RESEARCH ARTICLE

Molecular Profiles of Contrasting Shade Response Strategies in Wild Plants: Differential Control of Immunity and Shoot Elongation

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Short title: Contrasting phytochrome responses in wild plants

One-sentence summary: Geranium species from different habitats show contrasting molecular patterns and physiological responses to phytochrome-mediated detection of neighbour cues.

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ABSTRACT

Plants growing at high densities elongate their shoots to reach for light, a response known as the shade avoidance syndrome (SAS). Phytochrome-mediated detection of far-red light reflection from neighbouring plants activates growth-promoting molecular pathways leading to SAS. It is however unknown how plants that complete their life cycle in the forest understory and are shade tolerant prevent SAS when exposed to shade. Here we show how two wild Geranium species from different native light environments regulate contrasting responses to light quality cues. A comparative RNA sequencing approach unveiled the molecular underpinnings of their contrasting growth responses to far-red light enrichment. It also identified differential phytochrome control of plant immunity genes and confirmed that far-red enrichment indeed contrastingly affects resistance against Botrytis cinerea between the two species. Furthermore, we identify a number of candidate regulators of differential shade avoidance. Three of these, the receptor-like kinases FERONIA and THESEUS1 and the non-DNA binding bHLH protein KIDARI, are functionally validated in Arabidopsis thaliana through gene knockout and/or overexpression studies. We propose that these components may be associated with either showing or not showing shade avoidance responses.
**Introduction**

Plants have evolved a variety of strategies to deal with environmental stresses. Oftentimes, the underlying regulatory circuits are very well understood in model plants, while alternative strategies in species with a different evolutionary history are not. One example of this is the way in which de-etiolated plants adjust to light quality changes.

Plants absorb blue (B, 400–500 nm) and red (R, 600–700 nm) light, but not far-red (FR, 700–800 nm) light for photosynthesis. Due to this preferential absorption by leaves the red : far-red light ratio (R:FR) declines in dense vegetation, which is a signal for neighbour proximity. In response, shade intolerant plants prioritize enhanced elongation of their leaf-bearing organs (stems, petioles) over branching, lift their leaves to a more vertical position (hyponasty) to bring their leaves to the top of the canopy and flower early, a phenomenon known as: the shade avoidance syndrome (SAS, reviewed in Pierik and Testerink, 2014; Fraser et al., 2016). Low R:FR-induced shade avoidance is absent in shade-tolerant plants that thrive on the forest floor and cannot outcompete the tall trees surrounding them (Gommers et al., 2013; Valladares and Niinemets, 2008).

Changes in R:FR light are sensed by the phytochrome family of photoreceptors in all higher plants. These photoconvert from the active, FR-absorbing form (Pfr) to the inactive, R-absorbing form (Pr) and vice-versa. In direct sunlight (R:FR = 1.1), Pfr localizes to the nucleus, where it binds to a set of basic Helix-Loop-Helix (bHLH) transcription factors; the PHYTOCHROME INTERACTING FACTORS (PIFs). In canopy shade (R:FR < 0.4), PIFs are released when Pfr is photoconverted to Pr, and regulate cell elongation by enhancing transcription of growth-promoting genes (Quail, 2002). PIFs directly regulate the expression of genes encoding cell wall modifying proteins, such as XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE15 (XTH15) (Hornitschek et al., 2009). PIFs also directly target genes encoding proteins involved in controlling auxin homeostasis and signalling, which is a major hormone controlling hypocotyl elongation of *Arabidopsis thaliana* seedlings during shade.
avoidance (Tao et al., 2008; Keuskamp et al., 2010; Li et al., 2012; Hornitschek et al., 2012; Nozue et al., 2015). PIF4 interacts with growth repressing DELLA proteins (Djakovic-Petrovic et al., 2007; de Lucas et al., 2008) and BRASSINAZOLE RESISTANT1 (BZR1; Oh et al., 2012), which are targets of two other growth-regulating hormones, gibberellin (GA) and brassinosteroids (BRs), respectively.

SAS is a widespread strategy that is accompanied by controlled suppression of jasmonic acid (JA) and salicylic acid (SA) mediated defenses against pathogens and herbivores (de Wit et al., 2013; Moreno et al., 2009), thus prioritizing consolidation of light capture over other stresses. *A. thaliana* plants exposed to low R:FR light are more susceptible to the necrotrophic pathogen *Botrytis cinerea*, as a consequence of stabilization of Jasmonate ZIM-domain (JAZ) transcriptional repressors, when DELLA proteins are degraded (Chico et al., 2014; Cerrudo et al., 2012; de Wit et al., 2013).

