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Perception of low red:far-red ratio compromises both salicylic acid- and jasmonic acid-dependent pathogen defences in Arabidopsis

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SUMMARY

In dense stands of plants, such as agricultural monocultures, plants are exposed simultaneously to competition for light and other stresses such as pathogen infection. Here, we show that both salicylic acid (SA)-dependent and jasmonic acid (JA)-dependent disease resistance is inhibited by a simultaneously reduced red:far-red light ratio (R:FR), the early warning signal for plant competition. Conversely, SA- and JA-dependent induced defences did not affect shade-avoidance responses to low R:FR. Reduced pathogen resistance by low R:FR was accompanied by a strong reduction in the regulation of JA- and SA-responsive genes. The severe inhibition of SA-responsive transcription in low R:FR appeared to be brought about by the repression of SA-inducible kinases. Phosphorylation of the SA-responsive transcription co-activator NPR1, which is required for full induction of SA-responsive transcription, was indeed reduced and may thus play a role in the suppression of SA-mediated defences by low R:FR-mediated phytochrome inactivation. Our results indicate that foraging for light through the shade-avoidance response is prioritised over plant immune responses when plants are simultaneously challenged with competition and pathogen attack.

Keywords: shade avoidance, plant immunity, phytochrome, phosphorylation, NPR1, *Arabidopsis thaliana*.

INTRODUCTION

At high density, either in nature or in agriculture, plants are at risk of becoming shaded by the surrounding vegetation. To avoid getting cut off from the light plants in dense vegetation try to outgrow neighbours through increased leaf angles, stem elongation, apical dominance and early flowering; an escape strategy known as the shade-avoidance syndrome (Vandenbussche *et al.*, 2005; Franklin, 2008). The first signal known to announce the presence of surrounding plants is a decrease in the ratio red:far-red light (R:FR) (Morgan and Smith, 1976; Ballaré *et al.*, 1990). This cue is specific for plants, as red light (R) is absorbed for photosynthesis, whilst far-red light (FR) is reflected by green tissue (Smith, 2000). Red and far-red levels are perceived through the family of phytochrome photoreceptors (phyA-E in Arabidopsis), which exist in two photo-convertible forms: the active, far-red light absorbing form Pfr, and the inactive, red light absorbing form Pr (Smith and Holmes, 1977). PhyB is the main phytochrome involved in shade-avoidance responses, with redundant roles for phyD and phyE (Clack *et al.*, 1994; Franklin *et al.*, 2003). Active phyB is thought to bind the elongation promoting phytochrome interacting factors (PIF) 4, 5 and 7, members of a subfamily of basic helix–loop–helix (bHLH) transcription factors, in the nucleus, thereby targeting them for degradation

through the proteasome and thus preventing induction of the shade-avoidance signalling cascade (Lorrain *et al.*, 2008; Li *et al.*, 2012). Far-red light inactivates phytochrome, thus alleviating phytochrome-mediated PIF degradation. This situation leads to rapid induction of gene expression, including genes encoding other transcription factors such as PIF-like (PIL)1 and several homeodomain/leucine zipper (HD-zip) proteins (Salter *et al.*, 2003; Sessa *et al.*, 2005) and stimulates biosynthesis and signalling of various growth-promoting hormones including auxin (Tao *et al.*, 2008; Keuskamp *et al.*, 2010; Li *et al.*, 2012), gibberellins (Peng and Harberd, 1997; Djakovic-Petrovic *et al.*, 2007), brassinosteroids (Kozuka *et al.*, 2010) and ethylene (ET) (Finlayson *et al.*, 1999; Pierik *et al.*, 2004) ultimately resulting in enhanced elongation growth.

Another threat to plant survival in dense stands is the spreading of infectious diseases from one plant to another. There are two major hormonal pathways involved in plant immune responses: the salicylic acid (SA) and the jasmonic acid (JA) pathway. The SA and JA defence pathways each induce a different set of response genes and are often mutually antagonistic, which enables plants to fine-tune their defence response to a specific pathogen (Spoel and Dong, 2008; Pieterse *et al.*, 2012).

A key transcriptional regulator of SA-induced defence is the ankyrin-repeat and BTB/POZ protein–protein interaction domains containing protein NPR1 (Cao *et al.*, 1997; Ryals *et al.*, 1997). Under un-induced conditions NPR1 resides in the cytosol as an oligomer linked through intermolecular disulfide bridges between cysteine residues. Upon accumulation of SA an increase in cellular reducing capacity releases NPR1 monomers, which subsequently translocate to the nucleus (Kinkema *et al.*, 2000; Mou *et al.*, 2003; Tada *et al.*, 2008). NPR1 interacts with TGA transcription factors to co-activate transcription of primary defence-associated genes (Després *et al.*, 2000; Fan and Dong, 2002), and NPR1 may be part of an SA receptor complex (Fu *et al.*, 2012; Wu *et al.*, 2012). NPR1 phosphorylation and subsequent proteasome-mediated turnover of NPR1 are required for full induction of SA-induced transcription (Spoel *et al.*, 2009).

Induction of the JA pathway depends on the alleviation of transcriptional repression by proteins of the jasmonate ZIM domain (JAZ) family. The biologically active JA conjugate (+)-7-*iso*-jasmonoyl-L-isoleicine (JA-Ile) binds to the JA receptor complex coronatine insensitve (COI)1/JAZ (Fonseca *et al.*, 2009). COI1 is an F-box protein that is part of the E3 ubiquitin-ligase complex SCF^{COI1} (Xu *et al.*, 2002). Upon binding of JA-Ile, the SCF^{COI1} complex targets the JAZ proteins for degradation via the 26S proteasome (Chini *et al.*, 2007; Thines *et al.*, 2007). This situation allows for JA-induced gene expression through the bHLH transcription factors MYC2, MYC3 and MYC4, which are associated with herbivore resistance (Lorenzo *et al.*, 2004; Fernandez-Calvo *et al.*, 2011). Resistance against necrotrophic pathogens is regulated by the combined action of JA and ET and depends on the Apetala/Ethylene response factor (AP2/ERF) transcription factors ORA59 and ERF1 (Lorenzo *et al.*, 2003; Pré *et al.*, 2008).

Shading and pathogen attack are studied elaborately as single stresses. There are, however, several reports that highlight interplay between plant light signalling and pathogen defence. Total light intensity and the light period after infection have been shown to be important for the level of induction of SA-dependent defence (Genoud *et al.*, 2002; Griebel and Zeier, 2008). Light quality has also been shown to affect defence responses. Red light, but not light of other wavelengths, stimulates disease resistance against both biotrophic and necrotrophic pathogens in a variety of plant species (Islam *et al.*, 1998, 2008; Rahman *et al.*, 2010; Wang *et al.*, 2010), suggesting that phytochrome signalling may play a role in pathogen defence. The constitutively shade-avoiding Arabidopsis phytochrome mutants *phyA-phyB* and *phyB* and the *constitutive shade avoidance (csa) 1* mutant display reduced basal resistance against the hemi-biotrophic pathogen *Pseudomonas syringae* (Genoud *et al.*, 2002; Faigón-Soverna *et al.*, 2006), further suggesting a role for phytochromes in SA-dependent defence. The *phyAphyB* mutant furthermore had decreased SA-dependent systemic immunity in tissues away from the infection site (Griebel and Zeier, 2008).

