New Phytologist

Research

Trading direct for indirect defense? Phytochrome B inactivation in tomato attenuates direct anti-herbivore defenses whilst enhancing volatile-mediated attraction of predators

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Received: *4 August 2016*
Accepted: *11 August 2016*

New Phytologist (2016) **212:** 1057–1071
doi: 10.1111/nph.14210

Key words: defense, indirect defense, jasmonate, phytochrome, R : FR ratio, tomato (*Solanum lycopersicum*), tritrophic interaction, volatile.

Summary

- Under conditions of competition for light, which lead to the inactivation of the photoreceptor phytochrome B (phyB), the growth of shade-intolerant plants is promoted and the accumulation of direct anti-herbivore defenses is down-regulated. Little is known about the effects of phyB on emissions of volatile organic compounds (VOCs), which play a major role as informational cues in indirect defense.
- We investigated the effects of phyB on direct and indirect defenses in tomato (*Solanum lycopersicum*) using two complementary approaches to inactivate phyB: illumination with a low red to far-red ratio, simulating competition, and mutation of the two *PHYB* genes present in the tomato genome.
- Inactivation of phyB resulted in low levels of constitutive defenses and down-regulation of direct defenses induced by methyl jasmonate (MeJA). Interestingly, phyB inactivation also had large effects on the blends of VOCs induced by MeJA. Moreover, in two-choice bioassays using MeJA-induced plants, the predatory mirid bug *Macrolophus pygmaeus* preferred VOCs from plants in which phyB was inactivated over VOCs from control plants.
- These results suggest that, in addition to repressing direct defense, phyB inactivation has consequences for VOC-mediated tritrophic interactions in canopies, presumably attracting predators to less defended plants, where they are likely to find more abundant prey.

Introduction

Plants, like other organisms, need to make choices with regard to the allocation of finite resources between multiple physiological processes and alternative developmental programs. A well-known case of allocation tradeoffs, which has received much attention in plant ecology, is that associated with the distribution of resources between growth and defense (often referred to as 'the dilemma of plants'; Herms & Mattson, 1992; Cipollini, 2004). Evidence for the occurrence of this tradeoff comes from many sources, which show that the activation of plant defenses often correlates with reduced growth rate or competitive ability (Baldwin, 1998; Redman *et al.*, 2001; Zavala *et al.*, 2004; Zavala & Baldwin, 2006; Cipollini, 2007; Yan *et al.*, 2007; Ballhorn *et al.*, 2014), whereas fast growth is commonly associated with low levels of chemical defense and increased susceptibility to herbivory and pathogen attack (Cipollini, 1997; Kurashige & Agrawal, 2005; Donaldson *et al.*, 2006; Izaguirre *et al.*, 2006). Under conditions of crowding and high levels of competition, shade-intolerant plant species typically down-regulate defense responses (Ballaré, 2014), which is similar to the response described for many animals (Tollrian *et al.*, 2015).

Allocation decisions in plants are controlled by sophisticated mech3anisms that allow the plant to acquire information about its environment, including information on the risks posed by consumer organisms (herbivores and pathogens) and competitors. Detection of attack by plant consumers is achieved by a variety of sensing mechanisms, which include specific receptors or sensory systems for 'non-self' (i.e. pathogen- and herbivore-associated molecular patterns) and 'damaged self' (damage-associated molecular patterns) (Jones & Dangl, 2006; Panstruga *et al.*, 2009; Wu & Baldwin, 2010; Bonaventure, 2012; Heil *et al.*, 2012). Downstream of these sensory mechanisms, a complex network of hormonal pathways, including the jasmonic acid (JA) and salicylic acid (SA) pathways, orchestrates the activation

†This paper is dedicated to the memory of our colleague and friend Hernán E. Boccalandro.

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of a repertoire of induced defenses that is specific to the attacker (Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012; Stam *et al.*, 2014).

Perception of plant competitors is achieved in a variety of ways, and light is one of the most important sources of information used by plants to detect the proximity of other plants (Ballaré, 1999; Pierik *et al.*, 2012). Of the various light signals perceived by plants using specific photoreceptor proteins, the ratio of red (R, 660 nm) to far-red (FR, 730 nm) radiation (i.e. the R : FR ratio) is of major significance in the detection of competition (Smith, 1995). Because chlorophyll absorbs very efficiently in the R region of the solar spectrum, but not in the FR region (which is either transmitted or reflected), low R : FR ratios are indicators of direct shading (in association with reduced total irradiance) (Holmes & Smith, 1977) or the proximity of FR-reflecting neighbors (future competitors) (Ballaré *et al.*, 1990). The photoreceptor phytochrome B (phyB) is inactivated under low R : FR ratios, and this inactivation unleashes the expression of growth-related pathways, controlled by hormones, such as auxin (Tao *et al.*, 2008) and gibberellins (Feng *et al.*, 2008; de Lucas *et al.*, 2008), which, in turn, promote the elongation and projection of photosynthetic organs towards well-lit areas within the canopy (the shade-avoidance syndrome, SAS).

Activation of the SAS in response to low R : FR ratios is often associated with the down-regulation of plant defense (McGuire & Agrawal, 2005; Izaguirre *et al.*, 2006), presumably because the plant prioritizes growth over defense under conditions of competition (Ballaré, 2009, 2014; Havko *et al.*, 2016; Smakowska *et al.*, 2016). This down-regulation of defense under low R : FR ratios has been linked with the repression of both JA (Moreno *et al.*, 2009; Agrawal *et al.*, 2012; Cerrudo *et al.*, 2012; Izaguirre *et al.*, 2013) and SA (de Wit *et al.*, 2013) signaling, and the molecular mechanisms behind this repression are becoming increasingly well understood, at least for JA in Arabidopsis (Chico *et al.*, 2014; Leone *et al.*, 2014).

A major mechanism of plant defense against insect herbivory is based on the emission of volatile organic compounds (VOCs). Plants of > 20 families are known to emit VOCs in response to feeding or oviposition by herbivorous insects (Dicke & Baldwin, 2010; Mumm & Dicke, 2010; Heil, 2014; Karban *et al.*, 2014; Hilker & Fatouros, 2015), and it has been demonstrated in laboratory and field studies that many of these induced VOCs play a role in defense by attracting carnivorous arthropods that attack the herbivores (Dicke & Sabelis, 1988; Turlings *et al.*, 1990; Kessler & Baldwin, 2001; Schuman *et al.*, 2012). Very little is known about the regulation of herbivore-induced VOC emissions by environmental factors (Kegge & Pierik, 2010; Mumm & Dicke, 2010; Pierik *et al.*, 2014). The available evidence suggests that the emission of most VOCs involved in plant anti-herbivore defense is controlled by JA (Arimura *et al.*, 2005; Mumm & Dicke, 2010; Ponzio *et al.*, 2013) and, given that phyB is an important modulator of JA signaling (Ballaré, 2014), it is reasonable to expect that the VOC profiles emitted by plants in response to herbivory could be affected by the canopy light environment. However, very little direct evidence is available to support this assumption (Kegge & Pierik, 2010; Kegge *et al.*, 2013; Pierik *et al.*, 2014). Recent work in Arabidopsis (Kegge *et al.*, 2013) has demonstrated that low R : FR and canopy shade affect the volatile blend of both non-induced and methyl jasmonate (MeJA)-induced plants.

Here, we investigated the effects of phyB inactivation in tomato (*Solanum lycopersicum*), which represents a classic model for studies of plant–insect interactions (Ryan, 2000; Howe & Jander, 2008; Scranton *et al.*, 2013) and has a well-characterized repertoire of direct and indirect (VOC-mediated) defenses (Thaler *et al.*, 2002; Scranton *et al.*, 2013; Lins *et al.*, 2014; Alba *et al.*, 2015). We found that phyB inactivation by low R : FR ratios or by mutation of the two *PHYB* genes in the tomato genome (*PHYB1* and *PHYB2*) resulted in a significant repression of direct anti-herbivore defenses, and attenuation of defense responses induced by the application of MeJA. Strikingly, phyB inactivation also altered the patterns of VOCs emitted by MeJA-induced tomato plants, making them more attractive to the generalist predator *Macrolophus pygmaeus,* which feeds on herbivores of tomato (Lins *et al.*, 2014). Our results suggest that the R : FR ratio might have important consequences for VOC-mediated tritrophic interactions in plant canopies, and it is tempting to speculate that plants under competition to some extent trade direct for indirect defenses.

