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Linking phytochrome to plant immunity: low red : far-red ratios increase Arabidopsis susceptibility to *Botrytis cinerea* by reducing the biosynthesis of indolic glucosinolates and camalexin

Miriam D. Cargnel[1]*, Patricia V. Demkura[1]* and Carlos L. Ballaré[1,2]

[1]IFEVA, Consejo Nacional de Investigaciones Científicas y Técnicas–Universidad de Buenos Aires, C1417DSE Buenos Aires, Argentina; [2]IIB-INTECH, Consejo Nacional de Investigaciones Científicas y Técnicas–Universidad Nacional de San Martín, B1650HMP Buenos Aires, Argentina

Author for correspondence:
Carlos L. Ballaré
Tel: +54 11 4524 8070
Email: ballare@ifeva.edu.ar

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Summary

- Shade-intolerant plants respond to low red : far-red (R : FR) ratios, which signal the proximity of potential competitors, by down-regulating immune responses. Here we investigated the mechanisms underlying this immune suppression in Arabidopsis.
- We used genetic, transcriptomic and metabolomic approaches to examine the functional connections between R : FR ratio and Arabidopsis resistance to the fungus *Botrytis cinerea*.
- Low R : FR ratios reduced the concentration of indol-3-ylmethyl glucosinolate (I3M) (an indolic glucosinolate, iGS) and camalexin in plants inoculated with *B. cinerea*, and attenuated the I3M response triggered by jasmonate elicitation. These effects on metabolite abundance correlated with reduced expression of iGS and camalexin biosynthetic genes. Furthermore, the effect of low R : FR increasing Arabidopsis susceptibility to *B. cinerea* was not present in mutants deficient in the biosynthesis of camalexin (*pad3*) or metabolism of iGS (*pen2*). Finally, in a mutant deficient in the JASMONATE ZIM DOMAIN-10 (JAZ10) protein, which does not respond to low R : FR with increased susceptibility to *B. cinerea*, supplemental FR failed to down-regulate iGS production.
- These results indicate that suppression of Arabidopsis immunity against *B. cinerea* by low R : FR ratios is mediated by reduced levels of Trp-derived defenses, and provide further evidence for a functional role of JAZ10 in the link between phytochrome and jasmonate signaling.

Introduction

Under natural conditions, plants have to deal with multiple biotic stressors, including pathogens, herbivores and competitors. Plant fitness depends on appropriate responses to these stressors. Because these adaptive responses entail physiological and ecological costs, trade-offs often exist among them. For example, allocating resources to defense may entail an opportunity cost (or trade-off) in terms of reduced growth potential and competitive ability, and vice versa. Therefore, plant fitness requires good decisions between alternative resource allocation pathways. In shade-intolerant species, elongation and other shade-avoidance responses are rapidly activated when the plant is challenged by competitors (Ballaré *et al.*, 1990), and these responses often correlate with reduced expression of chemical defenses against pathogens and herbivores (McGuire & Agrawal, 2005; Izaguirre *et al.*, 2006). The reduced investment in defenses may free up resources that the plant could devote to growth to escape competition, which makes functional sense given the strong penalties associated with losing the race for light in rapidly growing canopies (Ballaré *et al.*, 1988; Schmitt *et al.*, 1995).

Repression of defense responses in plants or plant parts that experience a high risk of competition in crowded stands is a consequence of changes in the light environment sensed by the plant's informational photoreceptors. Both phytochrome B (phyB) and UVR8 are positive regulators of plant immunity (Ballaré *et al.*, 2012). The role of phyB as a modulator of plant defense is well characterized. Plants grown under sunlight in the absence of competition receive a ratio of red : far-red radiation (R : FR ratio) of *c.* 1.2. Under these conditions, most phyB molecules are in the active, FR-absorbing form (Pfr). When the density of a plant canopy increases, the R : FR ratio drops, as a consequence of the strong absorption of R photons by chlorophylls. In turn, this reduction in the R : FR ratio determines that an increasing fraction of the phyB molecules are photoconverted into the inactive, R-absorbing form of the photoreceptor (Pr) (Smith, 1995; Ballaré, 2009; Casal, 2012; Pierik & de Wit, 2014). Pfr is a positive regulator of the two principal hormonal regulators of plant immunity: jasmonate (JA) and salicylic acid (SA) (Moreno *et al.*, 2009; Cerrudo *et al.*, 2012;

*These authors contributed equally to this work.

De Wit *et al.*, 2013; Izaguirre *et al.*, 2013). Therefore, low R : FR ratios lead to attenuation of JA and SA signaling, resulting in increased susceptibility to pathogens and herbivorous insects (reviewed in Ballaré, 2014). Similarly, solar ultraviolet-B radiation, perceived by the photoreceptor UVR8 (Rizzini *et al.*, 2011), activates the synthesis of defense-related secondary metabolites (Demkura & Ballaré, 2012). Therefore under conditions of shading, the low levels of ultraviolet-B radiation (Grant, 1991; Flint & Caldwell, 1998), could limit the accumulation of chemical defenses, thereby contributing to plant susceptibility to pathogen infection. These effects of canopy light quality, down-regulating plant defense under crowded conditions, may have important implications for our understanding of density-dependent mortality under natural conditions (Bell *et al.*, 2006) and plant health in modern (high density) agricultural settings (Ballaré *et al.*, 2012).

The connection between phyB and JA signaling has been studied in some detail. JA plays a critical role orchestrating plant defenses against herbivorous insects and necrotrophic pathogens (Howe & Jander, 2008). JA is perceived by a co-receptor complex formed by the ubiquitin E3 ligase complex Skip1-Cullin1-F-box protein CORONATINE INSENSITIVE 1 (SCF^{COI1}) and JASMONATE ZIM DOMAIN (JAZ) proteins (Browse, 2009). JA-Ile, the bioactive form of JA, stimulates the specific binding of COI1 and JAZ proteins, which leads to ubiquitination of JAZs by SCF^{COI1} and their subsequent degradation by the 26S proteasome. JAZs are repressors of transcription factors that are positive regulators of JA-responsive genes. Therefore, degradation of JAZs activates JA-induced defense mechanisms (Browse, 2009; Pauwels & Goossens, 2011; Kazan & Manners, 2012). Recent work in several species has shown that low R : FR ratios reduce plant sensitivity to JA, leading to reduced defense responses against pathogens and insects (Moreno *et al.*, 2009; Cerrudo *et al.*, 2012; De Wit *et al.*, 2013; Izaguirre *et al.*, 2013; Kegge *et al.*, 2013). The mechanism linking phyB with JA signaling has received significant attention in recent years (Ballaré, 2011, 2014; Kazan & Manners, 2011; Moreno & Ballaré, 2014), and appears to involve changes in the abundance of JAZ repressor proteins, DELLA proteins (which interfere with JAZs, Hou *et al.*, 2010), and key transcription factors involved in the activation of JA responses, such as MYC2 (Chico *et al.*, 2014; Leone *et al.*, 2014).

Phytochrome modulation of JA sensitivity is, therefore, at the heart of a mechanism that balances the distribution of resources between growth and defense as a function of the perceived risk of competition. However, although reduced JA sensitivity has been shown to correlate with reduced expression of a large number of metabolites with potential anti-herbivore and antifungal activities (Ballaré, 2014), the specific causal connections that link phyB with plant resistance to herbivores and pathogens have not been identified.