Functional evidence for interaction between competition for light and JA signalling has been shown in studies combining shade with herbivore attack. *Chenopodium album* plants exposed to simulated canopy shade were less resistant against subsequent herbivore attack than plants in control light (Kurashige and Agrawal, 2005). Inactivation of phytochromes was further shown to increase susceptibility to herbivores, as both Arabidopsis and *Nicotiana longiflora* plants pre-treated with low R:FR, as well as *phyB* mutants of Arabidopsis and tomato (*Solanum lycopersicum*), supported higher caterpillar growth (Izaguirre *et al.*, 2006; Moreno *et al.*, 2009). Recently, it was shown that FR pre-treatment also enhances susceptibility to the necrotrophic pathogen *Botrytis cinerea* in Arabidopsis (Cerrudo *et al.*, 2012).

In the current research we studied the interaction between shade avoidance and SA-dependent defence, on the one hand, and JA-dependent defence, on the other. We investigated how shade avoidance and pathogen defence interact when both stresses are induced simultaneously at the physiological and genome-wide transcriptional levels. We found enhanced disease susceptibility and reduced gene expression profiles for both the SA- and JA-dependent pathways under low R:FR. Interestingly, shade-avoidance traits were not affected by defence induction, implying dominance of phytochrome signalling over defence induction. The SA-dependent transcript profile was affected radically

by addition of a low R:FR signal. We provide evidence that suggests that this suppression is brought about by a mechanism in which the low R:FR signalling pathway targets SA-inducible phosphorylation cascades and compromises the balance between monomerization and phosphorylation of the transcriptional co-activator NPR1.

RESULTS

Shade avoidance increases susceptibility to both a biotrophic and a necrotrophic pathogen

The effect of phytochrome signalling on pathogen defence was tested both by using the phytochrome mutant *phyB* and through subjecting plants grown in control light to low R:FR, achieved by supplementing control white light with FR-emitting light emitting diodes (LEDs). Constitutively shade-avoiding *phyB* plants inoculated with the virulent hemi-biotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) supported significantly higher bacterial proliferation than the Col-0 wild-type (Figure 1a). Mutant *phyB* plants inoculated with the necrotrophic pathogen *Botrytis cinerea* (*Bc*) were also more susceptible than Col-0 plants (Figure 1b), showing that both SA- and JA-dependent basal resistance are attenuated when constitutive expression of shade avoidance is genetically programmed. Resistance against *Bc* was also attenuated in plants that had been subjected to shading conditions through growth in high density (Figure S1). When Col-0 plants were placed in low R:FR immediately after inoculation with *Pst* or *Bc* they also showed reduced resistance as compared with inoculated plants kept in control light (Figure 1c,d). This finding indicates that defence against both a hemi-biotrophic and a necrotrophic pathogen is suppressed by the shade-avoidance response even when the two responses are induced simultaneously.

Pathogen defence does not affect the shade-avoidance response

To study the effect of induced defence on the shade-avoidance response, we subjected Col-0 plants inoculated with *Pst* or *Bc* to a low R:FR signal 1 day post-inoculation (dpi) and measured the petiole elongation in infected leaves over 24 h. Growth was inhibited in control light due to *Pst* infection, but the low R:FR-induced elongation response remained intact in *Pst*-inoculated plants (Figure 1e). Petiole growth in control light was not affected in leaves on which *Bc* spores had been placed (Figure 1f), although defence was induced in the petioles as measured by the induction of the marker gene *PDF1.2* (Figure S2). Petiole elongation in response to low R:FR, however, was not affected by *Bc* inoculation (Figure 1f). These results demonstrate that an induced defence response against either a biotrophic or necrotrophic pathogen does not inhibit the low R:FR-induced elongation response.

The interaction between shade avoidance and defence is not a direct consequence of resource partitioning

It is possible that one response overrules the other through resource partitioning (Cipollini, 2004). Here, we measured the response of the defence overexpression mutants *cpr1*, which has constitutively induced SA-dependent defence, and *cev1*, which has constitutively activated JA and ET signalling, to low R:FR. As these two mutants constitutively express defence, induction of shade avoidance would indicate that there is no immediate energetic restraint for both responses to occur simultaneously. First, we verified that expression of the SA-responsive marker gene *PR1* was unaffected by low R:FR in c*pr1* (Figure 2a), indicating that the genetically programmed constitutive expression of SA-dependent defence in this mutant was not compromised by induction of a shade-avoidance response. Expression of the JA defence marker gene *PDF1.2* was reduced in *cev1* under low R:FR as compared with control light (Figure 2b). However, *PDF1.2* expression was still over a 100-fold higher in *cev1* than in its wild-type Col-5, showing that the JA pathway is still highly expressed in this mutant under low R:FR. Interestingly, although both *cpr1* and *cev1* were more disease resistant than wild-type, low R:FR increased susceptibility in both mutants (Figure S3). This result suggests that a response to phytochrome inactivation by low R:FR can affect pathogen defence even when the defence machinery is induced constitutively. We then measured whether the defence-overexpressing mutants were still able to respond to low R:FR. Both mutants displayed stunted growth under control conditions (Figure 2c,d), suggesting that the constitutively expressed defence response goes at the expense of general growth. However, in response to the low R:FR treatment, petiole elongation was still enhanced in *cpr1* (Figure 2c), indicating that SA defence and shade avoidance can be expressed simultaneously. For *cev1* an interaction effect was found between genotype and light treatment (Figure 2d), but when the effect of light treatment was tested in the subset of the *cev1* genotype petiole elongation was increased significantly. Thus, constitutive activation of the JA-mediated defence pathway reduces low R:FR-induced petiole elongation, but does not inhibit fully the shade-avoidance response in the *cev1* mutant. This situation may be facilitated by the constitutive activation of ET signalling, a positive regulator of the shade-avoidance response, in the *cev1* mutant. Induction of the shade-avoidance marker gene *PIL1* by low R:FR remained unchanged in both defence overexpressors (Figure 2e,f), indicating that shade-avoidance signalling was not compromised by constitutive overexpression of SA- or JA-dependent defence. The low R:FR-induced petiole elongation and *PIL1* expression in combination with the overexpression of defence in *cpr1* and *cev1* shows that plants