Materials and Methods

Plant material and growth conditions

Tomato plants (*Solanum lycopersicum* L.) cv Moneymaker (accession number LA2706), as wild-type (WT) and a phyB double mutant (*phyB1phyB2*) (Weller *et al.*, 2000), were used in all the experiments. Seeds were sown in small plastic trays with a mixture of organic matter-enriched soil and perlite (3 : 1). One week after germination, seedlings were transferred to 3-l pots and grown in a temperature-controlled glasshouse under natural radiation (25 ± 2°C, 50 ± 10% relative humidity (RH)) until the experimental manipulations. We used two levels of the 'light' factor (ambient and FR), which were applied from the seedling stage, and two levels of the 'induction' factor (control and MeJA). Experiments were carried out at IBAM-CONICET, Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, Mendoza, Argentina (33°0′S, 68°52′W) and the Laboratory of Entomology, Wageningen University, the Netherlands (51°59′N, 5°39′E).

Insects

Caterpillars of *Mamestra brassicae* L. (Lepidoptera: Noctuidae) were obtained from the stock colony of the Laboratory of Entomology, Wageningen University, the Netherlands. Caterpillars were reared on Brussels sprout plants (*Brassica oleracea* var. *gemmifera* cv Cyrus) at 22 ± 1°C, 60 ± 5% RH, 16-h photoperiod. Nymphs and adults of the predator *Macrolophus pygmaeus* Rambour (Hemiptera: Miridae) were supplied by Koppert Biological Systems (Berkel en Rodenrijs, the Netherlands), and were reared on *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs

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as prey, without exposure to plants or tomato plant material. Thus, the predators were naïve with respect to tomato volatiles. After arrival at the laboratory, predators were kept in climate cabinets (25 ± 1°C, 70 ± 5% RH, 16-h photoperiod) inside cages (60 × 40 × 40 cm^3) containing two potted tomato plants, one of each genotype, and eggs of *E. kuehniella* as food.

FR irradiation

Wild-type seedlings kept in the glasshouse (peak photosynthetically active radiation = 1000 μmol m^{-2} s^{-1}) were divided into two groups. One group was exposed to supplemental FR radiation during the natural photoperiod, which was provided from one of the sides by LED bars (730 nm; Philips Green Power, Eindhoven, the Netherlands; 1 bar per plant) (FR treatment). Plants of the second group were fitted with identical LED bars, but the LEDs remained off during the course of the experiment. The LEDs reduced the R : FR ratio from 1.1 (ambient) to 0.1 (FR treatment), as measured with a Skye SKL 908 Spectrosense2+ R : FR detector (Skye Instruments, Llandrindod Wells, Powys, UK) with the sensor pointing in the direction of the LED bars.

Plant defense induction

Defenses were induced using MeJA. The plants used for the experiments were between 3 wk (gene expression) and 5 wk (VOC collection) old, and had between six and seven true leaves. Chemical elicitation was performed by spraying 50 ml of an aqueous solution containing 0 (control), 100 (gene expression) or 450 μM (VOC collection) of MeJA (Sigma, St Louis, MO, USA), as indicated in the relevant figure legends. These MeJA treatments were effective in inducing typical gene expression responses, but they did not cause visible growth inhibition. Plants were harvested 8 or 72 h after the elicitation treatment and immediately frozen in liquid nitrogen or lyophilized (FreeZone 2.5 Liter Benchtop, Labconco, Kansas City, MO, USA) for gene expression analysis and determinations of leaf phenolics, respectively. For volatile sampling and Y-tube olfactometer assays, we used whole plants at 24 h after induction.

Analysis of trichome density and index

Total leaf trichomes were counted in an optical microscope (Nikon Eclipse E200, Tokyo, Japan) using photographs (Micrometrics 318 CU Camera, Shanghai, China) of epidermal imprints of the adaxial surface of the fifth fully expanded leaf. For density quantification of individual types of trichomes, scanning electron microscopy was performed with a JEOL-6610LV microscope (Tokyo, Japan) as described in Kang *et al.* (2010b) with minor modifications. Briefly, tissues were fixed for 24 h in a solution of 2.5% paraformaldehyde, 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.4 (Sigma-Aldrich, Munich, Germany). Samples were dehydrated in a graduated acetone series, critical point dried with CO_2 (DCP-1, Denton Vacuum, Moorestown, NJ, USA), mounted and sputter coated with 30-nm gold particles (Desk IV, Denton Vacuum). Samples were examined with a 30 kV accelerating voltage and the resulting images were captured digitally. Stem samples were taken from the fifth internode; leaf samples were obtained from the apical zone of the terminal leaflet, avoiding the midvein.

Leaf phenolics and high-performance liquid chromatography (HPLC) analysis

Total soluble leaf phenolics were extracted using standard protocols (Mazza *et al.*, 2000). Leaf discs (1 cm in diameter; youngest fully expanded leaf) were placed in 1.4 ml of 1% (v/v) methanol–HCl solution and allowed to extract for 48 h at −20°C. The absorbance of extracts was read at 305 nm using a Cary 50 UV–VIS spectrophotometer (Varian Inc., Palo Alto, CA, USA) and 10-mm optical path cells. After extraction, leaf discs were dried at 65°C during 48 h and weighed using an analytical balance. Individual leaf phenolics were determined as described by Keinänen *et al.* (2001) with slight modifications. Briefly, *c.* 10–15 mg of lyophilized tissue without the midvein was ground in a mortar with liquid N_2 and transferred to an Eppendorf with 1.5 ml of a methanol–water–acetic acid mixture (2 : 3 : 0.0075). Samples were vortexed for 45 s and centrifuged at 12 000 ***g*** for 20 min. The supernatant was filtered through a 45-μm nylon syringe filter and kept at −20°C until use. Phenolics were separated by HPLC (Knauer Euroline, Berlin, Germany) on a Restek Pinnacle II C18 (5.0 μm, 4.6 × 150 mm^2) column with solvents A (0.25% aqueous H_3PO_4) and B (acetonitrile), eluted with an initial gradient of 8% B at 0 min, 12% B at 6 min, 20% B at 10 min, 50% B at 23–30 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 305 nm. The retention times and UV-VIS spectra of individual phenolics were compared with those of commercial standards. Peak areas at 305 nm were employed to calculate phenolic content using external calibration curves. Solvents used for the determination of leaf phenolics were purchased from Sintorgan (Buenos Aires, Argentina). Phenolic standards (chlorogenic acid, rutin and kaempferol) were purchased from Sigma-Aldrich.

Gene expression

Tomato leaves (fourth fully expanded) were harvested 8 h after MeJA treatment (100 μM), and total RNA was extracted using the LiCl–phenol–chloroform method (Izaguirre *et al.*, 2003). Quantitative real-time PCR analysis (qRT-PCR) was performed as described previously (Moreno *et al.*, 2009). qRT-PCR was carried out in a 7500 PCR Real System (Applied Biosystems, Foster City, CA, USA) with FastStart Universal SYBR Green Master (Rox; Roche). The *SGN-U346908* gene (Expósito-Rodríguez *et al.*, 2008) was used as an internal standard; the primers for the genes of interest are listed in Supporting Information Table S1. Normalized gene expression was expressed as the fold change relative to the wild-type under ambient light conditions and no MeJA treatment. The results are based on three independent biological replicates. Each replicate consisted of a pool of four individual plants.

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Collection and analysis of headspace VOCs

Volatiles from control and MeJA-induced (450 μM) plants were collected from WT and *phyB1phyB2* plants 24 h after the induction treatment. Headspace sampling was carried out in a controlled-environment room, under the same climatic conditions as used for the Y-tube olfactometer test (see later), between 10:00 and 13:00 h. When testing plants from the FR radiation treatment, the FR supplementation was maintained during VOC collection. Pots with plants were wrapped using aluminum foil and placed in 30-l glass jars and left for 30 min to acclimate before headspace volatile collection began. Air from jars containing the pots with soil wrapped in aluminum foil and no plants was collected to correct for background odors. Air was filtered through activated charcoal before reaching the glass jars with plants, and volatiles were collected by drawing air out of the jars with a suction pump through a stainless steel cartridge containing 200 mg of Tenax TA (20/35 mesh; CAMSCO, Houston, TX, USA) at a rate of 200 ml min^{-1} for 2 h. The aerial parts of each plant were weighed, and the total area of leaves was measured immediately. Before releasing the volatiles into the gas chromatograph, the Tenax TA cartridges were dry purged under a stream of nitrogen (50 ml min^{-1}) for 15 min at ambient temperature in order to remove moisture. The collected volatiles were then thermally released from the Tenax TA adsorbent using an Ultra 50 : 50 thermal desorption unit (Markes, Llantrisant, Glamorgan, UK) at 250°C for 10 min under a helium flow of 20 ml min^{-1}, whilst simultaneously re-collecting the volatiles in a thermally cooled universal solvent trap: Unity (Markes) at 0°C. Once the desorption process was completed, volatile compounds were released from the cold trap by ballistic heating at 40°C s^{-1} to 280°C, which was then kept for 10 min, whilst all the volatiles were transferred to a ZB-5MSi analytical column (30 m × 0.25 mm (internal diameter) × 0.25 μm (film thickness) with 5-m built-in guard column; Phenomenex, Torrance, CA, USA), placed inside the oven of a Thermo Trace GC Ultra (Thermo Fisher Scientific, Waltham, MA, USA), for further separation of plant volatiles. The gas chromatograph oven temperature was initially held at 40°C for 2 min and was immediately raised at 6°C min^{-1} to a final temperature of 280°C, where it was kept for 4 min under a constant helium flow of 1 ml min^{-1}. For the detection of volatiles, a Thermo Trace DSQ quadrupole mass spectrometer (Thermo Fisher Scientific) coupled to the gas chromatograph was operated in an electron impact ionization (EI) mode at 70 eV in a full scan with a mass range of 35–400 amu at 4.70 scans s^{-1}. The MS transfer line and ion source were set at 275 and 250°C, respectively. The tentative identification of compounds was based on the comparison of mass spectra with those in the NIST 2005 and Wageningen Mass Spectral Database of Natural Products MS libraries, as well as experimentally obtained linear retention indices (LRIs).