Glucosinolates (GS) are important defenses in members of the order Brassicales (Grubb & Abel, 2006; Halkier & Gershenzon, 2006). More than 100 GS have been identified. GS share a chemical structure consisting of a β-D-glucopyranose residue linked, via a sulfur atom, to an *N*-hydroximinosulfate ester and a variable R group, which is derived from an amino acid (Fig. 1). GSs are classified according to the R group. Aliphatic GS (aGS) are derived from Ala, Leu, Ile, Met, or Val; aromatic GS are derived from Phe or Tyr, and indole glucosinolates (iGS) are derived from Trp. Upon tissue damage (e.g. during herbivory or attack by a necrotrophic fungus) GS are activated by endogenous thioglucosidases (myrosinases), which generate several different hydrolysis products that can be toxic to the attacker (Grubb & Abel, 2006; Halkier & Gershenzon, 2006). aGS are the major class of GS in young rosette leaves in Arabidopsis (Brown *et al.*, 2003; Mewis *et al.*, 2005), and play a role in biotic defense and plant interactions with pathogens and herbivorous insects (Giamoustaris & Mithen, 1995; Tierens *et al.*, 2001). iGS, along with another secondary metabolite derived from Trp, camalexin (Glawischnig, 2007), are also important defenses (Ferrari *et al.*, 2003; Kliebenstein, 2004; Kliebenstein *et al.*, 2005; Lipka *et al.*, 2005; Bednarek *et al.*, 2009; Sanchez-Vallet *et al.*, 2010; Schlaeppi *et al.*, 2010; Bednarek, 2012). aGS and iGS were shown to have an additive effect reducing the attack of unadapted herbivores in Arabidopsis (Müller *et al.*, 2010). The regulatory networks that control the induction and expression of GS and camalexin by biotic stressors are largely unknown (Ahuja *et al.*, 2012). The information on the effects of photoreceptors on the biosynthesis and metabolism of these compounds is particularly limited. Work in watercress has shown that the constitutively high levels of gluconasturtiin in this species can be lowered by exposing plants to light enriched in FR radiation (Engelen-Eigles *et al.*, 2006), suggesting that phytochrome could modulate GS levels. Camalexin levels have been reported to be modulated by UV-C radiation (Mert-Turk *et al.*, 2003; Glawischnig, 2007), but the ecological significance of this effect is unclear, as UV-C quanta are not present in the natural daylight spectrum received at ground level.

Phenolic compounds, derived from Phe through the phenylpropanoid biosynthetic pathway (D'Auria & Gershenzon, 2005) can also play a role in pathogen defense. For example, sinapates can serve as precursors of syringyl lignin, which is involved in cell wall fortification (Kishimoto *et al.*, 2006; Quentin *et al.*, 2009; Lloyd *et al.*, 2011). The increased resistance of Arabidopsis plants to *Botrytis cinerea* caused by activation of the UVR8 receptor by ultraviolet-B radiation has been attributed to the accumulation of increased levels of sinapates (Demkura & Ballaré, 2012). Soluble sinapates were also shown to be involved in Arabidopsis defense against the fungus *Verticillium longisporum* (König *et al.*, 2014). Both JA and phytochrome are known to regulate the phenylpropanoid pathway (Mancinelli *et al.*, 1991; Creelman & Mullet, 1997). However, the significance of phyB regulation of phenolic compounds for Arabidopsis resistance to biotic stressors has not been investigated.

In this paper, we examined the effects of low R : FR ratios on Trp-, Met- and Phe-derived metabolites and their potential significance in mediating the effects of R : FR ratio on Arabidopsis resistance to the necrotrophic fungus *B. cinerea*. We found that low R : FR ratios reduced I3M accumulation (and I3M induction in response to methyl jasmonate (MeJA)), and lowered camalexin levels in plants inoculated with *B. cinerea*. These effects on

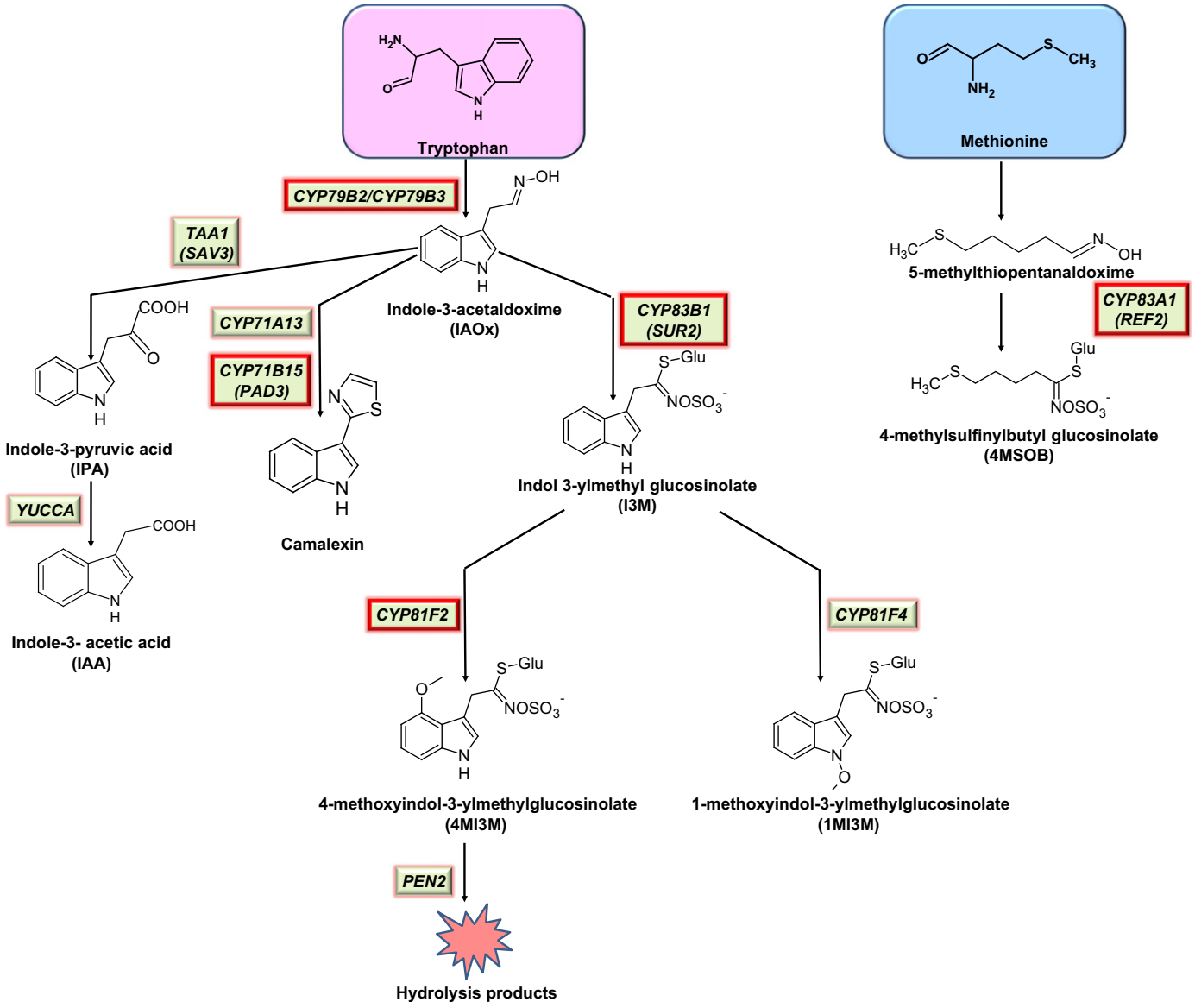

Fig. 1 Simplifed schematic representation of the pathways of glucosinolate (GS) and camalexin biosynthesis in *Arabidopsis thaliana* (according to Schlaeppi *et al.*, 2010; Yatusevich *et al.*, 2010); key genes whose expression was measured in this study are indicated in boxes with a red border. Auxin biosynthesis via the TAA1 pathway (Tao *et al.*, 2008) is also represented according to the model of Mashiguchi *et al.* (2011).