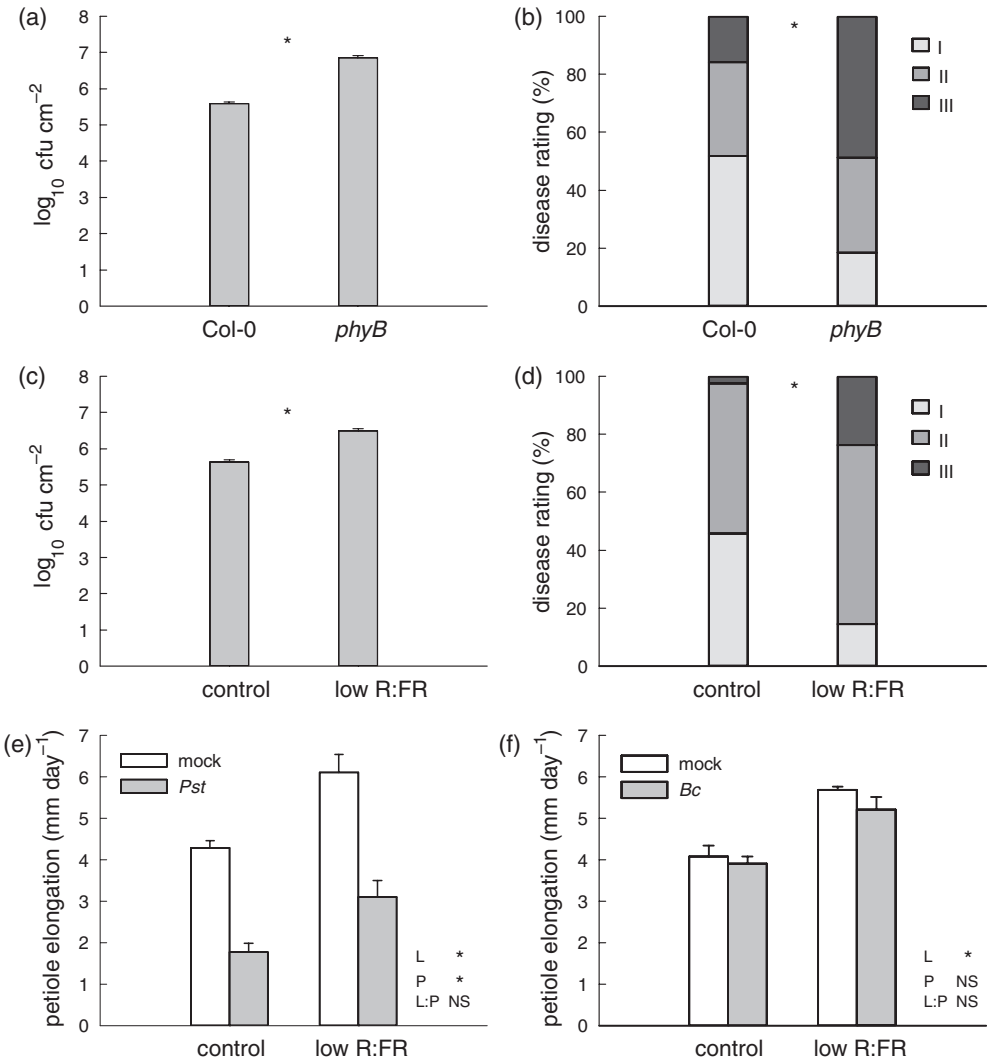

Figure 1. Disease incidence and petiole elongation of wild-type and *phyB* plants.
(a, c) Bacterial growth quantification 3 days post infection (dpi) of *Pst*-inoculated leaves of *phyB* mutant and Col-0 wild-type plants subjected to control light or low red:far-red (R:FR). Cfu = colony-forming units.
(b, d) Lesion diameters scored 3 dpi on leaves inoculated with a 5 μl droplet of *Bc* spore solution. I: lesion diameter <2 mm, II: lesion diameter ≥2 mm and ≤4 mm, III: lesion diameter >4 mm ($n > 20$ leaves).
(e, f) Petiole elongation of plants inoculated with *Pst* or *Bc*. Plants were transferred to control light or low R:FR 1 dpi. Data represent means ± standard error (SE). Asterisks indicate significant difference for (a, c) Student's *t*-test; (b, d) chi-squared test; (e,f) two-way analysis of variance (ANOVA); $P < 0.05$). L, light treatment; P, pathogen treatment; L:P, interaction between light treatment and pathogen treatment; NS, not significant.

are able simultaneously to direct resources to shade avoidance and defence responses.

Shade avoidance suppresses defence triggered by exogenous application of SA or MeJA

The increased susceptibility of shade-avoiding plants might be caused by conflicting responses of shade avoidance and pathogen defence. This outcome could be through antagonistic signal transduction steps or through downstream physiological consequences such as loosening cell walls for elongation versus cell wall fortification against pathogen penetration. Here, we investigated whether the interaction between pathogen defence and shade avoidance is also found when defence is induced by hormones. The SA pathway was induced with 0.5 mM SA, whereas the JA pathway was triggered with 0.1 mM methyl jasmonate (MeJA), and plants were then placed under low R:FR. SA-induced expression of *PR1* was strongly suppressed by low R:FR treatment (Figure 3a). Likewise, induction of *PDF1.2* was reduced in MeJA-treated plants placed under low R:FR (Figure 3b). Interestingly, basal levels of the defence genes were also reduced in mock-treated plants in low R:FR, as we found a significant effect of light treatment, but no interaction

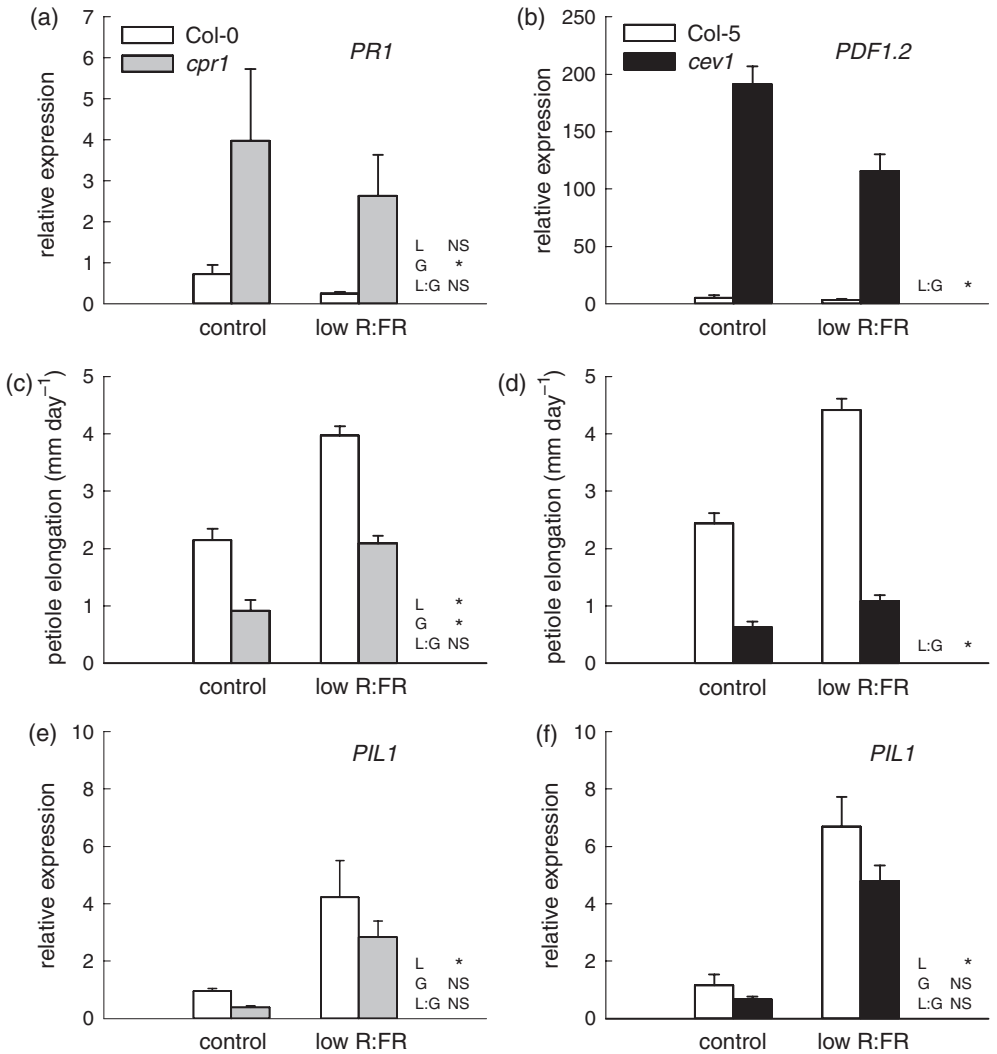

Figure 2. Marker gene expression and petiole elongation in the constitutive defence overexpressors *cpr1* and *cev1*.
(a) Defence marker gene expression in *cpr1* (light grey bars) and (b) *cev1* (black bars) and their wild-types Col-0 and Col-5, respectively (white bars) in control light and low red:far-red (R:FR) ($n = 4$).
(c, d) Petiole elongation after 24 h control light or low R:FR in *cpr1* and *cev1* ($n = 8$).
(e, f) Relative expression of the shade-avoidance marker gene *PIL1* ($n = 4$). Data represent means ± standard error (SE). Asterisks indicate significant difference (two-way analysis of variance (ANOVA), $P < 0.05$).). L, light treatment; G, genotype; L:G, interaction between light treatment and genotype; NS, not significant.

between light and hormone treatment (Figure 3a,b). Low R:FR-induced petiole elongation was not affected by application of SA, and neither was the expression of *PIL1* (Figure 3c,e). Petiole elongation both in control light and low R:FR was reduced by application of MeJA as compared with mock-treated plants (Figure 3d). However, elongation was still enhanced when MeJA-treated plants were subjected to low R:FR (Figure 3d) and the induction of *PIL1* was not affected by MeJA (Figure 3f). These results confirm that defence is inhibited by simultaneous induction of a shade-avoidance response, whereas the shade-avoidance response is not hindered by induction of defence responses.