Mamestra brassicae feeding experiment

Mamestra brassicae larvae (L_5) were placed on the third fully expanded, attached leaf of WT or *phyB1phyB2* plants (one larva per leaf), which was lightly wrapped with organza fabric. The caterpillars were allowed to feed for 24 h. Tissue consumption was quantified by non-destructive estimation of the area of the leaf before and after *M. brassicae* feeding using leaf photographs.

Y-tube olfactometer

Responses of predator females to plant volatiles were observed in a two-choice Y-tube olfactometer as described previously (Lins *et al.*, 2014) with minor modifications. A Y-shaped Pyrex tube (3.5 cm inside diameter), formed by an entry arm (26 cm in length) and two side arms (each 10 cm in length at a 70° angle), was used. Each arm was connected to a glass jar (30 l in volume) harboring the plants. Compressed air was provided to each plant-containing glass jar (2.5 l min^{-1}). Before reaching the glass jar, the air was passed through an activated charcoal filter. The glass jars with odor sources were kept behind a black panel, preventing insects from visually detecting the plants. A single plant was introduced in each glass jar. Single *M. pygmaeus* female predators (1–5 d old) were introduced at the downwind end of the entry arm and observed until they walked at least 6 cm into one of the side arms. Females not choosing a side arm within 10 min were considered to have made no choice and were excluded from data analysis. Each female was tested only once and then discarded. For each pair of odor sources, 50 females were tested during five different experimental days, 10 on each day with a new set of plants as odor sources. After testing a batch of five females, the odor sources were switched in order to minimize positional bias. Y-tube bioassays were carried out in a climate room at 23 ± 1°C, 50 ± 10% RH. Three pairs of plant groups were tested: control WT and *phyB1phyB2* plants grown under ambient light; MeJA-induced WT and *phyB1phyB2* plants grown under ambient light; and MeJA-induced WT plants grown under ambient light vs MeJA-induced WT plants supplemented with FR light (MeJA was applied 24 h before Y-tube olfactometer test).

Statistical analyses

For data on trichome numbers, gene expression and levels of phenolic compounds, Student's *t*-tests and two-way ANOVA, followed by Tukey's honestly significant difference (HSD) *post hoc* test, were used when appropriate in order to assess differences between means. Analyses were carried out with the InfoStat 2011 version software (Di Rienzo *et al.*, 2011). $P \leq 0.05$ was used as a threshold for statistical significance. Choice responses of the predator *M. pygmaeus* were analyzed by generalized linear models (GLMs) with a binomial distribution and a logit-link function. The response variable was the proportion of insects responding to one of the odor sources. For all experiments, we fitted a binomial GLM to estimate whether the proportional response of the predator was significantly different from a 50% distribution. The significance of the response was tested using a chi-squared Wald test, performed using Spss v.21.0 (SPSS Inc., Chicago, IL, USA). Before analysis, the volatile emission data, expressed as peak areas divided by the fresh mass of the plant, were tested for normality and homogeneity of variances using the Shapiro–Wilk and

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Bartlett tests, respectively. To test for significant differences among treatments, a non-parametric test (Kruskal–Wallis) was used, as data distribution did not meet the assumptions for standard parametric ANOVA. Compounds with variable importance in the projection (VIP) score values ≥ 1 were subjected to Mann–Whitney *U* analyses to test for significant differences between treatments. The statistical analyses were performed using R statistical software (R Core Team, 2014). The volatile emission data were log transformed and mean centered before being subjected to multivariate data analysis using the projection to latent structures-discriminant analysis (PLS-DA) and its extension, orthogonal projection to latent structures-discriminant analysis (OPLS-DA), functions of SIMCA-P + 12.0 software (Umetrics AB, Umeå, Sweden), as described previously (Weldegergis *et al.*, 2015). This projection method determines whether samples collected from the different plant groups can be separated on the basis of quantitative and/or qualitative differences in their volatile blends. The results of the analysis are visualized in score plots, which reveal the sample structure according to model components, and loading plots, which display the contribution of the variables (compounds) to these components, as well as the relationships among the variables, based on the influence of each variable in the projection (VIP values) (Wold *et al.*, 2001). Compounds were excluded if they were present in less than one-half of the samples of the treatment. The advantage of OPLS-DA compared with PLS-DA is that, in OPLS-DA, a single component can be used as a predictor for the class, whilst the remaining components describe the variation orthogonal to the first predictive component. Discrimination in the first component is between classes, and separations along the second component (orthogonal component) indicate metabolite differences between samples of the same class. When there is variation among samples of the same treatment group, OPLS-DA is a better choice as it takes within-class variations into consideration (Westerhuis *et al.*, 2010; Worley & Powers, 2013; Hadrévi *et al.*, 2015).

Results

phyB inactivation triggers SAS responses and results in lower levels of constitutive defenses

We used two complementary approaches to inactivate phyB in tomato: a genetic approach employing a mutant that harbors null mutations in the two *PHYB* genes present in the tomato genome (*phyB1phyB2*) (Weller *et al.*, 2000), and a physiological approach, using lateral FR to mimic the effect of neighboring plants in an even-height canopy (Ballaré, 1999). Both approaches resulted in strong SAS phenotypes (Fig. 1a).

Tomato plants produce abundant trichomes on leaves and stems, which may play a role as physical defenses against certain herbivorous insects (Kang *et al.*, 2010a) (Fig. 1b). The *phyB1phyB2* mutant showed reduced densities of stem (Fig. 1c) and leaf (Fig. 1d) trichomes compared with WT. This reduction in trichome density was essentially the result of a reduced number of epidermal cells per unit area in *phyB1phyB2*, as the trichome index (TI = number of trichomes/number of epidermal cells) was not affected in the mutant ($TI_{Abaxial} = 0.33$ in WT and $TI_{Abaxial} = 0.34$ in *phyB1phyB2*, $P = 0.66$; $TI_{Adaxial} = 0.23$ in WT and $TI_{Adaxial} = 0.22$ in *phyB1phyB2*, $P = 0.72$).

Soluble phenolic compounds, such as flavonoids, have been reported to provide chemical defense against some herbivores in tomato (Elliger *et al.*, 1981; Stamp & Yang, 1996). Levels of flavonoids (C_{15} compounds) tended to be lower in *phyB1phyB2* than in WT plants. By contrast, the mutant had slightly higher levels of some C_6-C_3 compounds, such as chlorogenic acid and ferulic acid, than the WT (Table S2), presumably reflecting a repression of the flavonoid pathway in response to phyB inactivation.

phyB inactivation represses JA-induced direct defenses

In Arabidopsis and other species, phyB inactivation is associated with a repression of JA responses (references in Ballaré, 2014). phyB inactivation in tomato led to a significant down-regulation of the effect of exogenous MeJA on the expression of genes involved in JA-induced defenses, such as *THREONINE DEAMINASE* (*TD*) and *PROTEINASE INHIBITOR II* (*PIN-II*) (Fig. 2); by contrast, the MeJA effect promoting *POLYPHENOL OXIDASE* (*PPO*) expression was not affected in the *phyB1phyB2* mutant (Fig. 2).

phyB1phyB2 plants support greater herbivore damage than WT plants

When *M. brassicae* caterpillars were allowed to feed on WT or *phyB1phyB2* plants in a no-choice experiment, leaf damage was much more severe in the mutant than in WT plants (Fig. 3). This result is consistent with experiments that monitored damage by insects belonging to other feeding guilds, such as piercing–sucking cell content-feeding thrips (Izaguirre *et al.*, 2006).