metabolite abundance correlated with reduced expression of genes involved in iGS and camalexin biosynthesis, such as *CYP79B2*, *CYP83B1 (SUR2)* and *PAD3*. Furthermore, the effect of low R : FR ratios increasing Arabidopsis susceptibility to *B. cinerea* was not present in mutants deficient in the biosynthesis of camalexin (*pad3*) or bioactivation of iGS (*pen2*). Finally, in a mutant deficient in *JAZ10*, in which the effect of low R : FR ratios increasing plant susceptibility to *B. cinerea* is known to be attenuated (Cerrudo *et al.*, 2012), supplemental FR failed to down-regulate I3M production. Taken together, these results indicate that the effect of low R : FR ratio increasing Arabidopsis susceptibility to *B. cinerea* is mediated by reduced biosynthesis of Trp-derived defense metabolites, and provide strong evidence of a functional role for JAZ10 in the link between phyB and JA signaling.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh seeds were germinated as described previously (Moreno *et al.*, 2009). Seven days after germination, seedlings were transferred to individual pots (0.11 l) with a vermiculite : perlite : peat 1 : 1 : 1 mixture and watered every 2 days with Hakaphos Rojo solution 18-18-18 (Compo). Plants were grown in a growth chamber under short-day conditions (10 h : 14 h, light : dark cycles, 22°C, 150 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) provided by fluorescent bulbs). Rosette-stage plants of similar age and size (typically 3–4-wk-old) were selected for experiments and randomly assigned to the treatments. The Columbia (Col-0) ecotype of *A. thaliana* was

used as the wild type control in all experiments. The *phyB-9* mutant (Reed *et al.*, 1993) and *jaz10* null mutant *(SAIL_92_D08)* were obtained from the ABRC (www.arabidopsis.org). The *sav3-2* mutant was obtained from the laboratory of Joanne Chory (Tao *et al.*, 2008), and the *pad3-1*, *pen2-1*, and *pad3 pen2* double mutant were kindly provided by Paul Schulze-Lefert (Max Planck Institute for Plant Breeding Research, Cologne, Germany).

Light treatments

Arabidopsis plants cultivated in the growth chamber under 150 μmol m^{-2} s^{-1} PAR from fluorescent bulbs were placed in front an array of water-cooled incandescent lamps covered with either opaque screens ('Amb, ambient' light treatment) or FR-transmitting filters ('FR' treatment), as described previously (Cerrudo *et al.*, 2012). The FR treatment reduced the R : FR ratio of the integrated horizontal radiation to *c.* 0.55. Previous studies in canopies of mustard (*Sinapis alba*) and chamico (*Datura ferox*) seedlings indicated that this R : FR ratio in the horizontal light flux corresponds to a leaf area index of *c.* 0.5, in which mutual shading among neighboring plants is negligible (Ballaré *et al.*, 1991). Therefore, this light treatment produces a realistic simulation of the light environment perceived by a plant grown in the proximity of non-shading neighbors, without affecting temperature or PAR levels. Plants were exposed to the light treatments (Amb or FR) for 4 days before the elicitation experiments and *B. cinerea* infection bioassay. The light treatments were maintained until harvest for gene expression or metabolite analysis, or until the completion of the infection bioassay.

Fungal culture and *B. cinerea* bioassays

Botrytis cinerea Pers.:Fr was grown and maintained on potato dextrose agar (1.5% agar, 2% potato extract, 2% dextrose). Conidia were collected from agar plates with distilled water and a glass rod, filtered and resuspended in a 0.1 M sucrose/0.07 M KH_2PO_4 solution to induce germination (Elad, 1991). In the infection bioassays, five leaves of 4-wk-old rosettes were inoculated on the adaxial surface with a 5 μl droplet of spore suspension (5×10^5 conidia ml^{-1}). Plants (control and inoculated) were kept in individual open-top chambers made of clear polyester to prevent desiccation. After 48–72 h, infected leaves were collected and scanned with a HP Scanjett 4500c (Hewlett-Packard, Palo Alto, CA, USA). Lesion areas were measured using Adobe Photoshop software (version 7.0; Adobe Systems, San Jose, CA, USA). For gene expression and metabolite analyses, 10–12 leaves of 4-wk-old rosettes were inoculated; rosettes were harvested and immediately frozen in liquid nitrogen at different time points after inoculation: 24 h (for gene expression analysis), 48 h (for quantification of GS and camalexin).

MeJA treatments

The effect of a reduced R : FR ratio on plant responses to JA was assessed by spraying 4-wk-old, soil-grown rosettes with a 200 μM MeJA (Sigma-Aldrich) solution. Plants not assigned to the JA treatment were sprayed with distilled water, which was supplemented with ethanol in the same proportion (0.04‰) as that used to dissolve MeJA in the solution used for the JA treatment. Rosettes were harvested and immediately frozen in liquid nitrogen at different time points after MeJA treatment: 24 h (for gene expression analysis), 48 h (for quantification of GS and camalexin) or 72 h (determination of phenolic compounds).

Leaf phenolics

Leaf phenolics were determined following established protocols (Demkura & Ballaré, 2012). Briefly, freeze-dried tissue without the midvein (10–15 mg) was ground in a mortar and transferred to an Eppendorf tube with 1.5 ml of a methanol: 0.25% acetic acid mixture (2 : 3 v/v). Samples were vortexed for 45 sec and centrifuged at 13 600 ***g*** for 20 min. The supernatant was filtered through a 45 μm syringe filter and kept at −20°C until use. Phenolics were separated by HPLC (Knauer Euroline) on a Restek Pinnacle II C18 (5.0 μm, 4.6 × 150 mm) column with solvents A (0.25% aqueous H_3PO_4) and B (acetonitrile), eluted with a gradient of 5% B at 0 min, 50% B at 22 min, 5% B at 25 min, with an equilibration time of 10 min and a flow rate of 1 ml min^{-1}. The injection volume was 20 μl, and elution was monitored with a diode array detector at 230, 305, and 320 nm. Sinapoyl malate, kaempferol and quercetin glycosides were identified by comparing their UV spectra and relative retention times with previously published data (Besseau *et al.*, 2007).

Leaf glucosinolates

GS were extracted and quantified using previously described protocols (Brown *et al.*, 2003). Freeze-dried tissue without the midvein (25–30 mg) was ground in a mortar and transferred to an Eppendorf tube. Each tube was filled with 1.2 ml of 70% methanol containing 0.125 μmol of sinigrin (2-propenyl- glucosinolate, Sigma-Aldrich) as internal standard. Samples were incubated for 5 min at 70°C in a water bath and centrifuged at 13 600 ***g*** for 2 min; two more aliquots of 70% methanol were added to repeat the extraction procedure and supernatants were combined. After extraction, supernatants were transferred into columns filled with 400 μl (75 mg) of DEAE-Sephadex A-25 (previously equilibrated with 600 μl of 70% methanol and 600 μl of MilliQ water); loaded columns were washed again with 600 μl 70% methanol and 600 μl of MilliQ water. To desulfate GSs retained in the column, 25 μl of arylsulphatase solution (Sigma-Aldrich; H-1 from *Helix pomatia*, prepared as described Graser *et al.*, 2001) resuspended in 600 μl of 0.02 M sodium acetate buffer pH 5.2 were added, and capped columns were incubated overnight at room temperature. After incubation, desulphoglucosinolates were eluted with 400 μl of MilliQ water.