Low R:FR suppresses both SA- and JA-dependent genome-wide transcript profiles

It is striking that both the SA- and JA-dependent pathways are overruled by shade avoidance, whereas the SA and JA pathways can act antagonistically to each other (Kunkel and Brooks, 2002; Pieterse *et al.*, 2012). As both shade avoidance and defence responses involve extensive transcriptional changes (Devlin *et al.*, 2003; De Vos *et al.*, 2005; Sessa *et al.*, 2005; Wang *et al.*, 2006), we used a genome-wide transcriptomics survey on plants simultaneously treated with low R:FR and SA or JA to study the interaction at the transcriptome level.

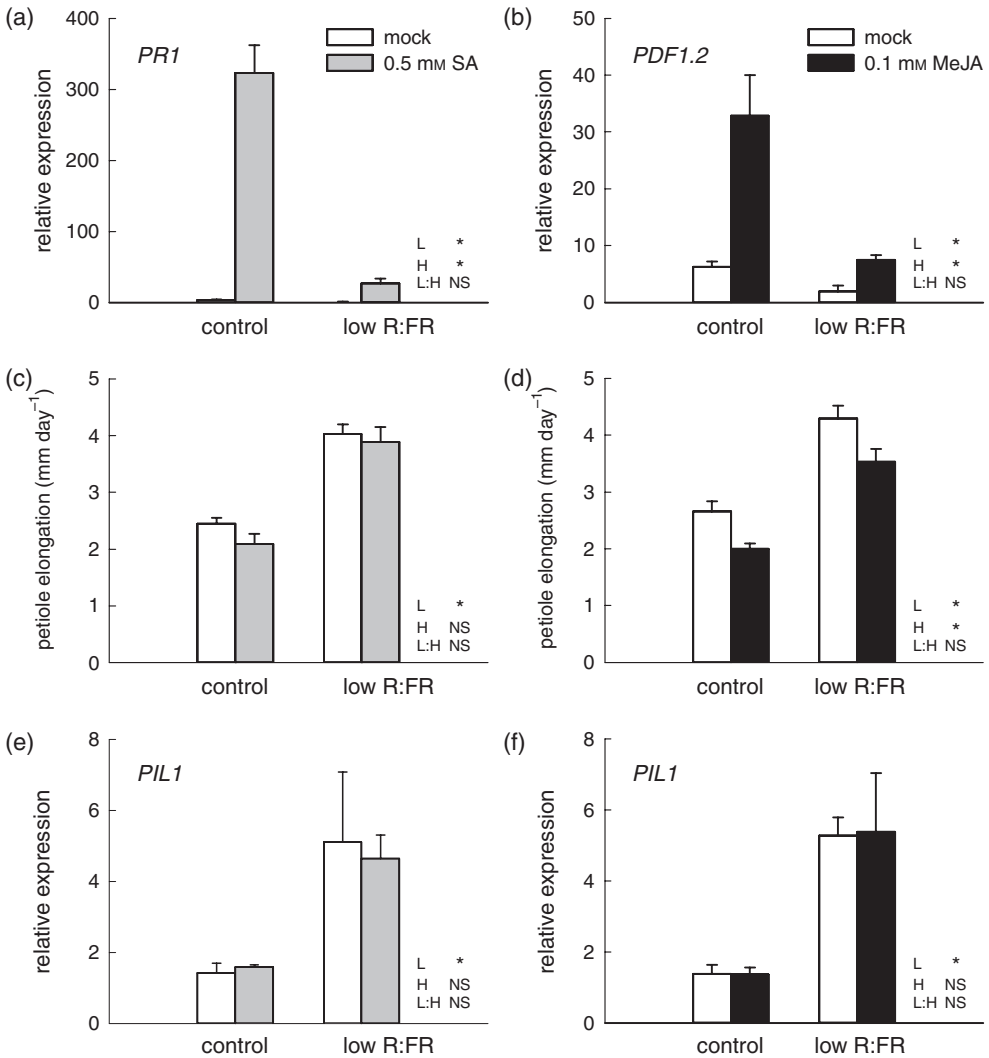

Figure 3. Hormone-induced marker gene expression and petiole elongation under low red:far-red (R:FR).
(a, b) Relative expression of defence marker genes in plants sprayed with 0.5 mM salicylic acid (SA) (light grey bars), 0.1 mM methyl jasmonate (MeJA) (black bars) or mock solution (white bars) and placed in control light and low red:far-red (R:FR) immediately after hormone treatment ($n = 4$).
(c, d) R:FR-induced petiole elongation after 24 h ($n = 8$).
(e, f) Relative expression of the shade-avoidance marker gene *PIL1* ($n = 4$). Data represent means ± SE. Asterisks indicate significant difference (two-way analysis of variance (ANOVA), $P < 0.05$). L, light treatment; H, hormone treatment; G, interaction between light treatment and hormone treatment; NS, not significant.

Two separate experiments were conducted for SA and MeJA treatments. In order to study events early in signalling while still having induction of the defence pathway, we harvested after 2 h of treatment. As expected, there was a large overlap in differentially expressed genes (DEGs) between the low R:FR treatment and the combined treatment of low R:FR and defence hormone (Figure 4). This situation was especially evident in the SA experiment, in which 78% of the genes regulated by low R:FR were also expressed differentially in the combined treatment with SA, as compared with 66% overlap in low R:FR-induced genes in the combined treatment with MeJA. The overlap in low R:FR-induced DEGs further confirms a minor effect of defence on the shade-avoidance response.

Changes in gene expression as induced by SA single treatment were massively reduced by the addition of a low R:FR signal, as only 15% of all DEGs regulated by SA were also expressed in the combined treatment with low R:FR (Figure 4a). A substantial number of DEGs appeared exclusively in the combined treatment of SA and low R:FR, however gene ontology (GO) analysis of this subset showed no over-representation of genes linked directly to defence (Table S1). We therefore conclude that the SA-induced transcriptional defence response is reduced severely by simultaneous perception of low R:FR. 66% of the DEGs by MeJA treatment (including a number of defence-related genes) were still expressed in the combined treatment with low R:FR, although at a lower level in the combined

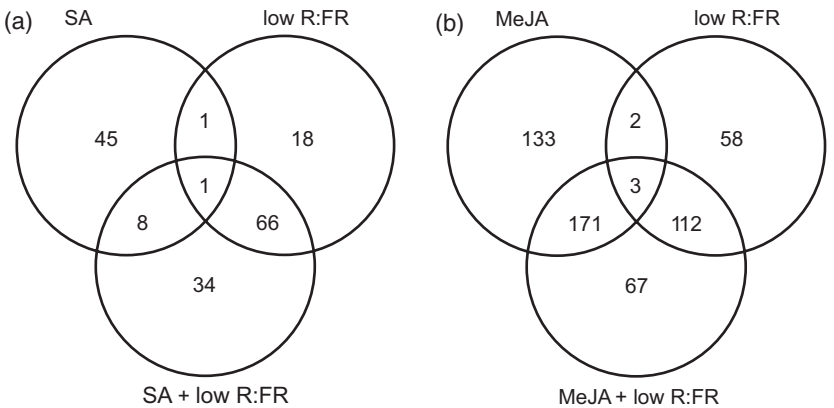

Figure 4. Microarray data analysis.
(a) Differentially expressed genes in microarray experiment 2 h after treatment with 0.5 mM salicylic acid (SA); or (b) 0.1 mM methyl jasmonate (MeJA), low red:far-red (R:FR), or combination of defence-inducing hormone and low R:FR. 3 replicate arrays were executed for each treatment.