phyB inactivation changes the blend of VOCs emitted by JA-induced plants

Having demonstrated significant effects of phyB inactivation on constitutive and induced direct defenses, we subsequently investigated whether changes in phyB can alter the emission of inducible volatile compounds, with potential consequences for indirect defense and tritrophic interactions. To this end, we ran a factorial experiment that combined two levels of phyB status with two levels of MeJA induction, resulting in four genotype × MeJA combinations: WT control plants (WC), *phyB1phyB2* control plants (pC), WT plants elicited with MeJA (WJ) and *phyB1phyB2* plants elicited with MeJA (pJ). Across the four genotype × treatment combinations, 69 different VOCs were detected in the headspace blends (Table S3); these compounds were present in > 50% of the samples of at least one genotype × treatment combination. In the control treatment (no MeJA), we detected 64 compounds in WT and *phyB1phyB2* plants, whereas, in the MeJA treatment, 69 VOCs were found for WT and *phyB1phyB2* plants. A multivariate data analysis (PLS-DA), including all the volatile compounds of all four

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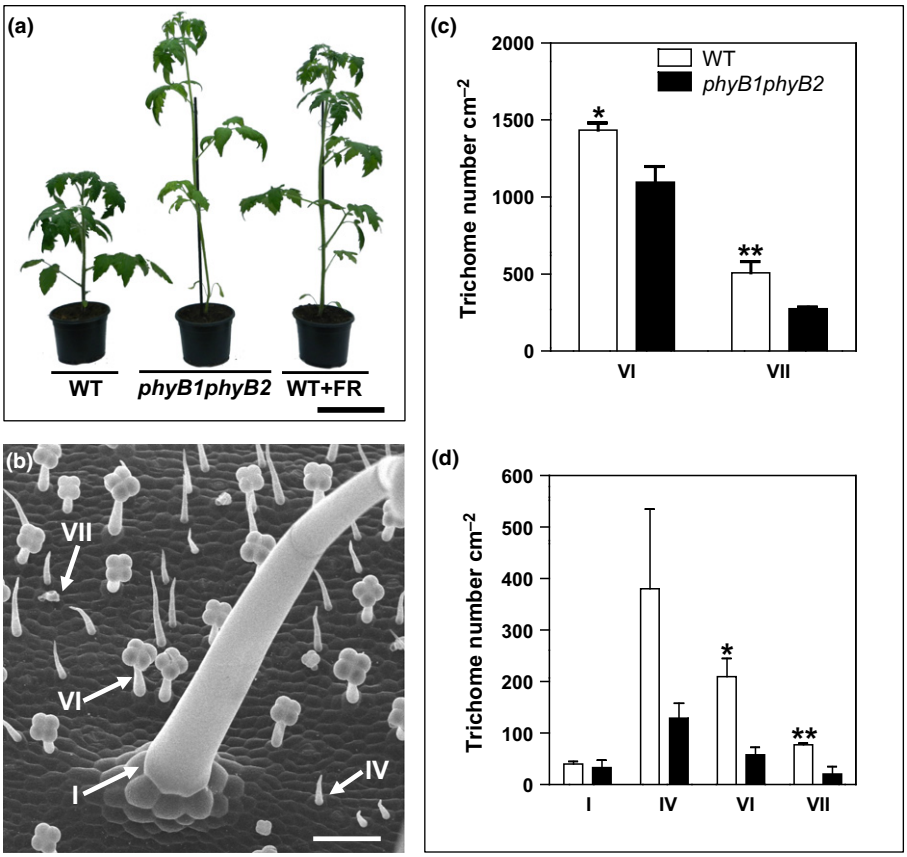

Fig. 1 Effects of phytochrome B (phyB) inactivation on the morphology and trichome attributes of tomato plants (*Solanum lycopersicum*). (a) Shoot morphology of wild-type (WT) plants (cv Money maker, WT), *phyB1phyB2* double mutant plants and WT plants irradiated from the side with far-red (FR) radiation (WT+FR), all grown under natural daylight in a glasshouse (bar, 15 cm). (b) Scanning electron micrographs of the leaf adaxial surface of WT plants. Trichome types I, IV, VI and VII are indicated by arrows (bar, 200 μm). (c, d) Trichome density on stems (types VI and VII) and leaves (types I, IV, VI and VII), respectively, from 4-wk-old WT and *phyB1phyB2* plants (mean (± SE) of three replicate plants). Asterisks indicate significant differences between WT and *phyB1phyB2* plants (unpaired *t*-test: *, $P < 0.05$;**, $P < 0.01$).

genotype × treatment combinations (i.e. those listed in Table S3), resulted in a model with three significant principal components, with the first two explaining 31.2% and 25.6% of the total variance, respectively (Fig. 4a). The third component explained 8.8% of the total variation. The PLS-DA based on the volatile blends showed two major clusters, control and MeJA-treated plants, regardless of plant genotype. For this model, 26 volatile compounds with VIP values ≥ 1.0 contributed most to the separation between headspace blends (Table S3). MeJA treatment influenced the emission of acyclic mono- and homoterpenes: (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene ((*E,E*)-TMTT), (*E*)-4,8-dimethylnona-1,3,7-triene ((*E*)-DMNT), linalool, (*E*)-β-ocimene, allo-ocimene and (*E,E*)-cosmene; cyclic monoterpenes: *trans*-2-caren-4-ol and *cis*-limonene oxide; and fatty acid-derived compounds ((3-pentanol and green leaf volatiles (GLVs): hexyl butanoate; (*Z*)-3-hexenyl isovalerate; (*Z*)-3-hexenyl butyrate; (*Z*)-3-hexen-1-ol isobutyrate and (*Z*)-3-hexen-1-ol propanoate) (Table S3; Fig. 4b), all with VIP values greater than 1.

A pair-wise comparison by OPLS-DA (an extension of PLS-DA in SIMCA-P software, in which variation within the same group of samples is taken into consideration) was carried out between WT and *phyB1phyB2* control plants (WC vs pC). The resulting two-dimensional score plot, based on the first predictive and first orthogonal components (Fig. 5a), revealed differences in the headspace composition between these groups. Twenty-five volatile compounds with a VIP value ≥ 1.0, almost 36% of the total VOCs found in the headspace, were the main determinants of the separation between the groups. The headspace of WC plants was characterized by 16 volatile compounds, most of which belonged to the group of cyclic monoterpenes, whereas the volatile blend of pC plants was determined by the remaining nine compounds (consisting of two cyclic monoterpenes, two sesquiterpenes, one homoterpene, an acyclic monoterpene and three fatty acid derivatives; Table 1; Fig. 5b). The two homoterpenes, (*E,E*)-TMTT and (*E*)-DMNT, correlated with different sample groups: (*E,E*)-TMTT was associated with WT plants, whereas (*E*)-DMNT was associated with *phyB1phyB2* plants (Fig. 5b). Despite the large number of VOCs identified in the headspace, with 25 compounds being instrumental in the separation of the two plant groups, only the homoterpene (*E,E*)-TMTT and the cyclic monoterpene ketone pipertone were emitted in significantly different amounts between WC and pC plants (Table 1).

The corresponding pair-wise PLS-DA comparison between WT and *phyB1phyB2* plants under MeJA treatment (WJ vs pJ) yielded a model with four significant principal components, with the first two explaining 26.6% and 28.7% of the total variance, respectively (Fig. 5c,d). A group of 24 compounds with VIP values ≥ 1.0 contributed most to the differentiation between the blends of MeJA-treated WT and MeJA-treated *phyB1phyB2* plants (Table 2). Most of these compounds were terpenoids and GLVs. Terpenoids, such as 3,7,7-trimethyl-1,3,5-cycloheptatriene, pulegon, anetofuran, *trans*-3(10)-caren-2-ol, *p*-mentha-1,3,8-triene and (*E,E*)-TMTT, were emitted in significantly higher amounts by WJ plants compared with pJ plants

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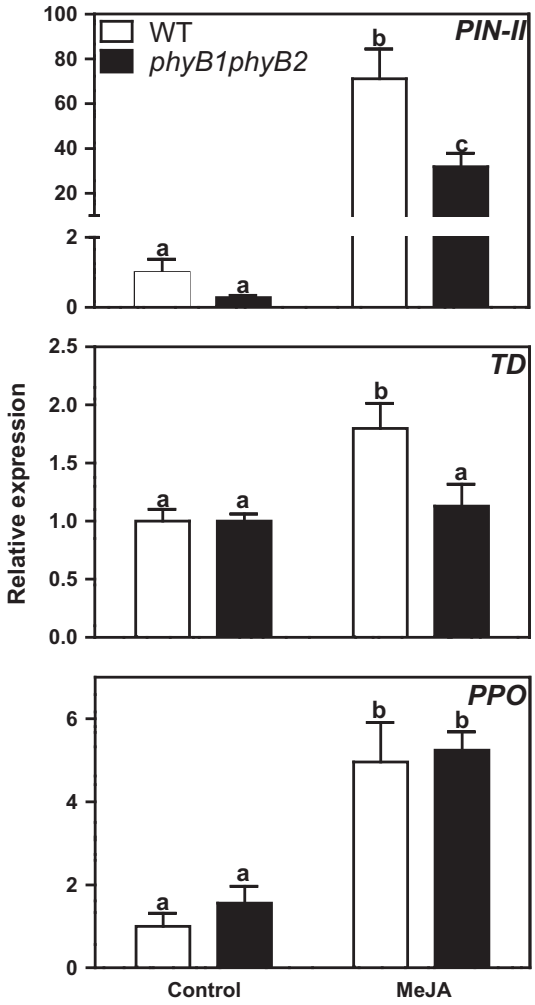

Fig. 2 Effect of phytochrome B (phyB) inactivation on gene expression responses to methyl jasmonate (MeJA, 100 μM) treatment. Tomato (*Solanum lycopersicum*) leaves (fourth fully expanded) were harvested 8 h after MeJA treatment. Mean values (+ SE) of three biological replicates are shown. Expression data are normalized to the expression level of the wild-type (WT) under ambient light conditions and no MeJA. Different letters indicate significant differences between treatment means as calculated using Tukey's honestly significance difference (HSD) statistical test ($P < 0.05$). *PIN-II*, *PROTEINASE INHIBITOR II*; *TD*, *THREONINE DEAMINASE*; *PPO*, *POLYPHENOL OXIDASE*.