The molecular pathways that stimulate elongation growth would be maladaptive in forest understory plants. Indeed, variation in low R:FR-induced stem elongation rates has been documented between (Morgan and Smith, 1978; Gilbert et al., 2001) and within (Sasidharan et al., 2009; Coluccio et al., 2011; Jiménez-Gómez et al., 2010; Filiault and Maloof, 2012) species. Species from forest understories, in addition to suppressing low R:FR-induced stem elongation have evolved shade tolerance strategies, such as optimal leaf morphology for low light photosynthesis (Morgan and Smith, 1978) and tolerance to biotic and abiotic stresses (reviewed in Valladares and Niinemets, 2008). Currently, it remains unknown how shade tolerant species inhibit SAS whilst being exposed to potent SAS-inducing light signals (Gommers et al., 2013). Identifying the molecular mechanisms underpinning suppression of SAS has great potential to i) show how different strategies of adaptive plasticity can be regulated and ii) inform crop-breeding programs targeted at minimizing yield losses, which are caused by wasteful carbon investments in non-harvestable stems (Carriedo et al., 2016) and suppressed immunity.

Here, we study responses to changing R:FR light ratios in two *Geranium* species from contrasting habitats: *G. robertianum* (herb Robert),
which grows in a wide amplitude of conditions including forest understories and *G. pyrenaicum* (hedge cranesbill), which occurs in open habitats. In response to low R:FR light conditions, these two species showed highly contrasting growth, transcriptome, and pathogen defense responses. We identified novel putative SAS regulators, which were subsequently confirmed in gene functional studies in *A. thaliana*.

**Results**

**G. pyrenaicum and G. robertianum express opposite growth responses to low R:FR.** To study the regulatory pathways that suppress shade avoidance, we exposed two wild *Geranium* species from contrasting habitats to FR-enriched (low R:FR = 0.2) versus control (R:FR = 1.8) white light conditions. *G. pyrenaicum* expressed the classic shade avoidance response by elongating its petioles, whereas this was not apparent in *G. robertianum* (Figure 1 A-B). Detailed petiole growth kinetics over 24 h (Figure 1 C) and 48 h (Supplemental Figure 1 A) show that both species initially enhance petiole elongation upon low R:FR treatment. Nevertheless, *G. robertianum* does not show enhanced petiole elongation in low R:FR towards the end of the day and during the night (Figure 1 C), regardless of the start time of the treatment (Figure 1 D, Supplemental Figure 2). This results in no net difference between the treatments after 24 h in *G. robertianum*. By contrast, *G. pyrenaicum* is always able to rapidly induce petiole elongation upon exposure to low R:FR, independent of the time of day, although it suppresses petiole elongation in the night period when treatments started at 17:30 or 20:00 (Figure 1 D, Supplemental Figure 2).

In both species, petioles elongated mainly at the apical part, just under the leaf lamina (Supplemental Figure 1 B-C). Based on these results, we harvested the most responsive apical part of the petiole after 2 and 11.5 h of low R:FR treatment and corresponding controls grown in white light, for a transcriptome analysis to investigate the gene expression profiles associated with these growth differences.
**De novo assembly of Geranium transcriptomes and comparison to plant model species.** Two Geranium reference transcriptomes were assembled *de novo* from combined Illumina-sequenced normalized and non-normalized libraries to establish maximum transcript coverage. Using the Markov Clustering Algorithm (OMCL (Enright et al., 2002)) with an inflation factor (I) of 3.0, transcripts were clustered into putatively orthologous transcript groups. All low-abundant OMCL groups (< 20 reads) were removed before further analysis, without affecting the number of differentially expressed OMCL groups during further analysis (Figure 2 A-B). Of the 26,434 OMCL groups, 51\% was shared by the two species (Figure 2 B), and lowering the I increased this to 74\%, at the cost of solely *G. robertianum* OMCL groups (~28\% with I = 3.0 to ~5\% with I = 1.1, Figure 2 B), indicating that this species has many transcript variants, whilst being considered a diploid, as is *G. pyrenaicum* (Warburg, 1938; Tofts, 2004). Accordingly, most OMCL groups contained only one *G. pyrenaicum*, but more than one *G. robertianum* transcript (Figure 2 D, I = 3.0 and < 20 reads).

*Geranium* transcriptomes were compared to model species *A. thaliana*, tomato (*Solanum lycopersicum*), beet (*Beta vulgaris*) and rice (*Oryza sativa*), using OMCL clustering with I = 1.1 (and > 20 reads for *Geranium* transcripts). As shown in Figure 2 C, 2251 OMCL groups were shared among all six species, 1480 covered only *Geranium* transcripts and only two OMCL groups were present in all species but not identified in *Geranium*. Our two species showed strong overlap with beet (~88\%), tomato (~82\%), and *A. thaliana* (~77\%).