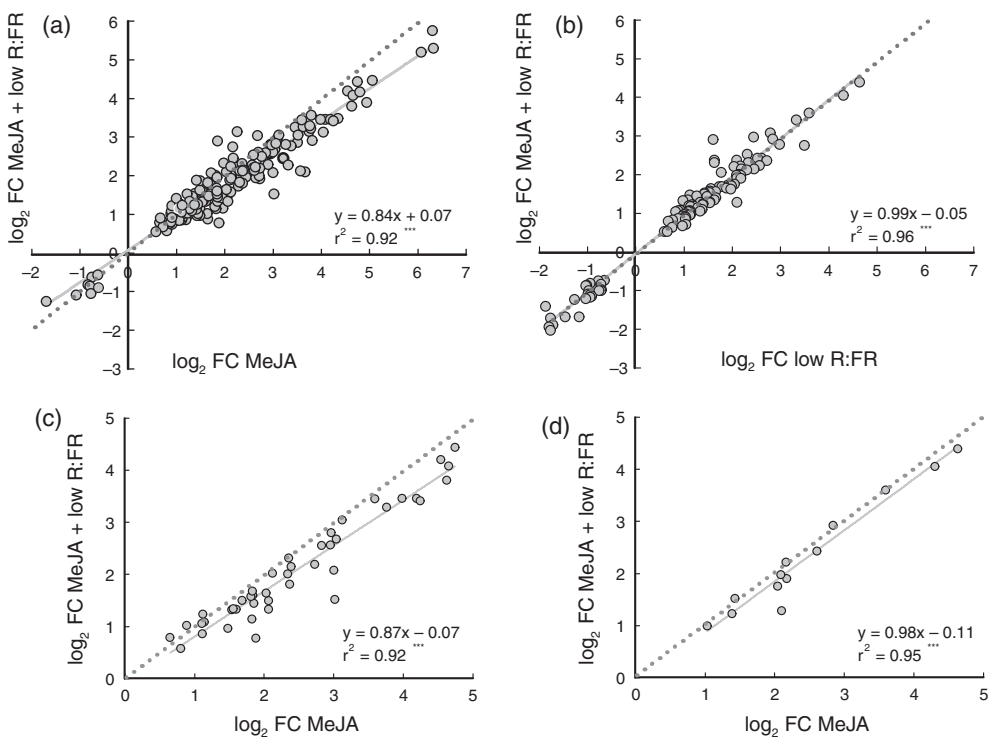

Figure 5. Linear regression of expression levels of two subsets of genes from the methyl jasmonate (MeJA) experiment in single and combined treatments.
(a) Linear regression on $\log_2$ fold changes ($\log_2$ FC) of genes induced by MeJA alone or combined with low red:far-red (R:FR); and (b) of genes induced by low R:FR alone or in combination with MeJA. (c) $\log_2$ FC of genes within GO categories belonging to jasmonic acid (JA) defence (GO IDs 9753, 9611, 51707, 19760, 9812 and 9695) as induced by MeJA alone or combined with low R:FR; and (d) of genes belonging to shade avoidance (GO IDs 9639, 9638, 10017, 10218 and 10202) as induced by low R:FR alone or in combination with MeJA. Genes plotted in (c) and (d) are specified in Table S2. Dotted line represents $y = x$. Asterisks indicate significant regression analysis (linear mixed model, $P \leq 0.001$).

treatment (Figure 5a,c and Table S2). Conversely, the expression levels of low R:FR-regulated genes that were also induced in the combined treatment of low R:FR and MeJA were highly similar in both treatments (Figure 5b,d and Table S2).

Surprisingly, there was hardly any functional over-representation in the genes that were significantly regulated exclusively in the combined treatments (Table S1; MeJA + FR only and SA + FR only). As both defence pathways may be suppressed by phytochrome signalling through a common mechanism, we compared the DEGs that were unique to the combined treatment of SA or MeJA with low R:FR. There was no overlap between the DEGs in the combined treatment of low R:FR with SA and those in the combination of low R:FR with MeJA (Figure S4), suggesting that low R:FR affects the transcriptome of the different defence pathways without inducing common transcriptional responses in the two combined treatments. Together, the microarray data indicate that low R:FR suppresses both the SA- and JA transcript profiles, whereby

the MeJA-responsive transcript profile is more moderately reduced in expression level than the SA-responsive profile, which is almost completely abolished in low R:FR.

Low R:FR affects SA-dependent phosphorylation

The massive phytochrome-mediated reduction in DEGs of the SA-regulated transcript profile prompted us to investigate whether NPR1, the central activator of SA-induced transcription, is a target of low R:FR signalling. We compared the list of the 45 SA-responsive genes that were repressed by low R:FR with the microarray data from Wang *et al.* (2006), for which the NPR1-dependency of transcripts induced by the SA analogue benzothiadiazole *S*-methylester (BTH) was determined. This comparison revealed that the majority of low R:FR-inhibited DEGs was NPR1 dependent, suggesting that NPR1 may indeed (but not exclusively) be targeted by low R:FR signalling. This suggestion was confirmed by the reduction of the SA-induced expression of the direct targets of NPR1-mediated transcription *WRKY18, WRKY38* and *WRKY62* in low R:FR (Figure 6a). Additionally, GO analysis of the SA arrays indicated that the expression of a number of SA-inducible kinases was repressed by low R:FR (Table S1 and S3). Phosphorylation has been shown to be important for NPR1-dependent transcription (Spoel *et al.*, 2009) and we hypothesized that low R:FR inhibits SA-dependent defence by targeting SA-induced phosphorylation cascades. We assessed whether induction of SA-responsive genes requires phosphorylation in a kinase inhibitor assay. SA-induced expression of both NPR1-dependent and -independent genes was inhibited by the global kinase inhibitor staurosporine (Figure 6b), which suggests that low R:FR signalling could indeed

