treatment of wild-type, *phyB-9*, respiratory burst oxidase protein D, and respiratory burst oxidase protein F plants.

Fig. S7 Phytochrome B, respiratory burst oxidase protein D, and respiratory burst oxidase protein F regulate the expression of hundreds of transcripts in response to excess red light stress.

Fig. S8 Gene ontology annotation of the 2217 (Dataset S22; A) white light-, and 393 (Dataset S28; B) red light-, response transcripts identified as described in Figs 2 and S7.

Fig. S9 Cytosolic and nuclear localization of phytochrome B (phyB) in *phyB-9* and *phyB-9* PHYB-GR1 plants in the presence or absence of dexamethasone.

Fig. S10 *phyB-9* PHYB-GR1 phenotype in response to different illumination regimes and application of dexamethasone.

Fig. S11 *phyB-5* YHB phenotype in response to different illumination regimes.

Dataset S1 Significant upregulated transcripts in wild-type plants following 10-min white light.

Dataset S2 Significant upregulated transcripts in phytochrome B plants following 10-min white light.

Dataset S3 Significant upregulated transcripts in respiratory burst oxidase protein D plants following 10-min white light.

Dataset S4 Significant upregulated transcripts in respiratory burst oxidase protein F plants following 10-min white light.

Dataset S5 Significant downregulated transcripts in wild-type plants following 10-min white light.

Dataset S6 Significant downregulated transcripts in phytochrome B plants following 10-min white light.

Dataset S7 Significant downregulated transcripts in respiratory burst oxidase protein D plants following 10-min white light.

Dataset S8 Significant downregulated transcripts in respiratory burst oxidase protein F plants following 10-min white light.

Dataset S9 Significant upregulated transcripts in wild-type plants following 10-min red light.

Dataset S10 Significant upregulated transcripts in phytochrome B plants following 10-min red light.

Dataset S11 Significant upregulated transcripts in respiratory burst oxidase protein D plants following 10-min red light.

Dataset S12 Significant upregulated transcripts in respiratory burst oxidase protein F plants following 10-min red light.

Dataset S13 Significant downregulated transcripts in wild-type plants following 10-min red light.

Dataset S14 Significant downregulated transcripts in phytochrome B plants following 10-min red light.

Dataset S15 Significant downregulated transcripts in respiratory burst oxidase protein D plants following 10-min red light.

Dataset S16 Significant downregulated transcripts in respiratory burst oxidase protein F plants following 10-min red light.

Dataset S17 Exclusive wild-type (WT) transcripts from overlap between WT altered transcripts and phytochrome B (*phyB*) altered transcripts following white light (phyB-dependent transcripts).

Dataset S18 Exclusive wild-type (WT) transcripts from overlap between WT altered transcripts and respiratory burst oxidase protein D altered transcripts following white light (RBOHD-dependent transcripts).

Dataset S19 Overlap between phytochrome B- and respiratory burst oxidase protein D-dependent transcripts in wild-type following white light.

Dataset S20 Exclusive wild-type (WT) transcripts from overlap between WT altered transcripts and respiratory burst oxidase protein F altered transcripts following white light (RBOHF-dependent transcripts).

Dataset S21 Overlap between phytochrome B- and respiratory burst oxidase protein F-dependent transcripts in wild-type following white light.

Dataset S22 Overlap between phytochrome B-, respiratory burst oxidase protein D-, and respiratory burst oxidase protein F-dependent transcripts in wild-type following white light.

Dataset S23 Exclusive wild-type (WT) transcripts from overlap between WT altered transcripts and phytochrome B (*phyB*) altered transcripts following red light (phyB-dependent transcripts).

Dataset S24 Exclusive wild-type (WT) transcripts from overlap between WT altered transcripts and respiratory burst oxidase protein D altered transcripts following red light (RBOHD-dependent transcripts).

Dataset S25 Overlap between phytochrome B- and respiratory burst oxidase protein D-dependent transcripts in wild-type following red light.

Dataset S26 Exclusive wild-type (WT) transcripts from overlap between WT altered transcripts and respiratory burst oxidase protein F altered transcripts following red light (RBOHF-dependent transcripts).

Dataset S27 Overlap between phytochrome B- and respiratory burst oxidase protein F-dependent transcripts in wild-type following red light.

Dataset S28 Overlap between phytochrome B-, respiratory burst oxidase protein D-, and respiratory burst oxidase protein F-dependent transcripts in wild-type following red light.

Dataset S29 Primers sequences used for qRT-PCR.

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