**Transcriptome analysis of *G. pyrenaicum* and *G. robertianum* exposed to low R:FR light.** To identify transcriptional changes upon 2 or 11.5 h of low R:FR in the two species, we mapped Illumina sequenced reads of the non-normalized libraries (constructed from the apical half of petioles in control or low R:FR light at the two time points) to the newly constructed transcriptomes. Read counts of all contigs in an OMCL group were summed before further statistical analysis.

In *G. pyrenaicum*, 533 OMCL groups were differentially regulated after 2 h of low R:FR light and this increased after 11.5 h to 6.357 (Figure 3 A). In
G. robertianum, the numbers of differentially expressed OMCL groups were comparable at the two time points (1.482 at t = 2 h; 1.396 at t = 11.5 h, Figure 3 A). Matching the contrasting petiole elongation phenotypes of these species in low R:FR light, expression of Geranium orthologs of several SAS-associated genes (binned to the gene ontology (GO) terms ‘R or FR light signaling pathway’, ‘response to R or FR light’, ‘shade avoidance’, ‘response to R light’, ‘response to FR light’, ‘R light signaling pathway’ and/or ‘FR light signaling pathway’ in A. thaliana), was different between the two species, especially at time point t = 11.5 h (Figure 3 B). GO clustering of all up- and down-regulated OMCL groups (Supplemental Figure 3) shows that both species up-regulate ‘shade avoidance’-related, as well as several hormone- and cell-wall-related genes. Photosynthesis-associated ontologies were enriched among down-regulated OMCL groups, which would be in accordance with previous studies that show how phytochrome inactivation can repress photosynthetic capacity (Toledo-Ortiz et al., 2010; Yang et al., 2016).

As shown by the large number of OMCL groups with a significant treatment*species interaction, most differences in low R:FR-induced expression between the species occurred in the later time point (1.397 OMCL groups, compared to 287 at t = 2 h, Figure 3 C), matching the growth patterns shown in Figure 1.

A GO analysis on the OMCL groups with a significant treatment*species interaction (Supplemental Figure 4) revealed that after 2 h of low R:FR, the OMCL groups with a species*treatment interaction larger than zero (which represents significantly higher log₂ fold changes for G. robertianum than G. pyrenaicum) were overrepresented in the GO category for shade avoidance. At the second time point, the OMCL groups with a negative interaction were overrepresented in SAS-related GO categories, such as BR metabolic process, cell wall compounds and response to light stimulus.

JA-mediated defenses are repressed by low R:FR in G. pyrenaicum, but enhanced in G. robertianum. Among the GO categories with a strong
positive interaction effect in the evening, we identified several defense-associated ontologies (Supplemental Figure 4 and Figure 4 A). Many OMCL groups with possible functions in the JA-mediated defense against herbivores or necrotrophic pathogens appeared to be down-regulated after 11.5 h of low R:FR treatment in G. pyrenaicum, but not, or to a lesser extent, in G. robertianum (Figure 4 B and Supplemental Figure 5 A). Down-regulation of defense pathways by low R:FR has also been described in A. thaliana (Cargnel et al., 2014; Cerrudo et al., 2012; de Wit et al., 2013) and tomato (Cortés et al., 2016). Geranium orthologs of JA-inducible genes JASMONIC ACID RESPONSIVE3 (JR3), TRANSPARENT TESTA7 (TT7) and PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1) were transcriptionally induced by a methyl-JA treatment, but this induction was suppressed by simultaneous exposure to low R:FR light in G. pyrenaicum (Figure 4 C), similar to what was previously shown in A. thaliana (de Wit et al., 2013; Supplemental Figure 5 B). Interestingly, G. robertianum lacks the reduction in defenses observed in G. pyrenaicum. These data hint at differential control of immunity by R:FR between the two species. To examine the biological consequences of these differences, cotyledons of both species were infected with spores of the necrotrophic pathogen B. cinerea under control and low R:FR light. Consistent with the gene expression patterns, lesion diameters caused by the pathogen increased in the low R:FR treatment in G. pyrenaicum, but decreased in G. robertianum (Figure 4 D). These data indicate that the divergence of R:FR responses between these species extends to pathogen resistance. G. robertianum shows higher background susceptibility to B. cinerea than G. pyrenaicum and this may be associated with differences in leaf structure between the two species. Low R:FR-mediated petiole elongation in the seedlings (cotyledon petioles), however, remained unaffected by the B. cinerea treatment, and was pronounced in G. pyrenaicum, and absent in G. robertianum (Supplemental Figure 5 C).