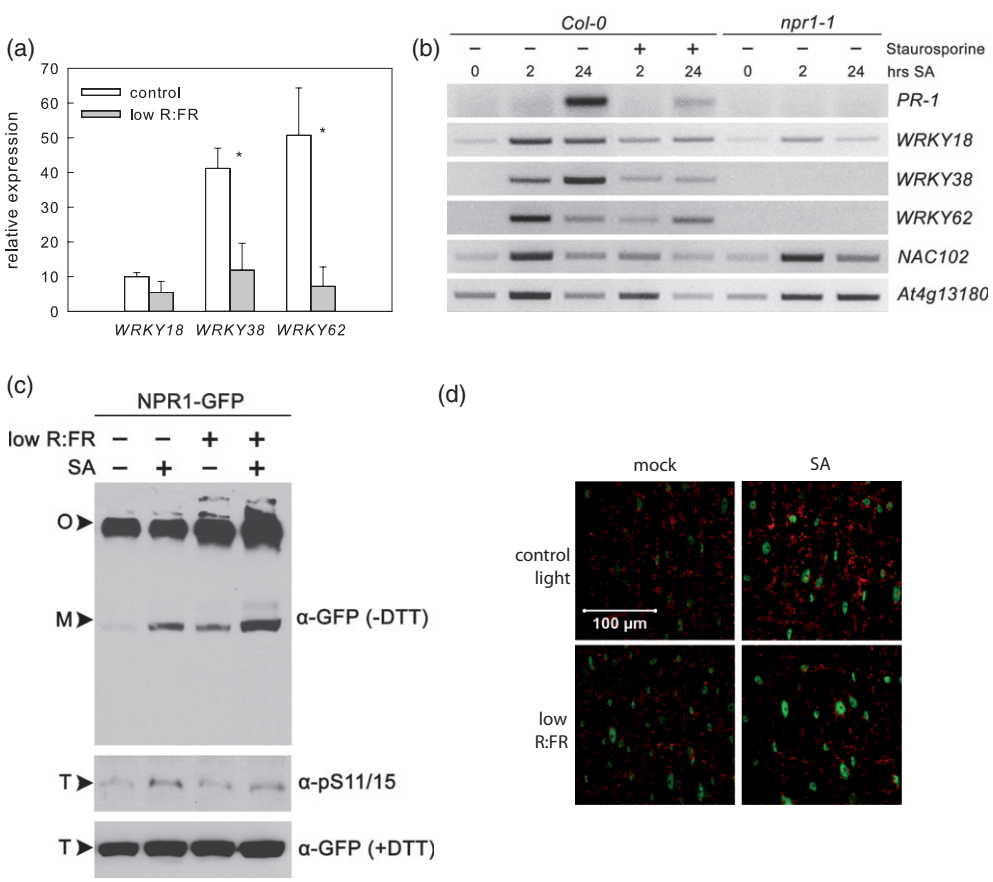

Figure 6. Phosphorylation-dependent gene expression and NPR1 protein analysis.
(a) *WRKY g*ene expression in petioles from Col-0 plants sprayed with 0.5 mM SA after 24 h of control light or low red:far-red (R:FR). Data represent means ± standard error (SE) ($n = 3$). Asterisks indicate significant difference.
(b) Semi-quantitative salicylic acid (SA)-induced expression of NPR1-dependent (*PR1, WRKY18, WRKY38,WRKY62*) and NPR1-independent [*NAC102* (Blanco *et al.*, 2005), *At4 g13180* (Wang *et al., 2006*)] genes in Col-0 and *npr1-1* seedlings in liquid medium. Here, 4.2 µM staurosporine was added to inhibit kinase activity.
(c) Total protein extracts from *35S::NPR1-GFP* plants sprayed with mock solution or 0.5 mM SA in control light or low R:FR after 8 h of treatment. Protein separation by SDS-PAGE was done in the presence or absence of 50 mM DTT and analysed by western blotting using an anti-GFP antibody or an antibody specific against NPR1 phosphorylated at Ser11 and Ser15 (Spoel *et al.*, 2009). O, NPR1 oligomer; M, NPR1 monomer; T, total NPR1.
(d) Confocal imaging in petioles of *35S::NPR1–GFP* plants sprayed with mock solution or 0.5 mM SA in control light or low R:FR after 8 h of treatment. Pictures represent a Z-stack from the epidermis inwards.

inhibit SA-induced transcription through suppression of SA-inducible kinases. To verify whether SA-induced phosphorylation is targeted by the shade-avoidance signalling pathway, we studied NPR1 phosphorylation in low R:FR. As NPR1 phosphorylation in the nucleus is preceded by NPR1 monomerization in the cytosol and translocation to the nucleus, we first studied whether SA-induced monomerization was affected by low R:FR. Surprisingly, NPR1 monomerization occurred in low R:FR even in the absence of SA and SA-induced monomerization was further enhanced by low R:FR than in control light (Figure 6c). The NPR1 monomer subsequently translocated to the nucleus as shown by confocal imaging (Figure 6d), showing that the SA pathway is inhibited by low R:FR despite NPR1 presence in the nucleus and an activating SA signal. Using an antibody specific against phosphorylated NPR1, we found that phosphorylation of NPR1 was clearly enhanced in plants treated with SA (Figure 6c). However, although plants in low R:FR contained increased amounts of monomer in the nucleus, phosphorylation was not enhanced proportionally in these plants, also not in the combined treatment with SA. These results indicate that NPR1 phosphorylation is compromised in low R:FR, confirming the hypothesis that SA-induced phosphorylation cascades could be important targets for the interaction between SA defence and shade avoidance.

DISCUSSION

Plants have developed a variety of plastic responses to survive adverse environmental conditions and our knowledge of the mechanisms underlying plant responses to single stresses is forever expanding. In the natural environment, however, especially combinations of stressful circumstances might occur. As crosstalk between inducible signalling pathways helps to fine tune the most suitable response in a complex environment, the understanding of a plant's response to its complex environment requires that stress responses be studied in combination (Mittler, 2006; Sultan, 2010). A few reports have described that plants that are shade avoiding are more susceptible to pathogens and, in particular, herbivores (Genoud *et al.*, 2002; Izaguirre *et al.*, 2006; Griebel and Zeier, 2008; Moreno *et al.*, 2009; Cerrudo *et al.*, 2012). Here, we extend these observed interactions and describe how the shade-avoidance pathway may affect defence signalling.

Shade avoidance is prioritised over pathogen defence

Whereas previous reports on interaction between light signalling and pathogen defence have focused mainly on the defence response, we have also measured the effect on shade avoidance. We found that when both stresses are induced simultaneously the shade-avoidance response to low R:FR is hardly affected, both at the levels of transcription and of the final elongation response (Figures 1–5). This finding implies that competition for light is prioritised over pathogen defence. Indeed, the shade-avoidance response is crucial for the plant's survival during competition for light, as a small difference in height with neighbouring vegetation results in an excessive difference in light capture (Ballaré *et al.*, 1988; Pierik *et al.*, 2003). Plants thus seem to have evolved a mechanism that ensures they can keep up with neighbours and maintain light interception for photosynthesis even when they are under attack by pathogens.

Pathogen defence is compromised by shade avoidance

In accordance with previous reports, we have found that the constitutive shade-avoidance mutant *phyB* has reduced SA-dependent resistance against *Pst* as well as reduced JA-dependent defence against *Bc*. Even when phytochrome signalling was modified through supplemental FR light simultaneously with pathogen inoculation, plants were more susceptible to both the SA- and JA-resisted pathogens (Figure 1). This result is important as it shows that the inhibition of the defence responses (which we observed in the *phyB* mutant, and others in *phy* mutants or prolonged FR pre-treatment) is not necessarily because shade avoidance was induced first – so the defence response could not be induced due to, for instance, resource partitioning. Indeed, even with constitutively induced defence, the *cev1* and *cpr1* mutants were still able to display enhanced elongation in low R:FR (Figure 2). Accordingly, *C. album* plants with induced defence due to previous herbivore attack were still able to elongate stems upon shading (Kurashige and Agrawal, 2005). These data show that under experimentally manipulated conditions plants can, in principle, express both a shade avoidance and a defence response. It remains to be investigated whether this situation also holds for a long-standing pathogen infection, in which the plant's resources might be more severely depleted or redirected compared with in our short-term experiments. Previously, it has been shown that the *sav3-2* shade-avoidance mutant which does not display a shade-avoidance response to low R:FR still has reduced resistance against herbivores in low R:FR, despite the fact that this mutant does not direct resources towards elongation growth upon low R:FR (Moreno *et al.*, 2009). This situation further hints towards a low R:FR-mediated reduction of defence that is not directly through resource partitioning.