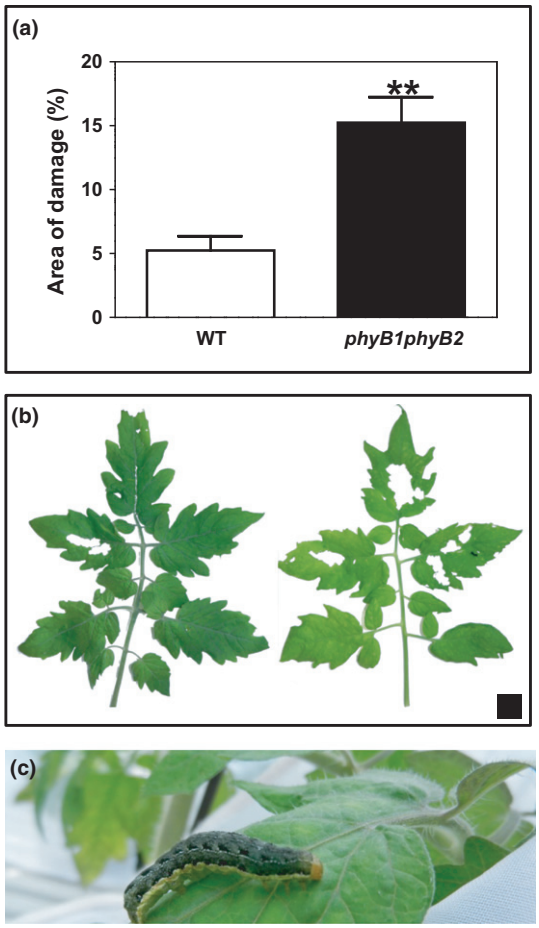

Fig. 3 Effect of phytochrome B (phyB) inactivation in tomato (*Solanum lycopersicum*) on leaf herbivory. (a) Herbivore damage caused by L_5 caterpillars of *Mamestra brassicae* (cabbage moth) to wild-type (WT) and *phyB1phyB2* plants in a no-choice bioassay carried out in a glasshouse under natural daylight. Bars indicate the percentage leaf damage after 24 h of feeding (mean (+ SE) of nine 4-wk-old plants per genotype). The asterisks denote a significant difference between WT and *phyB1phyB2* plants (unpaired *t*-test: **, $P < 0.01$). (b) Representative photographs of leaves of WT (left) and *phyB1phyB2* (right) at the end of the feeding experiment (the black square indicates 5 cm^2). (c) Image of an *M. brassicae* caterpillar feeding on tomato leaves.

(Table 2). By contrast, α-terpinene was measured at significantly higher levels in pJ samples (Table 2). These results demonstrate that genetic inactivation of phyB can significantly affect the blends of VOCs emitted by MeJA-induced plants.

As a complementary approach to test the effect of phyB status on VOC profiles emitted by JA-induced plants, we sampled the headspace of MeJA-treated WT plants grown under either ambient light or ambient light supplemented with FR radiation (which inactivated phyB by depleting the active Pfr form of the photoreceptor). The results (Table S4; Fig. S1) clearly demonstrate that VOC emissions of JA-induced plants are significantly affected by FR. More importantly, the physiological inactivation of phyB led to a re-arrangement in the pattern of abundance of monoterpenes that is consistent with the effect of inactivating phyB by mutation (cf Table 2 with Table S4). In both cases, phyB inactivation led to reduced emissions of *p*-mentha-1,3,8-triene (compound 25), *trans*-3(10)-caren-2-ol (compound 36) and pulegon (compound 34), and increased emissions of α-terpinene (compound 16). In addition, phyB inactivation by FR or mutation led to reduced emissions of the homoterpene (*E,E*)-TMTT (compound 69).

phyB inactivation increases the attractiveness of JA-induced plants to the mirid predatory bug *M. pygmaeus*

To test whether the differences in VOC emissions caused by phyB inactivation could be functionally significant in indirect defense, we used a choice test with the mirid predatory bug *M. pygmaeus*. The Y-tube olfactometer set-up used in this study worked well for this insect: 94% of the tested

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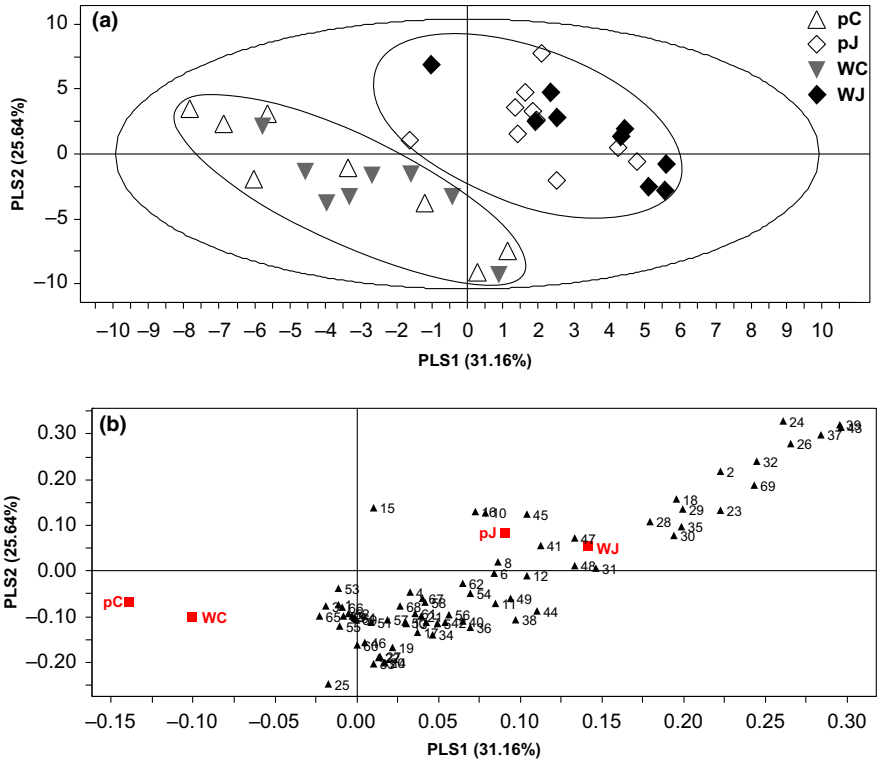

Fig. 4 Effects of methyl jasmonate (MeJA) treatment and genetic inactivation of phytochrome B (phyB) on the blend of volatile compounds collected in the headspace of tomato (*Solanum lycopersicum*) plants. (a) Separation of the headspace composition of different plant groups (wild-type control (WC, $n = 8$); *phyB1phyB2* control (pC, $n = 8$); wild-type with MeJA (450 μM) (WJ, $n = 9$); *phyB1phyB2* with MeJA (450 μM) (pJ, $n = 10$)) using projection to latent structures-discriminant analysis (PLS-DA), depicted as a two-dimensional score plot using the first two PLS components. The ellipses represent 95% confidence intervals using Hotelling T^2 statistics. (b) Loading plot indicating the contribution of each volatile compound to the separation between groups. For compound identity in relation to the numbering in the loading plot, please refer to Supporting Information Table S3.

M. pygmaeus females displayed a behavioral response. No influence of experimental day was found on the response of the predator, and this conclusion was valid for all treatments (GLM, $P > 0.05$).