**Candidate OMCL group selection for differential low R:FR-induced growth patterns.** To find OMCL groups that might be functionally associated with the low R:FR-induced growth patterns of the Geraniums species, we selected the groups up-regulated in both time points in G. pyrenaicum, but
only in the early time point in G. robertianum (Figure 5 A). Among this selection of 31 OMCL groups (Figure 5 B) were orthologs of some known downstream SAS components, such as Gibberelllic Acid 20 Oxidase2 (GA20OX2), Xyloglucan Endotransglycosylase/Hydrolase9 (XTH9) and several Small Auxin Regulated RNAs (SAURs). In a search for potentially upstream differential components, we selected bHLH transcription factor Kidari (KDR) and two members of the family of Catharanthus roseus Receptor-Like Kinases (CrRLKs): Theseus1 (THE1) and Feronia (FER) (Figure 6 A). Transcription of these OMCL groups was analysed using RT-qPCR in more detailed kinetics and plotted together with petiole elongation rates (Figure 6 B). KDR expression was rapidly and strongly up-regulated by low R:FR in G. pyrenaicum, and dropped during the night. Low R:FR-induced transcription of THE1 and FER was slower and weaker than KDR, peaking at the beginning of the night. All three OMCL groups are hardly up-regulated in the low R:FR-treated G. robertianum petioles, and expression is even slightly inhibited in the night. Expression of a number of other OMCL groups from the cluster of 31, including GA20OX2 and transcription factors BZIP61 and CIB2 was also confirmed with RT-qPCR to match the RNA seq patterns (Supplemental Figure 6 A-B).

Heterologous studies in A. thaliana confirm a role for KDR, THE1 and FER in SAS. To functionally test the importance of KDR, THE1 and FER in the regulation of SAS, we continued in the model species A. thaliana because transgenic methods are currently unavailable in Geranium species. Petioles of a KDR knock-out (kdr-1, Supplemental Figure 7 A) and knock-down (kdr-2, Supplemental Figure 7 A) line had reduced responses to low R:FR treatment, whereas the activation tagged line KDR-D (Supplemental Figure 7 B) was similar to Col-0 wild type (Figure 7 A-B). In young seedlings, these differences were more extreme. Hypocotyls of kdr-1 and kdr-2 were strongly inhibited in low R:FR-induced elongation and KDR-D was hypersensitive to low R:FR (Figure 7 C).

The THE1 knock-out the1-4, loss-of-function missense mutant the1-1, as well as the HERKULES1 (HERK1), a related RLK that was previously shown to act redundantly to THE1 in regulating hypocotyl, petiole and lamina
cell growth (Guo et al., 2009)) THE1 double mutant herk1 the1 showed significantly reduced low R:FR-induced petiole elongation (Figure 7 A-B, Supplemental Figure 8 A-B). The THE1ox on the other hand responded similarly to the wild type (Figure 7 A-B). Hypocotyls of the1-1, the1-4 and herk1 the1 seedlings showed severe low R:FR-induced elongation defects, whereas THE1ox showed an exaggerated response compared to Col-0 (Figure 7 C, Supplemental Figure 8 C). Consistent with the known THE1-HERK1 redundancy, the herk1 single mutant responded similar to Col-0 (Supplemental Figure 8 A-C).

Adult FER knock-down (fer-5) and knock-out (fer-4) plants, as well as the mutant of the FER co-receptor LORELEI-LIKE GPI-AP1 (LLG1 (Li et al., 2015)) llg1-2, all displayed petiole elongation responses to low R:FR that were similar to wild type (Figure 7 A-B). However, these mutations dramatically reduced low R:FR-induced hypocotyl elongation: fer-4, fer-5 and llg1-2 seedlings lacked this response completely (Figure 7 C). Interestingly, although all our candidate genes were found in petiole tissue in rosette-stage Geranium, their functional role is particularly striking in A. thaliana seedlings.

We exposed the1-1, the1-4, THE1ox, fer-5 and llg1-2 seedlings to blue-depleted light (Figure 8 A), another shade cue that induces strong elongation (Keuskamp et al., 2011), or 15 μM indole-acetic-acid (IAA, auxin, Figure 8 B). Even though variation in the responses existed, all genotypes would still strongly elongate hypocotyls in these treatments with some variation between different mutants for the same gene, but without a consistent reduction as seen in the low R:FR treatment. This indicates that these RLKs are particularly important for low R:FR-induced hypocotyl elongation, rather than being generic hypocotyl elongation regulators.

Finally, we established whether these novel SAS regulators are transcriptionally regulated in response to low R:FR in A. thaliana as observed in G. pyrenaicum. We analysed the expression of the KDR, THE1, FER and SAS marker genes IAA19 and ATHB2 in petioles of wild-type A. thaliana, as well as pif7, pif4 pif5 and pif4 pif5 pif7 mutants, using RT-qPCR. KDR expression was induced in petioles and was abolished solely in the pif4 pif5 pif7 triple mutant, while IAA19 and ATHB2 expression in low R:FR was partly,
but not completely dependent on PIF4, PIF5 and PIF7 (Figure 9 A). THE1 transcription increased only marginally in low R:FR light in Col-0 and pif7, while FER, HERK1 and HERK2 expression remained completely unchanged by the light treatment (Figure 9 A and Supplemental Figure 8 D) in A. thaliana.