The fact that both SA- and JA-induced pathogen defence responses are overruled by the shade-avoidance response is quite remarkable, as SA- and JA-dependent defence signalling have been shown often to be mutually antagonistic (Pieterse *et al.*, 2012). The inhibition of both defence responses could be brought about through a common low R:FR-induced process or might be regulated through a specific mechanism for both defence pathways. The lack of any shared DEGs in the combined treatment of SA or

MeJA with low R:FR that could indicate the induction of a similar downstream mechanism (Figure S4 and Table S1) implies that both defence pathways are each targeted specifically by the phytochrome pathway.

Low R:FR-induced repression of JA-dependent defence

The transcript profiles of SA- and JA-mediated defence were both suppressed by low R:FR, but in different ways. The numbers of genes in the JA profile were not reduced as rigorously as in the SA profile, but the expression levels of MeJA-dependent DEGs were generally decreased by low R:FR (Figure 5 and Table S2). A reduction in JA defence marker gene expression by low R:FR-induced phytochrome inactivation has been shown in Arabidopsis by Moreno *et al.* (2009) and in *Lotus japonicus* (Suzuki *et al.*, 2011). JA-induced gene expression can also be inhibited by cytosolic NPR1 upon SA treatment (Spoel *et al.*, 2003). Interestingly, this SA-dependent suppression of the JA response was shown to be dependent on SA-induced redox changes (Koornneef *et al.*, 2008), which suggests that the enhanced monomerization of NPR1 that we observed in low R:FR might negatively affect the JA signalling pathway. However, decreased resistance to *Bc* after FR-pre-treatment was maintained in *npr1* plants and in the SA biosynthesis mutant *sid2* (Cerrudo *et al.*, 2012), showing that suppression of JA-dependent pathogen defence by phytochrome inactivation is not dependent on SA and NPR1.

Reduced JA biosynthesis would be an obvious candidate for a reduced JA response by phytochrome signalling. JA levels in the *phyAphyB* Arabidopsis mutant, however, have been found to be comparable with wild-type controls, both in control conditions and after wounding (Zhai *et al.*, 2007). From the five DEGs in our transcript profiling that are regulated by MeJA and fall into the GO category 'JA biosynthesis' (GO ID 9695) only *ALLENE OXIDE CYCLASE (AOC)1*, which encodes an enzyme that catalyzes an essential step in JA biosynthesis, is repressed. However, this situation could be due either to direct suppression of JA biosynthesis or a suppressed feed-forward loop in low R:FR.

The JAZ repressors of JA-induced transcription are likely targets for attenuation of the JA response. JAZs are degraded upon JA perception to allow JA-induced gene expression; the expression of the JAZ genes themselves is rapidly upregulated by a positive feedback loop to JA, herbivory and wounding (Thines *et al.*, 2007; Chung *et al.*, 2008). Moreno *et al.* (2009) found increased MeJA-induced expression of *JAZ10* in low R:FR-pre-treated plants, although only at a relatively high MeJA concentration of 450 μM. *Bc* resistance was not found to be suppressed in two low R:FR-treated *JAZ10* RNAi lines (Cerrudo *et al.*, 2012), suggesting that JAZ10 indeed plays a role in the interaction. It will be interesting to see how phytochrome signalling affects protein stability of JAZ10 and other JAZs. In our microarrays without prolonged FR pre-treatment and a MeJA concentration of 100 μM, the expression of all MeJA-induced *JAZs* (including *JAZ10*) were somewhat reduced in the simultaneous treatment with low R:FR, except for *JAZ1* (Figure S5).

Low R:FR-induced repression of SA-dependent defence

Transcription of SA-responsive genes was strikingly reduced in low R:FR (Figure 4). NPR1 has long been established as the central regulator of SA-induced defence, as *npr1* mutants fail to raise an SA response. In the presence of SA, NPR1 oligomers in the cytosol are monomerized due to a change in the redox state of the cell and translocated to the nucleus. As the SA response is inhibited by low R:FR, it could be expected that NPR1 monomerization or translocation to the nucleus would be inhibited by phytochrome inactivation. However, we found the opposite: NPR1 monomerization was enhanced in low R:FR both with and without SA (Figure 6). This puzzling result implies that low R:FR affects the redox environment of the cell. It is well known that the two light harvesting complexes photosystems (PS) I and II have distinct pigment protein complexes, which preferentially absorb different wavelengths of the incident light. When the light environment becomes FR-enriched, this outcome will lead to enhanced excitation of the preferentially FR-absorbing PSI over PSII, which is more sensitive to R wavelengths. The imbalance of the relative number of photons captured by the two photosystems causes a shortage in electron supply from PSII to PSI. This outcome can lead eventually to adjustment of the stoichiometry of the photosystems to optimize photosynthesis quantum yield but may initially affect the redox state of the electron transport chain (Chow *et al.*, 1990; Walters, 2005), which might lead to changes in the redox state of the cell and subsequently affect NPR1 monomerization.

Previously, it has been shown that two NPR1 cysteine mutants had constitutive NPR1 monomer accumulation and enhanced *PR1* expression in the absence of elevated SA, indicating that monomeric NPR1 is sufficient to induce *PR* gene expression (Mou *et al.*, 2003). Here, we show that, in low R:FR, NPR1 monomer accumulates and translocates to the nucleus but does not induce defence-associated genes (Figure 6). SA-induced transcription is inhibited even when SA is applied together with low R:FR, indicating that defence-associated transcription is repressed by low R:FR-induced phytochrome inactivation. NPR1 does not have a DBD and transcription of defence genes depends on its interaction with TGA transcription factors, whose binding activity to promoter elements requires interaction with NPR1 (Després *et al.*, 2000; Fan and Dong, 2002). Whether in low R:FR the physical interaction between NPR1 and TGAs, the binding capacity of the TGAs to target genes, or recruitment of the initiation

complex and RNA polymerase II are affected remains to be investigated.

Phosphorylation of NPR1 in the nucleus has been shown to be necessary for full expression of NPR1-mediated target genes (Spoel *et al.*, 2009). We indeed found that phosphorylation of NPR1 did not increase proportionally to the increase in NPR1 monomers in low R:FR (Figure 6). If NPR1 is not phosphorylated and thus not cleared from the promoters of target genes, the transcription cycle might not be re-initiated and transcription could be stalled (Spoel *et al.*, 2009). This situation would explain why NPR1-dependent transcription is inhibited by low R:FR, even though NPR1 monomer is present in the nucleus.

The repression of SA-induced transcription through repression of phosphorylation cascades appears not to be restricted to NPR1-dependent genes. GO analysis of the DEGs revealed that a number of SA-regulated genes whose products are involved in protein phosphorylation were no longer expressed in combination with low R:FR (Table S1). The fact that several SA-induced kinases were inhibited by low R:FR (Table S3) suggests that defence-related phosphorylation is a general target for the shade-avoidance pathway. Accordingly, we found that SA induction of NPR1-independent genes can also be repressed by inhibition of kinase activity (Figure 6b). Phosphorylation cascades play an important role in both SA and JA defence, as well as in abiotic stress signalling (Colcombet and Hirt, 2008) and are thus important for directing stress responses. Targeting of kinases specific for certain responses might be an important aspect of interactions between signalling pathways and thus in the regulation of plant multiple stress responses.