Desulphoglucosinolates were analyzed by high-performance liquid chromatography (HPLC, KnauerEuroline) on a Restek Pinnacle II C18 (5.0 μm, 4.6 × 150 mm) column with solvents A (water) and B (20% acetonitrile), eluted with a gradient of 1% B at 0 min, 10% B at 10 min, 75% B at 22–24 min, with an

equilibration time of 10 min and a flow rate of 1 ml min^{-1}. The injection volume was 20 µl, and elution was monitored with a diode array detector at 229 nm. The major aGS in the Col-0 background, 4-methylsulfinylbutyl (4MSOB), and the iGS indol-3-ylmethyl (I3M), 4-methoxy-indol-3-ylmethyl GS (4MI3M), and 1-methoxy-indol-3-ylmethyl GS (1MI3M) were identified on the basis of their relative retention times and UV spectra. To calculate molar concentrations of individual GS, relative response factors (Brown *et al.*, 2003) were used to correct for absorbance difference between the internal standard and other compounds. Solvents used for determination of leaf phenolics and GS were purchased from Sintorgan (Buenos Aires, Argentina).

Camalexin

Camalexin levels were determined by HPLC following the protocol described by Beets & Dubery (2011) with minor modifications. Freeze-dried tissue without the midvein (10–12 mg) was ground in a mortar and transferred to an Eppendorf tube with 400 µl of 80% methanol. Samples were vortexed for 45 sec and centrifuged at 13 600 ***g*** for 5 min. The supernatant was recovered and a second aliquot of 80% methanol was added to the pellets. The extraction process was repeated, the supernatants were combined and filtered through a 45-µm syringe filter and kept at −20°C until use. Camalexin was analyzed by HPLC (Knauer-Euroline) on a Restek Pinnacle II C18 (5.0 µm, 4.6 × 150 mm) column with solvents A (water) and B (acetonitrile), eluted with a gradient of 10% B at 0 min, 98% B at 13–20 min, with an equilibration time of 10 min and a flow rate of 1 ml min^{-1}. The injection volume was 20 µl, and elution was monitored with a diode array detector at 318 nm. Identity of the camalexin peak in the samples was determined by comparing its retention time and UV spectrum with that of a purified camalexin standard (donated by Klaus Schlaeppi; Max Planck Institute for Plant Breeding Research, Cologne, Germany), and by confirming the absence of this peak in the *pad3-1* mutant.

Gene expression

Total RNA was extracted from 100 mg of frozen tissue using the LiCl–phenol/chloroform method (Izaguirre *et al.*, 2003). Purified fractions of total RNA were subjected to RQ1 (RNase-free) DNase treatment (Promega) to avoid contamination with genomic DNA. For cDNA synthesis, fractions of 1 µg of RNA were reverse transcribed using oligo(dT) as primer and M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The obtained cDNA samples were diluted 1 : 10 before use. Quantitative real-time polymerase chain reaction (qPCR) was performed in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) following the manufacturer's standard method for absolute quantification using FastStart Universal SYBR Green Master Mix (Roche Applied Science, Penzberg, Germany) and primers at a final concentration of 500 nM. The *A. thaliana UBIQUITIN* (*UBC*) gene was used to normalize for differences in concentrations of cDNA samples. Forward and reverse primer sequences were the following: 5′-CTGCGACTCAGGGAATCTTCTA-3′ and 5′-TTGTGCCATTGAATTGAACCC-3′ (*UBC*); 5′-TCACGCCATATCTACCAGC-3′ and 5′-TGACGTTGACCGTTGTTCCT-3′ (*CYP83A1*); 5′-CTCGCGAGACTTCTTCAAGG-3′ and 5′-CCATAACCAACGGTTTAGCC-3′ (*CYP79B2*); 5′-AGTCACTTCCGAACACTCA-3′ and 5′-TCGCAGGTTACCATATTCC-3′ (*CYP79B3*); 5′-TGGCTATGCGTAAACTCGTG-3′ and 5′-CCGGTAAACTTCAAAATGGTG-3′ (*CYP81F2*).

Statistical analysis

Statistical analyses were carried out using INFOSTAT software (professional version 1.1). Infections bioassays were analyzed using analysis of variance (ANOVA) with light and genotype as factors. Data on gene expression and metabolites (soluble phenolic compounds, camalexin, and GS) were analyzed using a two-way ANOVA with light and MeJA or *B. cinerea* infection as factors; each genotype was analyzed separately. When interaction terms were significant ($P < 0.05$), differences between means were analyzed using Tukey comparisons. Appropriate transformations of the primary data were used when needed to meet the assumptions of the analysis.

Results

Low R : FR ratios reduce the accumulation of GS

Previous microarray studies (Cerrudo *et al.*, 2012; De Wit *et al.*, 2013) have demonstrated that low R : FR ratios can decrease the expression of important transcription factors that regulate genes involved in Trp and iGS biosynthesis, such as ATR1/MYB34 (Bender & Fink, 1998; Celenza *et al.*, 2005), and aGS biosynthesis (MYB28, MYB29, and MYB76) (Hirai *et al.*, 2007; Gigolashvili *et al.*, 2009; Sønderby *et al.*, 2010) (Supporting Information Fig. S1). We investigated the interactive effects of low R : FR ratios and elicitation with MeJA or *B. cinerea* on the expression of GS biosynthetic genes. *SUR2*, a target of MYB34, encodes the cytochrome P450 CYP83B1 that catalyzes a critical step in iGS biosynthesis from indole-3-acetaldoxime (IAOx) (Barlier *et al.*, 2000; Bak *et al.*, 2001) (Fig. 1). Both MeJA treatment and inoculation with *B. cinerea* increased the expression of *SUR2*. Low R : FR ratios reduced *SUR2* expression levels, and strongly depressed the transcriptional response induced by *B. cinerea* inoculation (Fig. 2a,b). *CYP79B2* and *CYP79B3*, which are involved in the synthesis of IAOx from Trp (Fig. 1), also tended to be up-regulated at the transcriptional level by MeJA, and down-regulated by FR (Fig. S2).

To gain insight into the effects of light quality on aGS biosynthetic genes, we measured mRNA levels of *CYP83A1/REF1*. CYP83A1 catalyzes a critical step in the synthesis of the aGS 4-methylsulfinylbutyl glucosinolate (4MSOB) from 5-methylthiopentanaldoxyme (Fig. 1), and it is regulated at the transcriptional level by MYB28, MYB29, MYB76 and other transcription factors (Yatusevich *et al.*, 2010). Transcript levels of *CYP83A1* were not affected by MeJA, and were marginally