Since kdr, the1 and fer mutations resulted in clear hypocotyl elongation defects in low R:FR-exposed seedlings, we next analysed the expression of these candidate genes, as well as of IAA19 and ATHB2 in hypocotyls of the wild type, pif7 (with severely suppressed SAS in seedlings (Li et al., 2012)), kdr-1, the1-1 and fer-5 (Figure 9 B). KDR and THE1 expression were, similar to IAA19, induced by low R:FR in all genotypes but pif7, which lacked this response completely. FER expression remained unchanged in all genotypes, but was slightly repressed in low R:FR-exposed pif7 seedlings. Surprisingly, ATHB2 induction by low R:FR light was absent in pif7, kdr-1 and fer-5, but not in the1-1.

Consistent with these expression data, results summarized from previously published transcriptome studies also indicated that low R:FR light, auxin treatments and pif mutations have marginal effects on expression of FER, THE1 and related RLKs (Supplemental Figure 9). These relatively weak inductions found in published data sets may be related to their use of whole shoots or seedlings: two recent studies on organ-specific gene expression also find more clear induction of THE1 and FER expression in A. thaliana hypocotyls (Das et al., 2016; Kohnen et al., 2016).

Discussion
Our transcriptomics approach in wild species with opposite ecologies and consequently contrasting light quality responses has identified molecular patterns associated with contrasting ecological strategies and novel regulators of shade avoidance responses. G. pyrenaicum and G. robertianum appear to be a promising comparative model system to study SAS and its suppression, both at a growth-physiological and a transcriptomic level.

The two species respond differently to low R:FR not only at the level of plant architecture, but also in immunity (summarized in Figure 10). It has been shown in A. thaliana, and other shade avoiding species, that low R:FR light conditions reduce defense responses against herbivorous insects and both
necrotrophic and biotrophic pathogens that involve JA- or SA-mediated signalling (de Wit et al., 2013; Cargnel et al., 2014; Cerrudo et al., 2012; Moreno et al., 2009; Izaguirre et al., 2006). We show here that G. robertianum, which occurs in forest understories, enhances its immunity against the fungal pathogen B. cinerea upon shade detection, rather than the reduced immunity observed in shade avoiding A. thaliana and G. pyrenaicum. The mechanism through which low R:FR reduces pathogen resistance in shade-intolerant plants is not yet fully established, but involves up-regulation of the JASMONATE ZIM-DOMAIN (JAZ) proteins, which are negative regulators of the JA pathway (Moreno et al., 2009; Cerrudo et al., 2012; Campos et al., 2016). Indeed, the Geranium ortholog of JAZ3 (OMCL7907) is induced by low R:FR in the evening in G. pyrenaicum, but not in G. robertianum (Figure 5B). We speculate that differential JAZ transcript accumulation between the two Geranium species in response to phytochrome inactivation under low R:FR could lead to the observed differential control of immunity.

From the Geranium transcriptome, we identified KDR, FER and THE1 as novel candidate genes regulating shade avoidance in response to low R:FR and their involvement was confirmed in heterologous gene functional studies in A. thaliana (Figure 7). The atypical non-DNA-binding bHLH transcription factor KDR has previously been shown to bind, and functionally inhibit, another atypical bHLH protein, Long Hypocotyl in Far-red light1 (HFR1) (Hyun and Lee, 2006; Hong et al., 2013), a well-studied suppressor of PIF4 and PIF5 function (Hornschek et al., 2009). Furthermore, KDR transcription is directly regulated by the PIF4-Brassinazole Resistance1 (BZR1) complex (Oh et al., 2012; Bai et al., 2012). Our expression data in A. thaliana pif mutants showed that KDR expression in petioles depends on functional PIF4, PIF5 and PIF7 in both white light and low R:FR (Figure 9A), while in hypocotyls of young seedlings it solely depends on PIF7. Nevertheless, KDR had not previously been identified as a low R:FR-inducible functional regulator of SAS. The strong differences in expression levels between the Geranium species and its presumed interaction with a modulator of PIF activity, HFR1, make KDR an interesting candidate regulating the two different strategies in the shade. However, since we could not identify HFR1 orthologs in the Geranium
transcriptomes, it is possible that KDR in these species interacts with other regulators, potentially other bHLH proteins, to suppress PIF-induced elongation in low R:FR. *HFR1* was also not detected in the transcriptome of another wild species, *Rumex palustris*, which shows pronounced elongation when flooded, accompanied by transcriptional *KDR* induction (van Veen et al., 2013).