EXPERIMENTAL PROCEDURES

Plant growth and treatments

Wild-type, *phyB* (Reed *et al.*, 1993), *cpr1* (Bowling *et al.*, 1994) and *35S::NPR1-GFP* (Kinkema *et al.*, 2000) plants were in the *A. thaliana* Columbia (Col-0) background, *cev1* was on the Col-5 background (Ellis and Turner, 2001). Seeds were sown on 1:2 potting soil:perlite substrate with additional nutrients (Millenaar *et al.*, 2005) and stratified at 4°C for 3 days. Ten-day-old seedlings were transferred to individual pots of 70 ml soil. Plants were watered daily and grown in a 9-h light period of 180 μmol m^{-2} sec^{-1} at 20°C and 70% RH. *35S::NPR1-GFP* plants were grown in a 16-h light period. Four-week-old rosettes were sprayed with 0.5 mM SA, 0.1 mM MeJA, or a mock solution containing 0.1% v/v ethanol such that the leaves were covered in a fine mist. Plants were left to dry before they were put in different light treatments. A red:far-red ratio (R:FR) of 0.2 was obtained through supplemental far-red LEDs (730 nm; Philips Green Power, http://www.philips.com) added to a control white light background (R:FR 2.2; Philips HPI-T Plus, 400 W), with PAR of 110 μmol m^{-2} sec^{-1}. For elongation growth experiments one petiole per plant of approximately 5 mm in length was measured with a digital calliper at the start of the light treatment and 24 h later. Petioles of the same developmental age were used in microarray and real-time reverse transcription polymerase chain reaction (RT-PCR) assays.

Pathogen assays

The hemi-biotrophic bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000 was grown overnight in liquid King's B medium (King *et al.*, 1954). Bacteria cells were collected and resuspended in 10 mM $MgSO_4$. Three upper leaves of 4-week-old plants were pressure-infiltrated with bacterial suspension of OD_{600} = 6×10^{-4}. Inoculated plants were transferred to the light conditions mentioned above and kept in 100% RH for 1 h to facilitate the infection. Two leaf discs (0.3 cm^2 each) per plant were harvested in 10 mM $MgSO_4$ 3 days post-inoculation (dpi). Appropriate dilutions of homogenized samples were plated on King's B agar supplemented with 50 mg L^{-1} rifampicin, from which the number of colony-forming units per cm^2 of infected leaf tissue could be determined after 48 h. For *cpr1* bioassay and elongation growth measurements in *Pst*-inoculated leaves the plants were dipped in a bacterial suspension of OD_{600} = 0.02 and 0.015% Silwet L-77 and kept at high RH throughout the experiment.

The necrotrophic fungus *Botrytis cinerea* strain B0510 was grown on half-strength potato dextrose agar (PDA; Difco Laboratories) at 22°C. After 2 weeks, conidia were harvested in half-strength potato dextrose broth. A 5 μl droplet of 5×10^5 spores/ml suspension was placed on leaf laminas of 4-week-old plants, which were kept at 100% RH throughout the experiment. Lesion diameters were scored 3 dpi and divided into classes according to size.

RNA isolation and real-time RT-PCR

Petioles were harvested and snap frozen 2 h (microarray) or 24 h (real-time RT-PCR) after the start of light treatments. Each biological replicate consisted of three petioles pooled from three different plants. Total RNA was isolated from homogenized material using the RNeasy plant mini kit (Qiagen, http://www.qiagen.com) with on-column DNA digestion following the company's instructions. Total RNA was transcribed to cDNA by Superscript III reverse transcriptase (Invitrogen, http://www.invitrogen.com) using random hexamers at 50°C in a total volume of 20 μl containing 100 units of reverse transcriptase III, 4 μl of first-strand buffer, 40 units of RNase inhibitor (Qiagen) and 1 μl of 0.1 M dithiothreitol. Real-time RT-PCR was performed on a 20 μl reaction mixture containing 10 μl SYBR Green Supermix (Bio-Rad, http://www.bio-rad.com) in a Bio-Rad MyIQ single-colour real-time PCR detection system. Gene-specific primers were designed using the PRIMER3PLUS software (Untergasser *et al.*, 2007; sequences in Table S4). Gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with *UBQ5* as internal standard.

Transcriptional profiling

cDNA synthesis, cRNA synthesis and hybridization to ATH1 Affymetrix Arabidopsis Gene Chips were performed commercially by Service XS Leiden, The Netherlands (authorized service provider Affymetrix, http://www.affymetrix.com). Three biological replicates were used for each treatment. Microarray data were analysed using the Bioconductor packages in R (www.bioconductor.org). Data were normalized with the RMA algorithm and differential expression was assessed using the empirical Bayes method in the R LIMMA package (Smyth, 2004) and Benjamini–Hochberg multiple testing correction (Benjamini and Hochberg, 1995). As the separately performed JA and SA microarray experiments hybridized differently (Figure S6), we used different statistical cut-offs for both experiments. For the JA experiment (see Cerrudo *et al.*, 2012), genes were considered differentially expressed when adjusted P-value < 0.003. For the SA experiment, an adjusted

P-value <0.05 combined with a $\log_2$ fold change >1 was used to define DEGs. Gene ontology (GO) analysis was done with the BiNGO plugin of Cytoscape (Maere *et al.*, 2005). Data are available at the NCBI gene expression and hybridization array data repository, Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo; accession no. GSE35700 and GSE45728).

Kinase inhibition assay

Col-0 and *npr1-1* plants were grown on plates containing Murashige and Skoog (MS) medium (pH 5.7, supplemented with 20 g L^{-1} sucrose, 0.8% (w/v) agar, and 1 × Gamborg's vitamin solution). After 12 days, seedlings were submerged in solutions with or without 0.5 mM SA. To inhibit kinase activity, solutions were supplemented with 4.2 μM staurosporine (in 2% DMSO), while controls were treated with 2% DMSO alone. Seedlings were harvested after 2 h and 24 h and gene expression was analysed with semi-quantitative RT-PCR using gene-specific primers (Table S5).

Protein analysis

Frozen leaf tissue of *35S::NPR1-GFP* plants was ground in extraction buffer (50 mM Tris–HCl (pH7.5), 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.2% Nonidet P-40) with inhibitors (40 μM MG132, 50 μg ml^{-1} TPCK, 50 μg ml^{-1} TLCK, 0.6 mM PMSF and 1% Sigma phosphatase inhibitor cocktail). Homogenized samples were then centrifuged (16 000 ***g***) at 4°C for 20 min. SDS sample buffer was added to protein extracts, as well as 50 mM DTT for the reducing gel. After heating for 10 min at 70°C the protein samples were separated on SDS-PAGE gels and transferred to nitrocellulose membranes, which were probed with anti-GFP (Roche, https://www.roche-applied-science.com/) or an antibody specific against phosphorylated Ser11 and Ser15 residues of NPR1 (α-pS11/15) (Spoel *et al.*, 2009).

Confocal imaging

NPR1–GFP fluorescence was visualised in *35S::NPR1–GFP* plants using an inverted confocal laser scanning microscope (Zeiss LSM Pascal, ×40 C-apochromat objective) with an excitation wavelength of 488 nm, a 505–530 bandpath filter for GFP emission and a 560 nm long pass filter for red fluorescence visualisation by chloroplasts.

Statistical analysis

Data were analysed through analysis of variance (ANOVA) or chi-squared test in the R statistical environment (R Development Core Team 2009).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. *Bc* infection in plants grown at high density.

Figure S2. Leaf response to *Bc*-inoculation.

Figure S3. Disease incidence of the constitutive defence overexpressors *cpr1* and *cev1* in control light and low R:FR.

Figure S4. Overlap in differentially expressed genes induced by combined treatment of SA and low R:FR and combined treatment of MeJA and low R:FR.

Figure S5. Heatmap of $\log_2$ Fold Change expression values of *JAZ* genes in MeJA treatment and MeJA combined with low R:FR.

Figure S6. Volcano plots of JA and SA microarrays.

Table S1. GO analysis of differentially expressed genes from SA- and MeJA microarrays.

Table S2. Gene IDs corresponding to Figure 5c,d.

Table S3. SA-induced kinases repressed by low R:FR.

Table S4. Primer sequences (5′→3′) used for Real-Time RT-PCR.

Table S5. Primer sequences (5′→3′) used for semi-quantitative RT-PCR.

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