Macrolophus pygmaeus females did not show a preference for any of the odor sources when exposed to volatiles from WT vs *phyB1phyB2* control plants ($\chi^2 = 2.69$; df = 1; $P = 0.72$). By contrast, when given a choice between volatiles from MeJA-treated WT and MeJA-treated *phyB1phyB2* plants, *M. pygmaeus* females showed a significant preference for the latter ($\chi^2 = 10.44$; df = 1; $P = 0.013$). Similarly, when the predators were offered a choice between volatiles from MeJA-treated WT plants grown under ambient light and MeJA-treated WT plants exposed to supplemental FR radiation, the insects showed a clear preference for the odors of FR plants ($\chi^2 = 6.25$; df = 1; $P = 0.001$) (Fig. 6). These results indicate that the mirid has no preference when offered non-induced WT and *phyB1phyB2* plants; however, if the plants are induced with MeJA, the predator displays a significant preference for the blend of volatiles emitted by plants in which phyB is inactivated.

Discussion

Our study shows that the inactivation of phyB in tomato results in attenuated levels of direct physical and chemical defenses and, at the same time, enhances the expression of induced indirect defenses. Inactivation of phyB leads to changes in the blends of volatiles that increase the attractiveness of JA-induced tomato plants to predatory insects. In the following sections, we discuss the mechanisms behind these effects, and their implications for plant–herbivore interactions.

phyB inactivation leads to reduced levels of direct defenses

Inactivation of phyB in the *phyB1phyB2* double mutant had a clear effect in reducing trichome density, as a consequence of the increased size of the epidermal cells. This is broadly consistent with results in other plant species, showing that phyB modulates epidermal cell size and differentiation (Boccalandro *et al.*, 2009; Casson & Hetherington, 2014). In addition to this effect on putative physical defense, we also observed a slight reduction in the concentration of C_{15} phenolic compounds in response to phyB inactivation. This reduction in levels of flavonoids has been observed in various species (Mazza & Ballaré, 2015), although, in tomato, the flavonoid response to FR radiation can vary between organs (Cagnola *et al.*, 2012). The molecular mechanism connecting flavonoid biosynthesis with phyB is not completely clear, but many steps in the synthesis and metabolism of phenylpropanoids are known to be regulated by light and phytochrome (Mancinelli *et al.*, 1991; Beggs & Wellmann, 1994). Soluble phenolic metabolites have been implicated in anti-herbivore defense in many species (Appel, 1993), including tomato (Elliger *et al.*, 1981; Stamp & Yang, 1996).

In addition to the changes in epidermal topography and profiles of phenolic metabolites caused by phyB inactivation, which presumably make the plants more susceptible to herbivory, we also found a significant repression of JA marker genes, including *PIN-II* and *TD*, which encode important proteins that provide anti-herbivore defense by disrupting digestive processes in the insect gut (Ryan, 1990; Chen *et al.*, 2005). Phytochromes and JA are known to interact in Arabidopsis (Moreno *et al.*, 2009;

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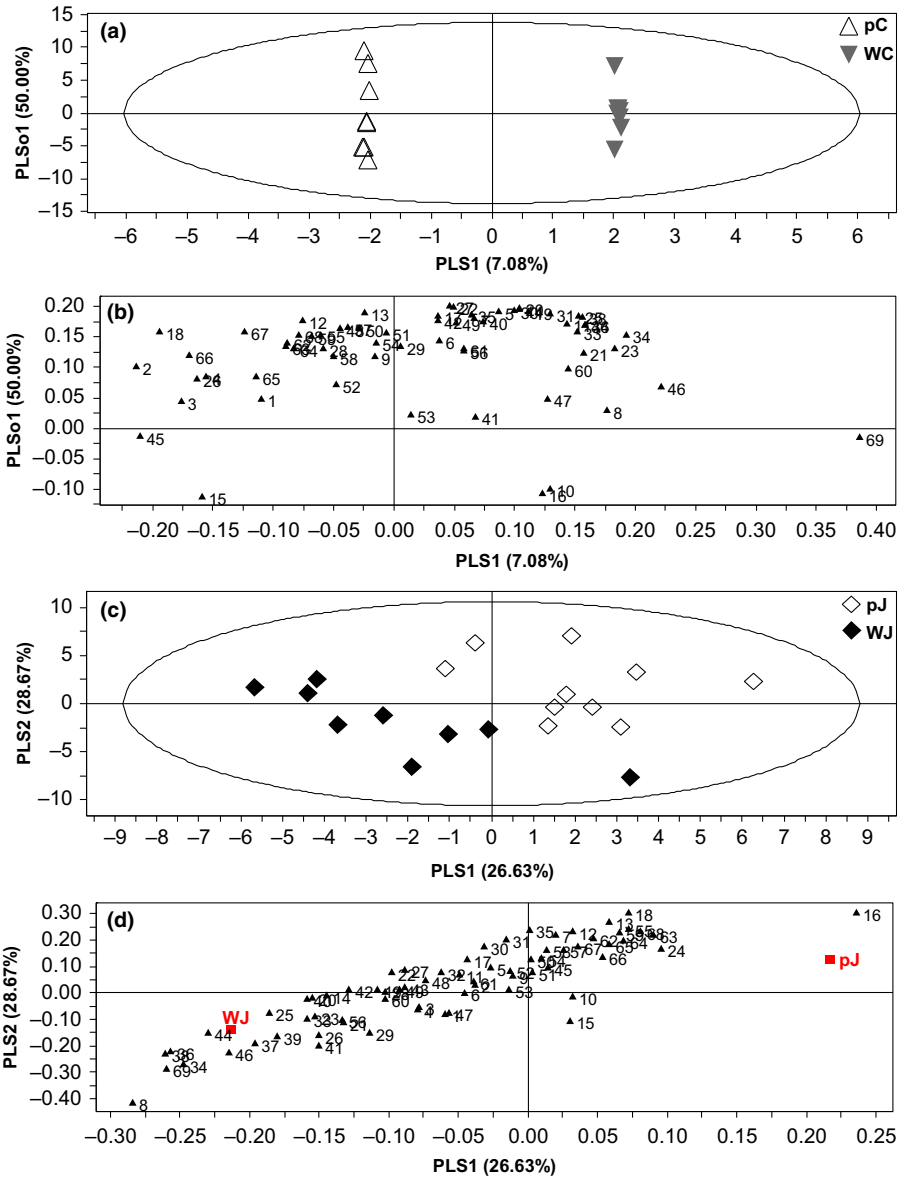

Fig. 5 Effects of genetic inactivation of phytochrome B (phyB) in tomato (*Solanum lycopersicum*) on the blend of volatile compounds emitted by control plants and plants induced with methyl jasmonate (MeJA). (a) Multivariate pair-wise comparison using orthogonal projection to latent structures-discriminant analysis (OPLS-DA) between control (not treated with MeJA) wild-type and *phyB1phyB2* plants (WC, pC, $n = 8$) based on the quantitative results of the volatiles in a two-dimensional score plot. (b) Loading plot indicating the contribution of each volatile compound to the separation between WC and pC. (c) Same analysis as in (a) comparing wild-type and *phyB1phyB2* plants after treatment with MeJA (450 μM) (WT plants elicited with MeJA (WJ), $n = 9$; *phyB1phyB2* plants elicited with MeJA (pJ), $n = 10$). (d) Loading plot indicating the contribution of each volatile compound to the separation between WJ and pJ sample groups. For compound identity in relation to the numbering in the loading plots, please refer to Supporting Information Table S3. The ellipses in (a) and (c) represent 95% confidence intervals using Hotelling T^2 statistics.

Robson *et al.*, 2010; de Wit *et al.*, 2013) and rice (Xie *et al.*, 2011). The functional link between phyB and JA responses in tomato is likely to involve the same molecular players as identified in the Arabidopsis model, where phyB inactivation represses JA signaling by tipping the balance between DELLA and JAZ repressor proteins in favor of the latter (Leone *et al.*, 2014), and by reducing the abundance of MYC transcription factors (Chico *et al.*, 2014). The general repression of defenses documented in our experiments (Figs 1, 2; Table S2) can explain the severe damage observed in *phyB1phyB2* plants (Fig. 3) and the enhanced performance of herbivores that has been documented in feeding bioassays (Izaguirre *et al.*, 2006).

Trading direct for indirect defense?

Although the concept that shade-light signals (particularly low R : FR ratio) repress the expression of direct defenses in shade-intolerant species is now well established and supported by data obtained in a variety of pathosystems (reviewed in Ballaré, 2014), the effects of light quality on indirect defenses have received very little attention. One exception is the recent work using the vine *Passiflora edulis*, which demonstrated that the production of extrafloral nectar, an indirect defense mechanism in *Passiflora* (McLain, 1983; Smiley, 1986; Apple & Feener, 2001; Xu & Chen, 2010) and many other species (Schoonhoven *et al.*, 2005; Heil, 2008, 2011), is down-regulated by low R : FR ratios (Izaguirre *et al.*, 2013). This down-regulation, as in the case of direct defenses, appears to be mediated by a reduction in plant sensitivity to JA (Izaguirre *et al.*, 2013).