A second novel node of regulation identified here for SAS is that of RLKs. The *THE1* and *FER* proteins regulate cell elongation (Lindner et al., 2012; Wolf and Höfte, 2014), but were not previously associated with photoreceptor responses. *THE1* acts during cell expansion (Guo et al., 2009; Hématy et al., 2007) and is considered a cell wall integrity (CWI) sensor, enhancing ROS production when CWI is lost (Denness et al., 2011). *THE1* is redundant to *HERK1* in regulating general vegetative growth (Guo et al., 2009). However, we show here that *HERK1* plays no apparent role in SAS, whereas *THE1* is a potent regulator independent of *HERK1*, as indicated by the single and double mutant data (Supplemental Figure 8 A–C). In *A. thaliana*, low R:FR light affected *THE1* expression in hypocotyls, possibly due to the presence of a PBE-box, a putative binding region for PIF1, PIF3, PIF4 and PIF5, in the *THE1* promoter (Oh et al., 2012; Pfeiffer et al., 2014; Martín et al., 2016). We show that this induction depends on PIF7, positioning *THE1* expression downstream of PIF7. Mutation of *the1* had no effect on the expression of the shade marker genes, suggesting that *THE1* might act in an independent branch of the PIF7-controlled pathways, possibly acting at the cell wall during low R:FR-induced cell expansion that drives hypocotyl elongation. Low R:FR-induced elongation requires cell wall modification (Sasidharan et al., 2014, 2011) and we speculate that released polysaccharides from the reorganizing cell wall could act as ligands to activate *THE1* (Lindner et al., 2012).

*FER* regulates female fertility through rupture of the growing pollen tube (Escobar-Restrepo et al., 2007; Duan et al., 2014), but has also been associated with vegetative growth (Guo et al., 2009; Li et al., 2015). Although *FER* expression is low R:FR-inducible in the two *Geranium* species, its expression in *A. thaliana* is unaffected by low R:FR. The *fer* and *llg1* mutant data, nevertheless, indicate an important role of this receptor complex in
shade avoidance of *A. thaliana* hypocotyls, and the loss of *ATHB2* induction in *fer-5* shows that FER is somehow connected to part of the canonical SAS-associated gene expression network in *A. thaliana*.

Our data suggest a specific role for these RLKs in low R:FR light-mediated elongation. Auxin levels are typically enhanced in low R:FR conditions (de Wit et al., 2015; Zheng et al., 2016; Tao et al., 2008; Keuskamp et al., 2010) and this is a key route to shoot elongation. However, IAA-induced hypocotyl elongation was not disturbed in any of the studied RLK mutants, nor was *IAA19* expression affected in *the1-1* and *fer-5*, suggesting that the identified RLKs are not in the established shade avoidance control module of PIF-mediated auxin synthesis and response. How the phytochrome-pathway regulates these RLKs, which putative ligands are controlling their activity in shade avoidance and which factors might be the kinase targets are all topics that future studies should elucidate.

Interestingly, FER has been directly associated with plant immunity (Kessler et al., 2010; Masachis et al., 2016) and *THE1* can regulate pathogen defense-related genes (Hématy et al., 2007). It remains to be determined if these factors also mediate shade avoidance–defense crosstalk.

In conclusion, our study using wild plants with different evolutionary histories has identified novel regulators of shade avoidance that had not been identified in *A. thaliana*. The studies in *A. thaliana* support the importance of these genes in SAS expression, but also hint that different organs and/or life cycle stages might express different modes of regulation. This study also shows that different species regulate the same components in different ways, consistent with differential control of shade avoidance.

**Methods**

**Plant material and growth conditions.** *G. pyrenaicum* (Cruydt-hoeck, Nijieberkoop, The Netherlands) and *G. robertianum* (Ecoflora, Halle, Belgium) seeds were germinated on polyethylene beads and water for six and eleven days, respectively, at saturated relative humidity, 20°C, under long-day conditions (16 h day, 8 h night; 180 μmol m⁻² s⁻¹ photosynthetically active
radiation (PAR); R:FR = 1.8). Seedlings were transferred to 70 ml pots with potting soil (mix Z2254, Primasta B.V., the Netherlands) and grown for two more weeks in long-day conditions (20°C; 70 % RH).