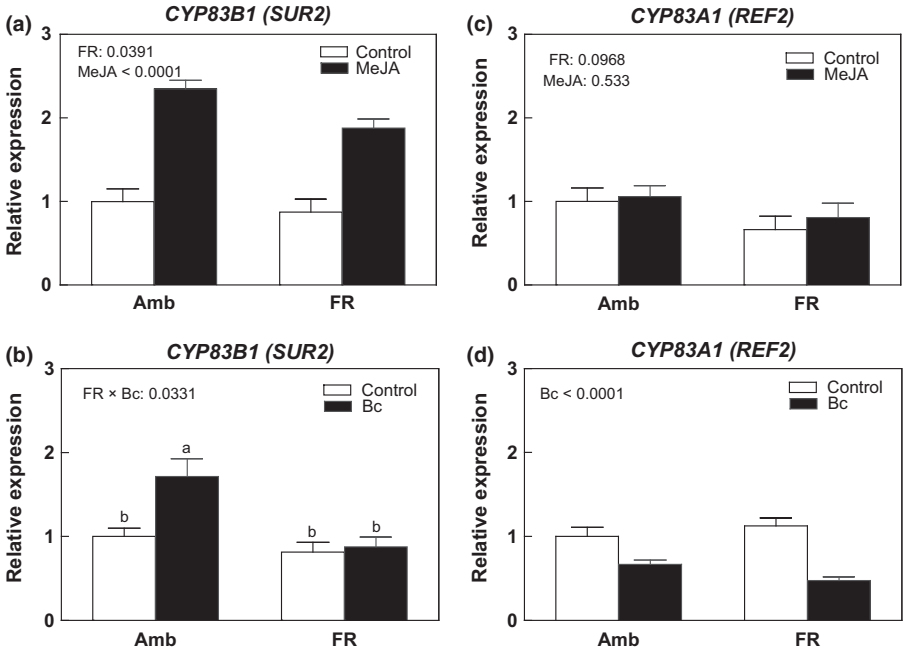

Fig. 2 Low red : far-red (R : FR) ratios down-regulate *CYP83B1/SUR2* (involved in indolic glucosinolate biosynthesis), with minimal effects on *CYP83A1* expression (involved in the biosynthesis of aliphatic glucosinolates) in *Arabidopsis thaliana*. (a, b) *CYP83B1/SUR2* mRNA levels; (c, d) *CYP83A1/REF2* mRNA levels. The experimental treatments resulted from a factorial combination of R : FR and methyl jasmonate (MeJA) (a, c) or R : FR and *Botrytis cinerea* (Bc) inoculation (b, d): Amb, white light; FR, white light supplemented with FR radiation (for details, see the Materials and Methods section). Samples for qPCR analysis were obtained 24 h after MeJA application or *B. cinerea* inoculation. Expression data are normalized to the expression level detected in the Control × Amb combination. Error bars, + 1 SE ($n = 3$; each biological replicate is a pool of three individual plants). The *P*-values for significant terms in the factorial ANOVA are indicated in each panel; differences between means are indicated by different letters in those cases in which the interaction term of the factorial analysis was significant ($P < 0.05$).

down-regulated in plants exposed to supplemental FR radiation (Fig. 2c), a finding that is consistent with the pattern described for the expression of *MYB28* and *MYB29* (Fig. S1). Inoculation with *B. cinerea* reduced *CYP83A1* mRNA levels, without a significant effect of supplemental FR on this response (Fig. 2d).

At the metabolite level, Arabidopsis plants responded to MeJA by increasing the accumulation of indol-3-ylmethyl GS (I3M), as reported previously in other studies (Brader *et al.*, 2001; Mewis *et al.*, 2005; Guo *et al.*, 2013). We found that this response was greatly reduced when the plants were exposed to low R : FR ratios (Fig. 3). Mutant *phyB* plants had constitutively low levels of I3M, and although these levels increased in response to MeJA, the induced levels were similar to those of non-induced Col-0 plants (*c.* 0.5 μmol g^{-1}; Fig. S3). Importantly, the effect of FR reducing JA-induced I3M accumulation was conserved in the *sav3-2* mutant (Fig. S4). This mutant is impaired in auxin biosynthesis via the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1) pathway (Fig. 1), which is activated in Arabidopsis in response to low R : FR ratios (Tao *et al.*, 2008). In healthy plants, neither MeJA nor supplemental FR radiation affected the levels of 4-methoxy-indol-3-ylmethyl GS (4MI3M) (Fig. 3), which is the substrate of the PEN2 (PENETRATION2) myrosinase (see Fig. 1). This is consistent with the observation that the expression of *CYP81F2*, which encodes a P450 monooxygenase that is essential for the pathogen-induced conversion of I3M into 4MI3M (Bednarek *et al.*, 2009) (see Fig. 1), was unaffected by MeJA or light quality (Fig. 4a).

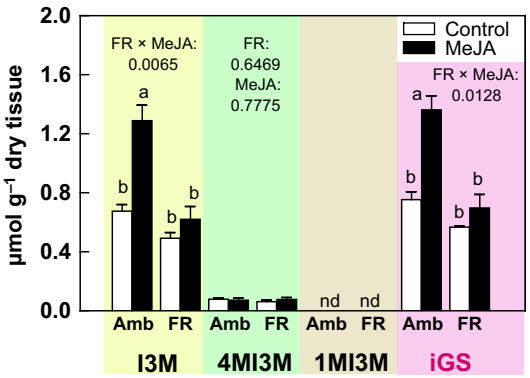

Fig. 3 Low red : far-red (R : FR) ratios decrease total indolic glucosinolate levels (iGS) and repress the iGS response triggered by jasmonate (JA) treatment. Indol-3-ylmethyl GS (I3M), 4-methoxy-indol-3-ylmethyl GS (4MI3M) and 1-methoxy-indol-3-ylmethyl GS (1MI3M) were quantified from *Arabidopsis thaliana* leaf tissue by HPLC 2 days after treatment with 200 μM methyl JA (MeJA). Open bars, control; closed bars, MeJA. Each bar represents the mean + 1 SE of four biological replicates (each replicate is a pool of three individual plants). Amb, white light; FR, white light supplemented with FR radiation (for details, see the Materials and Methods section). The *P*-value for the MeJA × FR interaction term of the ANOVA is shown. Different letters indicate significant differences between means ($P < 0.05$, Tukey test). nd, not detectable.

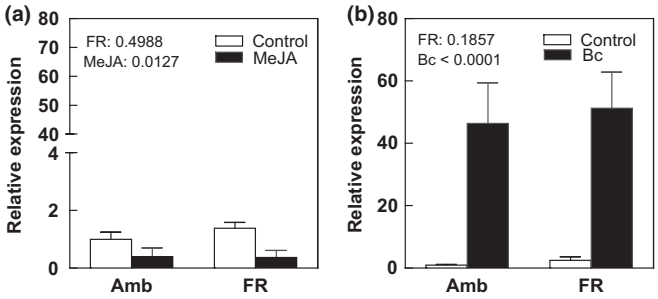

Fig. 4 Low red: far-red (R : FR) ratios do not affect mRNA levels of *CYP81F2*, which encodes a P450 monooxygenase that is essential for the pathogen-induced accumulation of 4-methoxy-indol-3-ylmethyl GS (4MI3M) and is strongly up-regulated in *Arabidopsis thaliana* plants infected with *Botrytis cinerea*. The experimental treatments resulted from a factorial combination of R : FR and methyl JA (MeJA) (a) or R : FR and *B. cinerea* (Bc) inoculation (b). Amb, white light; FR, white light supplemented with FR radiation (for details, see the Materials and Methods section). Samples for qPCR analysis were obtained 24 h after MeJA application or *B. cinerea* inoculation. Expression data are normalized to the expression level detected in the control × Amb combination. Error bars, + 1 SE ($n = 3$; each biological replicate is a pool of three individual plants). The *P*-values for significant terms in the factorial ANOVA are indicated in each panel.

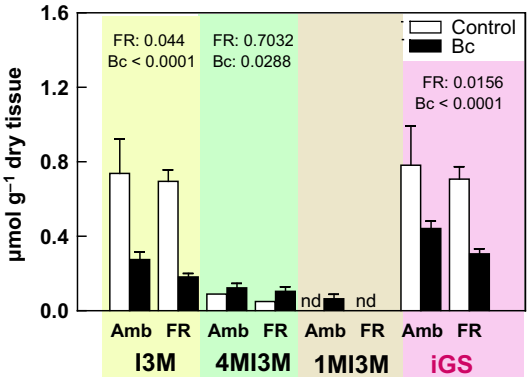

Fig. 5 Low red : far-red (R : FR) ratios down-regulate total indolic glucosinolate (iGS) levels in healthy and *Botrytis cinerea*-infected *Arabidopsis thaliana* plants. Indol-3-ylmethyl GS (I3M), 4-methoxy-indol-3-ylmethyl GS (4MI3M) and 1-methoxy-indol-3-ylmethyl GS (1MI3M) were quantified from leaf tissue by HPLC 2 days after inoculation with *B. cinerea* (Bc). Open bars, control; closed bars, Bc. Each bar represents the mean + 1 SE of four biological replicates (each replicate is a pool of three individual plants). Amb, white light; FR, white light supplemented with FR radiation (for details, see the Materials and Methods section). Note the significant reduction in the I3M pool in response to *B. cinerea* infection. The *P*-values for significant terms in the factorial ANOVA are indicated. nd, not detectable.