VOCs also play an important role in indirect defense (Dicke, 2009; Dicke & Baldwin, 2010; Turlings *et al.*, 2012; Hilker & Fatouros, 2015), but very little is known about the interconnection between light-mediated neighbor detection and volatile signaling (Pierik *et al.*, 2014). Competition between plants can

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Table 1 Variable importance in the projection (VIP) values from the projection to latent structures-discriminant analysis (PLS-DA) for the pair-wise comparison between wild-type control (WC, $n = 8$) and *phyB1phyB2* control (pC, $n = 8$) tomato (*Solanum lycopersicum*) plants

		VOC quantitative values[c]			
No.[a]	Compound[b]	WC ($n = 8$)	pC ($n = 8$)	*P* value	VIP value[d]
69	(*E*,*E*)-TMTT	24.07 ± 7.28	4.45 ± 0.28	**0.001**	3.09
46	Pipertone	2.18 ± 0.42	1.36 ± 0.33	**0.021**	1.78
2	3-Pentanol	4.94 ± 0.25	8.09 ± 1.71	0.105	1.72
45	3-Caren-2-one	0.27 ± 0.06	0.41 ± 0.07	0.083	1.69
18	*trans*-β-Ocimene	33.84 ± 913	70.25 ± 19.93	0.195	1.55
34	Pulegon	0.57 ± 0.06	0.43 ± 0.09	0.279	1.53
23	(*Z*)-3-Hexen-1-ol propanoate	0.35 ± 0.08	0.23 ± 0.09	0.374	1.46
8	3,7,7-Trimethyl-1,3,5-cycloheptatriene	287.71 ± 113.71	85.85 ± 20.10	0.161	1.42
3	(*E*)-2-Hexenal	0.13 ± 0.08	0.27 ± 0.09	0.161	1.40
66	Germacrene A	0.33 ± 0.08	0.44 ± 0.07	0.13	1.35
26	(*E*)-DMNT	0.69 ± 0.25	0.86 ± 0.17	0.083	1.30
15	3-Carene	6.35 ± 2.82	16.40 ± 3.54	0.052	1.28
21	Isoterpinolene	1.71 ± 0.53	1.44 ± 0.44	0.773	1.26
44	Cuminaldehyde	5.96 ± 0.71	4.79 ± 0.06	0.279	1.26
4	(*Z*)-3-Hexen-1-ol	2.87 ± 1.20	4.91 ± 1.16	0.235	1.25
36	*trans*-3(10)-Caren-2-ol	15.13 ± 1.10	12.88 ± 1.84	0.442	1.25
38	Anetofuran	75.98 ± 25.10	50.06 ± 16.51	0.279	1.25
25	*p*-Mentha-1,3,8-triene	5.05 ± 0.72	4.05 ± 0.79	0.279	1.23
33	Sirenin	1.14 ± 0.19	0.85 ± 0.17	0.195	1.22
60	Isobicyclogermacrene	0.40 ± 0.10	0.26 ± 0.03	0.382	1.16
11	*trans*-*m*-Mentha-4,8-diene	21.29 ± 3.17	17.32 ± 4.95	0.442	1.13
31	1,3,5-tris(Methylene) cycloheptane	15.98 ± 1.87	13.81 ± 2.52	0.382	1.03
47	2-Caren-10-al	0.32 ± 0.07	0.28 ± 0.08	0.721	1.03
10	β-Pinene	7.62 ± 0.60	6.20 ± 1.06	0.442	1.02
67	Bicyclogermacrene	0.62 ± 0.08	0.85 ± 0.18	0.645	1.00

[a]Numbers correspond to those displayed in the loading plots of Figs 4 and 5.
[b](*E*)-DMNT, (*E*)-4,8-dimethylnona-1,3,7-triene; (*E*,*E*)-TMTT, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.
[c]Quantitative measurements of volatile emissions (volatile organic compounds, VOC) from control WT (WC) and *phyB1phyB2* (pC) plants are given as mean peak area ± SE per gram fresh weight of foliage divided by 10^4. *P* values for the differences between treatments (Mann–Whitney *U*-test) are shown (in bold if $P < 0.05$).
[d]VIP values for the multivariate data analysis based on the predictive components are given. Values ≥ 1 are the most influential for separation of the treatments.

affect volatile profiles, although the mechanisms involved are poorly understood (Kegge & Pierik, 2010). Previous studies have focused on the effects of phyB and the R : FR ratio on the emission of the plant volatile hormone ethylene (Finlayson *et al.*, 1998; Pierik *et al.*, 2004), and have examined potential consequences of these effects on plant architecture and SAS responses (Pierik *et al.*, 2003). More recently, Kegge *et al.* (2013) examined the effects of low R : FR ratios on VOC profiles in Arabidopsis. They found that partial inactivation of phyB by supplemental FR repressed the emission of some (but not all) VOCs in JA-induced plants. Interestingly, the authors found that Arabidopsis plants induced with MeJA were more attractive to neonates of the specialist herbivore *Pieris brassicae* if they had been previously exposed to FR radiation. Regardless of the mechanism, which was not investigated, the results of Kegge *et al.* (2013) demonstrated that light quality can affect plant interactions with herbivores, not only by altering direct defenses, but also by altering the blend of odors that herbivores use during their host selection decisions.

To the best of our knowledge, no previous study has investigated how light signals that represent competition modulate the VOC-mediated attraction of insect predators to attacked plants. We used the mirid bug *M. pygmaeus* to test for phyB effects on predator attraction. This predator is known to be attracted by VOCs emitted by tomato plants in response to herbivory by some of its prey insects (such as *Tuta absoluta* or *Bemisia tabaci*) (Lins *et al.*, 2014). In our choice bioassay using MeJA-induced plants, we found a clear preference for plants in which phyB was inactivated by mutation (*phyB1phyB2* plants) or by exposure to supplemental FR radiation (mimicking the effect of neighboring plants) (Fig. 6). Chemical analysis clearly shows that the volatile blends of MeJA-induced plants are affected by phyB inactivation (Fig. 5c,d; Table 2 (*phyB1phyB2* effect) and Fig. S1; Table S4 (FR effect)). The fact that the mirids made a consistent choice between plants with contrasting phyB status, preferring those with low levels of active phyB (regardless of the method used to inactivate the photoreceptor) (Fig. 6), suggests that the effects of phyB on the VOC profiles of JA-induced plants are functionally important for tritrophic interactions. Pinpointing the specific compounds that are responsible for the mirid's choice is likely to be very difficult, as insects commonly respond to compound blends, rather than individual chemicals (Gols *et al.*, 2011; van Wijk *et al.*, 2011). However, it is interesting to note that both mutation of the *PHYB* genes (in *phyB1phyB2*) and

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Table 2 Variable importance in the projection (VIP) values for the projection to latent structures-discriminant analysis (PLS-DA) for the pair-wise comparison of wild-type (WT) and *phyB1phyB2* tomato (*Solanum lycopersicum*) plants treated with methyl jasmonate (MeJA, 450 μM)

		VOC quantitative values[c]			
No[a]	Compound[b]	WJ ($n = 9$)	pJ ($n = 10$)	*P* value	VIP value[d]
8	3,7,7-Trimethyl-1,3,5-cycloheptatriene	373.00 ± 88.84	95.00 ± 15.76	**0.008**	1.83
34	Pulegon	0.64 ± 0.08	0.40 ± 0.04	**0.017**	1.61
69	(*E,E*)-TMTT	56.02 ± 12.68	24.62 ± 2.78	**0.01**	1.55
36	*trans*-3(10)-Caren-2-ol	17.07 ± 1.30	12.72 ± 0.64	**0.002**	1.51
38	Anetofuran	98.59 ± 19.52	47.51 ± 4.48	**0.022**	1.51
16	α-Terpinene	230.50 ± 26.92	327.10 ± 22.53	**0.022**	1.43
44	Cuminaldehyde	7.04 ± 0.58	5.36 ± 0.33	0.065	1.35
46	Pipertone	1.81 ± 0.21	1.26 ± 0.13	0.054	1.33
37	(*Z*)-3-Hexenylbutyrate	12.17 ± 2.70	6.25 ± 1.97	0.094	1.31
39	Hexylbutanoate	0.30 ± 0.08	0.13 ± 0.03	0.129	1.23
35	*trans*-2-Caren-4-ol	24.98 ± 2.98	25.33 ± 3.32	0.905	1.22
32	(*Z*)-3-Hexen-1-ol isobutyrate	0.45 ± 0.17	0.30 ± 0.09	0.603	1.17
25	*p*-Mentha-1,3,8-triene	4.49 ± 0.43	3.40 ± 0.26	**0.035**	1.16
18	*trans*-β-Ocimene	5.65 ± 0.79	4.20 ± 0.79	0.497	1.13
23	(*Z*)-3-Hexen-1-ol propanoate	111.95 ± 17.22	123.53 ± 15.56	0.243	1.12
26	(*E*)-DMNT	5.58 ± 1.63	3.25 ± 0.97	0.315	1.11
33	Sirenin	1.15 ± 0.20	0.78 ± 0.09	0.113	1.08
40	Dihydrocarveol	4.47 ± 0.58	3.27 ± 0.31	0.095	1.05
56	γ-Elemene	0.28 ± 0.04	0.21 ± 0.03	0.400	1.05
20	γ-Terpinene	102.88 ± 15.12	74.98 ± 7.80	0.243	1.04
31	1,3,5-tris(Methylene) cycloheptane	0.74 ± 0.27	0.66 ± 0.19	0.72	1.04
43	(*Z*)-3-Hexenyl isovalerate	20.18 ± 1.88	14.57 ± 1.72	0.842	1.03
13	2-Carene	3052.95 ± 242.97	3145.95 ± 135.27	0.782	1.02
41	Methylsalicylate	0.80 ± 0.17	0.57 ± 0.21	0.395	1.00