Different *A. thaliana* genotypes (Col-0, *pif7* (Leivar et al., 2008), *pif4 pif5* (Lorrain et al., 2008), *pif4 pif5 pif7* (de Wit et al., 2015), *the1-1, THE1ox* (Hématy et al., 2007), *the1-4, herk1, herk1 the1* (Guo et al., 2009), *kdr-1* (SALK_048383C, Supplemental Figure 7 A), *kdr-2* (SALK_033495C, Supplemental Figure 7 A), *KDR-D* (Hyun and Lee, 2006) (Supplemental Figure 7 B), *fer-5* (SALK_029056C), *fer-4* and *llg1-2* (Li et al., 2015) were grown as previously described (de Wit et al., 2012), with a 9 h light (180 μmol m$^{-2}$ s$^{-1}$ PAR) / 15 h dark photoperiod until ready for experiments at 4 weeks. For seedling experiments, seeds of the same *A. thaliana* genotypes were surface-sterilized with chlorine gas, stratified (4 d dark, 4°C) and germinated on 0.5 MS agar (0.8% v/w) plates with MES buffer (1 g/L). Germination was started with a 2 h light pulse, followed by 24 h darkness and plates were then transferred to a long-day photoperiod as above. Treatments started 48 h after the light pulse.

**Light treatments.** Low Red:Far-red (R:FR) light conditions were obtained by supplementing standard growth chamber light (R:FR = 1.8, Philips HPI) with far-red LEDs (730 nm peak, Philips Greenpower Research modules), to obtain a R:FR of 0.2 without changing PAR. Blue light-depleted (B) (± 4 μmol m$^{-2}$ s$^{-1}$) conditions were obtained by filtering higher intensity white light using a Lee Medium Yellow 010 filter, to similar PAR as in controls (180 μmol m$^{-2}$ s$^{-1}$ for rosette plants, 110 μmol m$^{-2}$ s$^{-1}$ for seedlings). Light spectra are presented in Supplemental Figure 10. Treatments started at 10:00 am.

**Growth measurements.** *Geranium* and *A. thaliana* petiole elongation was measured over a set period with a digital calliper ($t_x - t_0$). In *Geranium*, petiole growth rate kinetics were monitored using linear variable displacement transducers (LVDTs); type ST 200; Schlumberger Industries, Bognor Regis, UK (Pierik et al., 2011; Voesenek et al., 2003). The LVDT was attached to the lamina-leaf junction of the second leaf 18 h prior to the start of the light treatment.
In *A. thaliana*, hypocotyl length was measured at the end of a four-day treatment using digital (600 dpi) images and ImageJ.

**Statistical analysis.** Growth and bio-assay data were analysed by two-way ANOVA preceded by Levene’s test to verify equal variances (p > 0.05), followed by a post-hoc Tukey test. *A. thaliana* gene expression data were analysed by a Student’s *t*-test preceded by an f-test to verify equal variances. If needed, data were ln transformed. All analyses were conducted in R and Microsoft Excel.

**RNA isolation and gene expression.** For RT-qPCR, the apical 1 cm of the petiole of the second *Geranium* leaf of three individual plants (Figure 6; petiole elongation response) or the whole second leaf (petiole and lamina) of two individual plants (Figure 4 C; pathogen defense of the leaf) were pooled as biological replicates. RNA was extracted using a Qiagen RNeasy Kit with on-column DNaseI treatment. cDNA was synthesised using the SuperscriptIII Reverse Transcriptase Kit (Invitrogen) with RNase inhibitors and random primers. Quantitative RT-PCR was performed with SyberGreen Supermix (Bio-rad) in a Viia7 PCR. The *Geranium* homolog of the PP2A subunit *PDF1* was used as a reference gene. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Primers are listed in Supplemental Table 1. For *A. thaliana* experiments in petioles, RNA was extracted from six pooled petioles (~5 mm) of three plants per biological replicate, after 4 h of light treatment. For *A. thaliana* experiments in seedlings, 40–45 hypocotyls were harvested and pooled per biological replicate, after 24 h of light treatment. Extraction and analysis were similar as for the Geraniums; the average Ct of *TUBULIN*, *APT1* and *AT1G13320* (petioles), or the CT of *At1G13320* solely (hypocotyls) was used as a reference. Expression data of RLK-encoding genes from previously published experiments on *A. thaliana* rosettes (heatmap in Supplemental Figure 9) was obtained using the Genevestigator programme and contains data from (Leivar et al., 2012; Hornitschek et al., 2012; Tao et al., 2008; de Wit et al., 2013; Sessa et al., 2005; Ciolfi et al., 2013; Delker et al., 2010; Goda et al., 2008; Armstrong et al., 2004; Okushima et al., 2005).
Illumina sequencing and bioinformatics. RNA was derived from the Geraniums as described. The replicates were a pool of 12 individual plants harvested in three separate experiments. The harvest time points were 2 and 11.5 h after the start of the far-red treatment (12:00 and 21:30 respectively). Normalized libraries for de novo assembly were constructed by Vertis Biotechnologie (Freising, Germany) from pooled material of control, low R:FR, blue light-depleted and green filter treated petioles and laminas, after 2, 11.5 and 24 h, for both species separately, using the kinetic denaturation-reassociation technique.