The effects of low R : FR reducing iGS accumulation persisted when Arabidopsis plants were inoculated with *B. cinerea* (Fig. 5). Interestingly, and as reported by other authors (Schlaeppi *et al.*, 2010), fungal inoculation caused a *reduction* in I3M concentrations (Fig. 5), presumably because I3M was rapidly converted into 4MI3M. In fact 4MI3M levels increased (Fig. 5), and the expression of *CYP81F2* was strongly up-regulated in response to infection with *B. cinerea* (Fig. 4b).

Aliphatic GS concentrations did not respond to MeJA (Fig. 6). This lack of response is generally consistent with previous

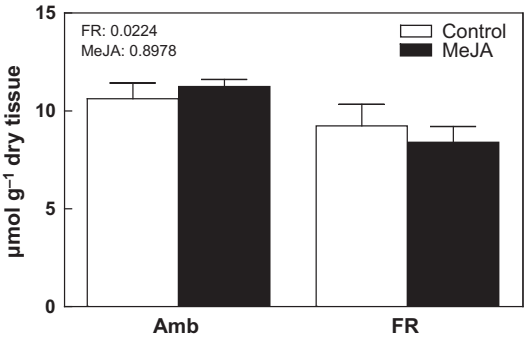

Fig. 6 Levels of aliphatic glucosinolates are not affected by jasmonate (JA), and slightly down-regulated by low red : far-red (R : FR) ratios. 4-Methylsulfinylbutyl (4MSOB) was quantified from *Arabidopsis thaliana* leaf tissue 2 days after treatment with 200 µM methyl JA (MeJA). Open bars, control; closed bars, MeJA. Each bar represents the mean + 1 SE of four biological replicates (each replicate is a pool of three individual plants). Amb, white light; FR, white light supplemented with FR radiation (for details, see the Materials and Methods section). The *P*-values for the main effects in the factorial ANOVA are shown.

findings (Brader *et al.*, 2001; Mewis *et al.*, 2005; Guo *et al.*, 2013). In our experiments, the concentration of 4MSOB was slightly reduced by low R : FR treatments (Fig. 6) or the *phyB* mutation (not shown), which is consistent with the expression patterns displayed by *CYP83A1* (Fig. 2c) and the relevant transcription factors (Fig. S1). Levels of 4MSOB were not affected by inoculation with *B. cinerea* (not shown).

Low R : FR ratios reduce camalexin accumulation in plants infected with *B. cinerea*

Studies of Arabidopsis mutants affected in their capacity to produce camalexin revealed that camalexin plays an important role in resistance to several necrotrophic pathogens (Glawischnig, 2007; Ahuja *et al.*, 2012; Bednarek, 2012). It is now generally accepted that camalexin biosynthesis requires an intact JA signaling pathway (Rowe *et al.*, 2010). However, camalexin accumulation is not normally induced by MeJA (Brader *et al.*, 2001). Camalexin accumulation is strongly induced in response to infection with *B. cinerea*, and plays a role in Arabidopsis defense against this pathogen (Ferrari *et al.*, 2003).

PAD3, a gene that encodes the cytochrome P450 monooxygenase CYP71B15, which catalyzes the final step in camalexin biosynthesis (Schuhegger *et al.*, 2006; Nafisi *et al.*, 2007; Böttcher *et al.*, 2009) (Fig. 1), was not significantly induced by MeJA (data not shown), but was clearly up-regulated in response to *B. cinerea*. The *CYP71B15/PAD3* expression response to infection was strongly attenuated in the presence of low R : FR ratios (Fig. 7).

Several pathogen-mimicking stimuli can induce the expression of camalexin biosynthetic genes; however, the gene expression changes do not always correlate with increased camalexin biosynthesis (references in Ahuja *et al.*, 2012). We investigated the effects of low R : FR ratios on camalexin accumulation in our infection bioassays. Under ambient light, levels of camalexin were undetectable in healthy plants (or plants treated with MeJA, data

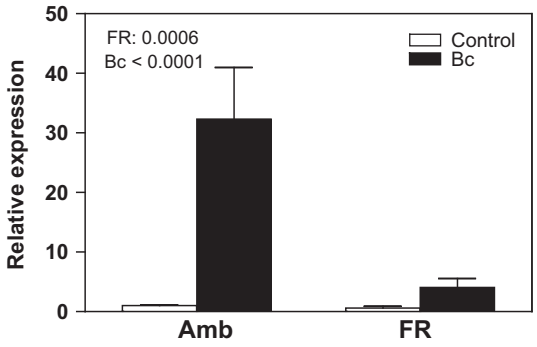

Fig. 7 Low red : far-red (R : FR) ratios down-regulate *CYP71B15/PAD3*, a key gene in the biosynthesis of camalexin, which is strongly expressed in response to inoculation with *Botrytis cinerea* in *Arabidopsis thaliana*. The experimental treatments resulted from a factorial combination of R : FR and *B. cinerea* (Bc) inoculation. Open bars, control; closed bars, Bc. Amb, white light; FR, white light supplemented with FR radiation (for details, see the Materials and Methods section). Samples for qPCR analysis were obtained 24 h after *B. cinerea* inoculation. Expression data are normalized to the expression level detected in the control × Amb combination. Error bars, + 1 SE ($n = 3$; each biological replicate is a pool of three individual plants). The *P*-values for significant terms in the factorial ANOVA are indicated.

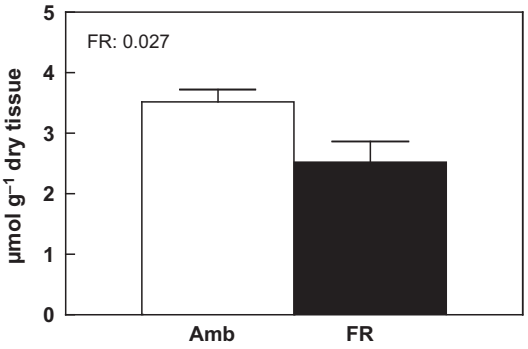

Fig. 8 Low R : FR ratios down-regulate camalexin levels in *Botrytis cinerea*-infected *Arabidopsis thaliana* plants. Camalexin was quantified from leaf tissue 2 days after inoculation with *B. cinerea*. Each bar represents the mean + 1 SE of seven biological replicates (individual plants). Amb, white light; FR, white light supplemented with FR radiation (for details, see the Materials and Methods section). The *P*-value for the FR in the ANOVA is shown. Camalexin was undetectable in non-inoculated plants.

not shown), but increased dramatically 2 days after *B. cinerea* inoculation. This response to infection was significantly attenuated when plants were exposed to FR supplementation (Fig. 8).

Low R : FR ratios do not reduce the levels of sinapates or flavonoids

Phenylpropanoid derivatives and other soluble phenolic compounds can play important roles in biotic defense. We measured sinapates because previous work had suggested that they mediate the effect of UVR8 increasing Arabidopsis resistance to *B. cinerea* (Demkura & Ballaré, 2012). Under our growth conditions, neither JA nor supplemental FR affected the concentration of sinapates (sinapoyl malate) or flavonoids (kaempferol glycosides) (Fig. S5). There was a tendency for the *phyB* mutant to have slightly reduced levels of two kaempferol glycosides (data not shown).