[a]Numbers correspond to those displayed in the loading plots of Figs 4 and 5.
[b](*E*)-DMNT, (*E*)-4,8-dimethylnona-1,3,7-triene; (*E,E*)-TMTT, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.
[c]Quantitative measurements of volatile emissions (volatile organic compounds, VOC) from MeJA-treated (450 μM) WT (WJ) and *phyB1phyB2* (pJ) plants are given as mean peak area ± SE per gram fresh weight of foliage divided by 10^4. *P* values for the differences between treatments (Mann–Whitney *U*-test) are shown (in bold if $P < 0.05$).
[d]VIP values for the multivariate data analysis based on the predictive components are given. Values ≥ 1 are the most influential for separation of the treatments.

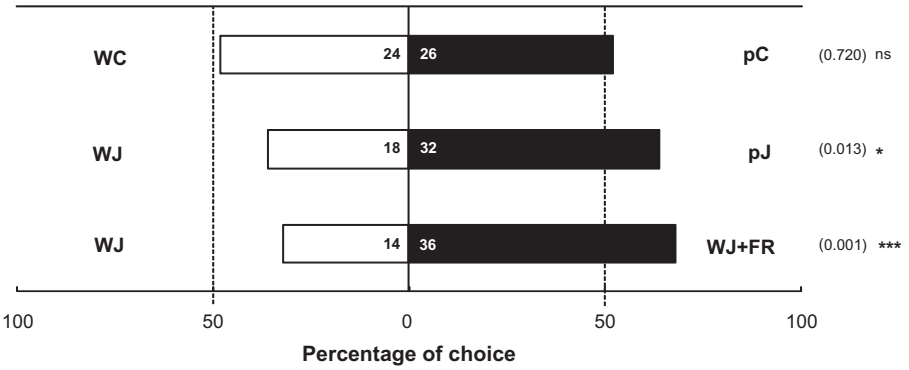

Fig. 6 Phytochrome B (phyB) inactivation increases the attractiveness of induced tomato (*Solanum lycopersicum*) plants to the mirid predator *Macrolophus pygmaeus*. The bars indicate the behavioral response of *M. pygmaeus* females in a Y-tube olfactometer when exposed to volatile blends emitted by plants with contrasting levels of active phyB and methyl jasmonate (MeJA) treatments. Wild-type control (WC) vs *phyB1phyB2* control (pC) plants indicates the contrast between WT and *phyB1phyB2* plants grown under ambient light and without MeJA treatment; WT plants elicited with MeJA (WJ) vs *phyB1phyB2* plants elicited with MeJA (pJ) indicates the contrast between WT and *phyB1phyB2* plants grown under ambient light and treated with MeJA (450 μM); WJ vs WJ+FR indicates the contrast between WT plants grown under ambient light and WT plants exposed to supplemental far-red (FR) radiation, when both groups of plants were treated with MeJA (450 μM). Asterisks indicate a preference differing significantly from a 50 : 50 distribution within a choice test (generalized linear model (GLM): *, $P < 0.05$; ***, $P \leq 0.001$; ns, not significant). Numbers in the bar segments represent the number of predators that chose the respective odor.

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photoinactivation of phyB (in FR-treated plants) led to a similar shift in the relative abundances of some of the monoterpenes emitted by MeJA-induced plants. This shift was characterized by repression of *p*-mentha-1,3,8-triene, *trans*-3(10)-caren-2-ol and pulegon, and increased emissions of α-terpinene (Tables 2, S4). Establishing whether or not this specific shift in the spectrum of monoterpenes is perceived by the mirid bug as information representing a 'shaded' plant under attack will require further investigation. Experiments testing the role of α-terpinene (alone or added to a blend from MeJA-treated WT plants) might help to explain the enhanced indirect defense.

Assuming that the responses documented in tomato represent evolved responses, which should not be taken for granted, given the pressure of artificial selection during the breeding process of this cultivated species, the effect of phyB inactivation on VOC profiles would appear to have adaptive value for both the emitting plant and the insect predator. For the plant, and provided that the biosynthetic cost of changing the VOC profile in response to phyB inactivation is not very high (Dicke & Sabelis, 1989), attracting more carnivores (Fig. 6) might partially compensate for the reduction in the investment in direct defenses (Figs 1, 2; Table S2). For the predator, directing its food search toward the VOCs emitted by less well-defended plants (i.e. plants exposed to low R : FR ratios) may increase the chances of finding more abundant and nutritious prey. It should also be kept in mind that some predators, including *M. pygmaeus*, are also facultative herbivores (Perdikis & Lykouressis, 2000; Lins *et al.*, 2014). Therefore, the mirid bug might also benefit from attraction to the VOCs emitted by plants with low levels of active phyB, as these plants are likely to have lower levels of anti-herbivore defenses. Finally, it is also reasonable to speculate that the bouquet of VOCs emitted by plants with low levels of active phyB might be used as a 'habitat cue' (Webster & Cardé, 2016) by insects seeking protection from excessive sunlight in shaded environments.

Research on plant responses to the R : FR ratio has traditionally focused on morphological changes that increase plant height and competitive ability in dense canopies (Smith, 1995; Ballaré, 1999; Casal, 2012; Pierik & de Wit, 2014; de Wit *et al.*, 2016). More recently, the role of phytochrome and other photoreceptors in the regulation of plant immunity against herbivores and pathogens has received increased attention (Ballaré *et al.*, 2012; Ballaré, 2014; Smakowska *et al.*, 2016). Our data do not only add to the emerging body of literature demonstrating intense crosstalk between light and defense signaling, but also show that changes in phyB status can affect the emission of plant VOCs, which are known to function as informational signals for a broad spectrum of canopy organisms. Therefore, we infer that the phenotypic responses elicited by changes in the R : FR ratio can have consequences that go well beyond the morphology of the plant that originally perceived a change in the light environment using its photoreceptor proteins. It seems that we are only beginning to scratch the surface of a complex world of interactions, in which plants not only adapt their morphology in response to light signals, but also modify the chemical composition of the canopy atmosphere in ways that are functionally meaningful for the sensory systems of herbivorous and predatory arthropods.

Acknowledgements

This research was financially supported by grants from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica) and UBACyT (Universidad de Buenos Aires Ciencia y Técnica) to C.L.B., and a Spinoza award of the Netherlands Organization for Scientific Research (NWO, SPI 87-313) to M.D. We thank Amy Austin for discussion and comments on the manuscript; Patricia Demkura and Miriam Izaguirre for their help with determination of phenolic compounds and PCR measurements; Patricia Fernández for her help with preliminary VOC experiments; Diego Bastos Silva for advice on Y-tube olfactometer assays and insect manipulations; Joop Woelke and André Gidding for rearing insects; and Unifarm (Wageningen, the Netherlands) for growing the plants.

Author contributions

L.E.C., H.E.B. and C.L.B. planned the original research. L.E.C. and B.T.W. performed the experiments and analyzed the primary data. L.E.C, B.T.W., M.D. and C.L.B discussed the analytical approach and results. L.E.C. and C.L.B. wrote the manuscript with input from all co-authors.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Effects of physiological inactivation of phytochrome B (phyB) by supplementary irradiation with far-red (FR) radiation on the blend of volatile compounds emitted by wild-type (WT) plants induced with methyl jasmonate (MeJA).

Table S1 Primer sequences used for quantitative real-time PCR

Table S2 Effect of phyB inactivation on the content of soluble phenolic compounds in tomato leaves

Table S3 Volatile compounds detected in the headspace of *Solanum lycopersicum* plants listed according to their elution order

Table S4 Variable importance in the projection values obtained from multivariate pair-wise comparison of wild-type (WT) plants exposed to methyl jasmonate (MeJA) under either ambient light or ambient light supplemented with far-red (FR) radiation

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