The effect of low R : FR ratios increasing Arabidopsis sensitivity to *B. cinerea* are missing in mutants deficient in Trp-derived defenses

We tested mutants impaired in camalexin biosynthesis (*pad3-1*) and iGS activation (*pen2-1*) for their resistance to *B. cinerea*. As expected (Ferrari *et al.*, 2003; Kliebenstein, 2004; Kliebenstein *et al.*, 2005; Lipka *et al.*, 2005; Bednarek *et al.*, 2009; Schlaeppi *et al.*, 2010; Demkura & Ballaré, 2012), both single mutants and the double *pen2-1 pad3-1* mutant were more susceptible to *B. cinerea* than Col-0 plants. In Col-0, FR increased susceptibility to the fungus, as reported previously by Cerrudo *et al.* (2012); however, no significant effects of low R : FR ratio were detectable in any of the mutants (Fig. 9).

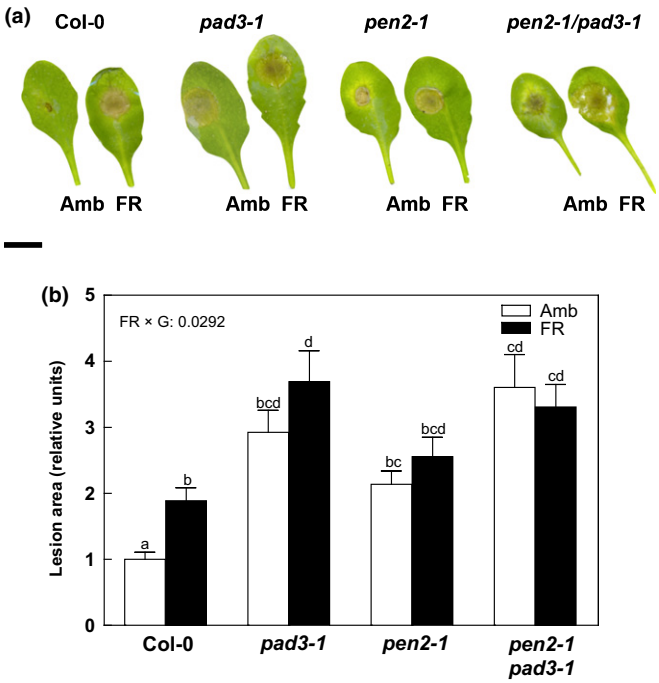

Fig. 9 Low red: far-red (R : FR) ratios increase *Arabidopsis thaliana* sensitivity to *Botrytis cinerea* in Col-0 (wild type) plants, but not in mutants deficient in Trp-derived defenses, which are hypersensitive to the fungus. Lesion areas were measured 48 h post inoculation (a, bar, 10 mm) and are given relative to the Col-0 wild type under white light conditions (b). Each bar represent the mean + SE of 16 infected plants (lesion area of each plant is the mean of five infected leaves). Amb, white light, open bars; FR, white light supplemented with FR radiation (for details, see the Materials and Methods section), closed bars. The *P*-value for the FR × genotype (G) interaction term of the ANOVA is shown. Different letters indicate significant differences between means ($P < 0.05$, Tukey test).

The effects of low R : FR ratios depressing I3M accumulation are missing in a *jaz10* mutant

Infection experiments (Cerrudo *et al.*, 2012) found that the effect of low R : FR ratios increasing Arabidopsis susceptibility to *B. cinerea* was missing in a *jaz10* mutant and in transgenic lines in which the *JAZ10* gene had been silenced by RNA interference, thereby implying that *JAZ10* plays an important role in mediating the effects of phyB inactivation on plant defense. We found that the effects of supplemental FR repressing I3M accumulation were also completely missing in the *jaz10* mutant (Fig. 10). By contrast, the effect of low R : FR reducing camalexin accumulation in Arabidopsis plants challenged with *B. cinerea* was conserved in the *jaz10* mutant (Fig. 11). Neither light quality (Fig. 5) nor the *jaz10* mutation (Fig. S6) affected the depletion of I3M caused by *B. cinerea*, which is consistent with the observation that *CYP81F2* expression is strongly up-regulated by fungal infection, but not regulated by JA (Fig. 4).

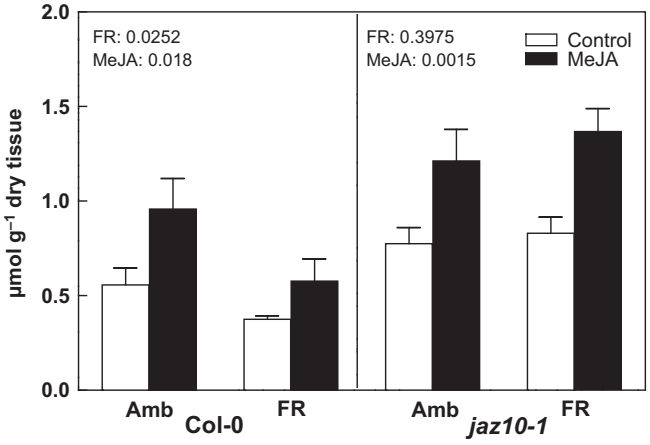

Fig. 10 The effect of low red : far-red (R : FR) ratios reducing indol-3-ylmethyl GS (I3M) accumulation requires *JAZ10*. I3M was quantified from *Arabidopsis thaliana* leaf tissue 2 d after treatment with 200 µM methyl jasmonate (MeJA). Open bars, control; closed bars, MeJA. Each bar represents the mean + 1 SE of four biological replicates (each replicate is a pool of three individual plants). Amb, white light; FR, white light supplemented with FR radiation (for details, see the Materials and Methods section). The *P*-values for the main effects in the factorial ANOVA are indicated for each genotype.

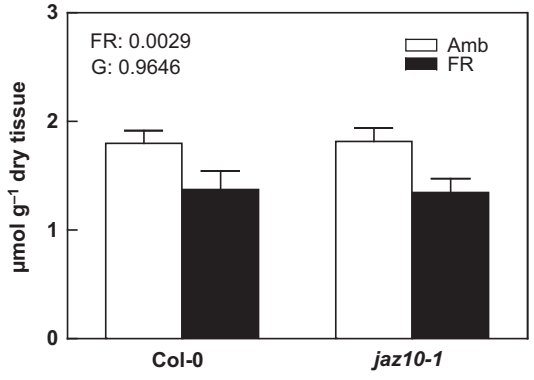

Fig. 11 Low red : far-red (R : FR) ratios reduce camalexin accumulation in *Botrytis cinerea*-infected *Arabidopsis thaliana* plants in a *JAZ10*-independent manner. Camalexin was quantified from leaf tissue 2 d after inoculation with *B. cinerea*. Each bar represents the mean + 1 SE of nine biological replicates (individual plants). Amb, white light, open bars; FR, white light supplemented with FR radiation (for details, see the Materials and Methods section), closed bars. The *P*-values for the FR and genotype (G) effects in the ANOVA are shown. Camalexin was undetectable in non-inoculated plants.

Discussion

In plant populations, the effect of neighbor proximity and shading increasing the incidence of fungal diseases is very well documented (Burdon & Chilvers, 1982; Augspurger & Kelly, 1984; Bell *et al.*, 2006; Roberts & Paul, 2006). Part of this effect is thought to be mediated by attenuation of plant immune responses under conditions that lead to partial inactivation of phyB (Ballaré *et al.*, 2012). Although the effects of phyB on defense hormone signaling have been previously described (reviewed in Ballaré, 2014), the metabolome responses to light quality that link phyB to plant defense against insects and pathogens are still poorly understood. Our study suggests that the main effect of phyB inactivation by low R : FR ratios on Arabidopsis defense is the down-regulation of the biosynthesis of Trp-derived defense metabolites (I3M and camalexin), and that this down-regulation can explain the increase in plant susceptibility to the fungus *B. cinerea*.

The general lack of effects of low R : FR ratio and JA on sinapates and flavonoids (Fig. S5) is consistent with previous experiments with Arabidopsis plants of this developmental stage (Demkura & Ballaré, 2012). However, effects of MeJA increasing the accumulation of other soluble phenolics, including anthocyanins, have been reported in other studies, and supplemental FR has been shown to attenuate those effects (Cerrudo *et al.*, 2012). Our observations suggest that, at least for sinapates and flavonoids, the amount of Pfr established by the R : FR ratio of our simulated-competition (FR) treatment was enough to maintain high levels of these phenolic compounds (Fig. S5). Previous experiments with mutants deficient in chalcone synthase (which is essential for the synthesis of C15 compounds, such as flavonoids and anthocyanins) or ferulic acid 5-hydroxylase (which is essential for sinapate biosynthesis) did not reveal susceptibility phenotypes in infection bioassays with *B. cinerea* under white light (Demkura & Ballaré, 2012). Taken together, the metabolite data and genetic evidence suggest that the effect of low R : FR ratios reducing Arabidopsis defense is unlikely to be mediated by reduced accumulation of soluble phenolic compounds.

Our study provides strong evidence to suggest that the R : FR ratio, acting through phyB, is a key modulator of Trp-derived defense metabolites, with important consequences for Arabidopsis immunity. Previous transcriptome analyses have shown that ANTHRANILATE SYNTHASE 1 (ASA1), which catalyzes the first step in Trp biosynthesis from chorismate is positively regulated at the transcriptional level by MeJA, and that this positive effect of MeJA can be cancelled by supplementing white light with FR radiation (Cerrudo *et al.*, 2012). *MYB34*, which encodes a transcription factor that positively regulates iGS biosynthesis is

regulated in a similar way (Cerrudo *et al.*, 2012; De Wit *et al.*, 2013; Fig. S1). We now demonstrate that key genes involved in iGS biosynthesis, such as *CYP79B2*, *CYP79B3* (Fig. S2) and *CYP83B1/SUR2* (Fig. 2), show a similar pattern of regulation (positive effect of JA, which is antagonized by low R : FR ratio), and that low R : FR ratios effectively cause a significant reduction in the levels of I3M and total iGS in plants treated with MeJA or infected with *B. cinerea* (Figs 3, 5). Whereas I3M is presumably not directly involved in Arabidopsis defense against *B. cinerea* (Buxdorf *et al.*, 2013), this reduction in the I3M pool is predicted to reduce the amount of precursors available to generate toxic hydrolysis products by endogenous thioglucosidases. Importantly, the effect of low R : FR ratios was conserved in the *sav3* mutant (Fig. S4), which fails to increase auxin levels and activate shade-avoidance responses under low R : FR ratios (Tao *et al.*, 2008; Moreno *et al.*, 2009). These results indicate that the repression of iGS accumulation by low R : FR ratios is not merely a consequence of a diversion of Trp precursors toward auxin biosynthesis. A similar inhibitory effect of low R : FR ratios was seen on the levels of camalexin in plants challenged with *B. cinerea* (Fig. 8), which correlated with a strong suppression of *PAD3* gene expression (Fig. 7). To our knowledge, this is the first demonstration of regulation of iGS and camalexin accumulation by photoreceptor-perceived light signals. I3M levels were slightly lower in *sav3* than in Col-0 (Fig. S4, cf Fig. 3). Interestingly, however, the iGS deficiency in *sav3* appeared to be compensated by significantly higher levels of camalexin: 2.2 μmol g^{-1} ($\pm$ 0.2) (*sav3*) versus 1.6 μmol g^{-1} ($\pm$ 0.1) (Col-0), $P < 0.05$ (48 h after infection with *B. cinerea*). These results suggest that the *sav3* mutation may lead to a reorganization of the metabolic fluxes between Trp-derived metabolites. Regarding aGS, the direction of the FR effect was similar to that found for Trp-derived metabolites, but of a much smaller magnitude (Fig. 6), which is consistent with the transcriptomic data for transcription factors (MYB28, MYB29, MYB76, Fig. S1) and biosynthetic enzymes (CYP83A1, Fig. 2c). The functional significance of the repression of iGS and camalexin biosynthesis by low R : FR ratios is demonstrated by the results of the bioassays with mutants impaired in the production or activation of these defenses, which showed that *pen2* and *pad3* simple and double mutants were highly susceptible to *B. cinerea*, both under low and high R : FR ratio (Fig. 9).

Recent work has begun to elucidate the connections between JA signaling and GS accumulation. The transcription factors MYC2, MYC3 and MYC4, which are direct targets of JAZ proteins and play a critical role activating JA responses (Fernández-Calvo *et al.*, 2011), have been shown to be essential for GS biosynthesis in Arabidopsis (Schweizer *et al.*, 2013). These MYC transcription factors interact, through their JAZ-interacting domain (JID), with all known MYB transcription factors that regulate GS biosynthesis genes (including MYB34 and MYB28, MYB29 and MYB76) (Schweizer *et al.*, 2013). Furthermore, MYC2 binds directly to the promoter region of key iGS biosynthesis genes, including *CYP79B2*, CYP79*B3* and *CYP83B1/SUR2* (Schweizer *et al.*, 2013). According to the model of Schweizer *et al.* (2013), both MYCs and MYBs are recruited to the promoter sites of GS biosynthesis genes to co-activate GS gene expression. This model provides clues to explain our observation of reduced iGS levels under low R : FR ratios. First, reduced expression of *MYC2* and *MYB34* (Cerrudo *et al.*, 2012; and potentially other MYBs, Fig. S1), and increased turnover of MYC2/3/4 (Chico *et al.*, 2014) under low R : FR ratios may result in reduced levels of MYC-MYB complexes. In addition, increased JAZ activity under low R : FR as a result of JAZ protein stabilization (Chico *et al.*, 2014; Leone *et al.*, 2014), and reduced abundance of DELLA proteins (Leone *et al.*, 2014), which are known to interfere with the activity of JAZ repressors (Hou *et al.*, 2010; Yang *et al.*, 2012), are predicted to inhibit the interaction between MYCs and MYBs, as JAZs bind to MYCs through the same interacting domain used by MYBs. In this context, it is interesting to note that the FR effect repressing I3M accumulation was missing in a *jaz10* mutant (Fig. 10), which is consistent with the lack of a FR effect increasing susceptibility to *B. cinerea* in Arabidopsis lines disrupted in *JAZ10* expression (Cerrudo *et al.*, 2012). This result suggests that FR may act by increasing *JAZ10* activity (see also Leone *et al.*, 2014), which by interacting with MYCs, may inhibit the formation of MYC-MYB complexes, thereby preventing the activation of GS biosynthesis.

Conclusion

Balancing resource allocation between competing physiological activities is a fundamental challenge for all living organisms. For plants, the trade-off between investment in growth or defense is critically important, particularly in situations of variable competition intensity (Ballaré, 2014; Huot *et al.*, 2014). Down-regulation of defense in plants (Izaguirre *et al.*, 2006; Moreno *et al.*, 2009; Cerrudo *et al.*, 2012; De Wit *et al.*, 2013; Kegge *et al.*, 2013) or plants parts (Izaguirre *et al.*, 2013) exposed to light signals of impending competition has been widely documented. Our results provide a mechanistic link between the main signal of competition (low R : FR ratio) and down-regulation of Arabidopsis immunity, mediated by the repression of Trp-derived secondary metabolites.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Interactive effects of MeJA and R : FR on expression profiles of transcription factors involved in GS biosynthesis.

Fig. S2 Effects of supplemental FR, MeJA and *B. cinerea* on *CYP79B2* and *CYP79B3* expression.

Fig. S3 The *phyB* mutant has low levels of I3M.

Fig. S4 The effect of low R : FR ratio reducing I3M levels is conserved in the *sav3-2* mutant.

Fig. S5 Sinapates and flavonoids are not regulated by MeJA or supplemental FR radiation.

Fig. S6 The effect of *B. cinerea* reducing the I3M pool is conserved in the *jaz10* mutant.

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