Illumina sequencing (paired-end, 101 bp reads) was carried out by Macrogen (Seoul, Korea), followed by de novo assembly (Trinity method (Grabherr et al., 2011)) of the reference transcriptomes and alignment of the experimental transcripts (RSEM-based abundance estimation).

Putatively orthologous transcript groups within the two species were constructed using OrthoMCL clustering (as described in (Li et al., 2003; Enright et al., 2002)), with inflation factor 1.1 (for kingdom-wide OMCL, to increase the ability to detect putatively orthologous groups) or 3.0 (for expression analysis) and the similarity matrix of an all-versus-all discontinuous megaBLASTn of the two (or six) transcriptomes as input. The best BLAST hit with the *A. thaliana* transcriptome was used to name *Geranium* OMCL groups.

For statistical analysis, read counts of all transcripts in an OMCL group were summed and a cut-off of minimally 20 reads in at least one treatment was applied to filter out low abundant families. To test for differentially expressed genes in single-factorial comparisons, a negative binomial model of OMCL group read numbers was made using the quantile-adjusted conditional maximum likelihood (qCML) method, followed by an exact test, including Benjamini and Hochberg’s algorithm to control false discovery rates (FDR), of the EdgeR package (Bioconductor) (Robinson et al., 2010). The Cox-Reid profile-adjusted likelihood (CR) method in combination with a generalized linear model (Likelihood Ratio Test, glmLRT) of the EdgeR package was used in multi-factorial comparisons (McCarthy et al., 2012). A P-value cut-off for differentially expressed genes was set at 0.01.
For gene ontology analysis, differentially expressed *Geranium* OMCL groups, with a BLAST E-value < 10^{-10} with *A. thaliana* genes, were tested for GO term enrichment using the R package GOseq (Young et al., 2010), with correction for the total length of all transcripts in the *Geranium* OMCL group.

**Pathogen assays.** Necrotrophic fungus *Botrytis cinerea* (strain B0510) was grown on half-strength potato dextrose agar (PDA; BD Difco) for two weeks before conidia were harvested in potato dextrose broth (PDB; BD Difco). *G. pyrenaicum* and *G. robertianum* seedlings (respectively six and eleven days after germination) were transferred to 19 ml pots with potting soil and on the following day, a 2 μl droplet of 2.5 * 10^5 spores / ml of *B. cinerea* was applied to each cotyledon. Plants were kept at saturated relative humidity in a control white or low R:FR treatment. Lesion diameters were measured 3 days after inoculation.

**Pharmacological treatments.** For the Methyl-Jasmonate treatment, 30 min prior to the start of the light treatments, a solution of 100 μM meJA or mock (0.1% EtOH) with 0.1% Tween-20 was sprayed on the plants. The Indole-acetic-acid (IAA) treatments of seedlings were performed as described previously (Keuskamp et al., 2011), with slight adjustments. Briefly, 200 μl of a concentrated, sterile IAA solution (600 μM) was applied to the plates to create a film on top of the agar, with a final concentration of 15 μM (0.1% DMSO).

**RNAseq data availability and accession numbers**

Raw sequencing files and transcript shotgun assemblies (accession numbers HAGG01000001-HAGG01168191 for *G. pyrenaicum*, and HAGH01000001-HAGH01300137 for *G. robertianum*) are stored at the European Nucleotide Archive (ENA), and combined under study accession number PRJEB18552 (http://www.ebi.ac.uk/ena/data/view/PRJEB18552). OMCL group assignment of the transcripts and RNAseq expression data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5371.
Supplemental Data

Supplemental Figure 1. A detailed analysis of low R:FR-induced growth over the time of day and in Geranium petioles.

Supplemental Figure 2. Low R:FR light suppresses petiole growth in G. robertianum, but not G. pyrenaicum, at a fixed time of the day.

Supplemental Figure 3. Gene ontology analysis on low R:FR induced and repressed Geranium OMCL groups.

Supplemental Figure 4. Plant processes differentially regulated in low R:FR by the two Geranium species

Supplemental Figure 5. Low R:FR light affects meJA-induced transcript abundance in the Geraniums and A. thaliana, and pathogen infection does not affect Geranium growth in the shade.

Supplemental Figure 6. Supplemental Figure 6. RT qPCR on Geranium transcripts and validation of RNA sequencing.

Supplemental Figure 7. Confirmation of kdr mutants.

Supplemental Figure 8. HERK1 has no function in the A. thaliana shade avoidance syndrome.

Supplemental Figure 9. RLK gene expression is hardly affected by low R:FR, pif mutations and IAA in previous transcriptome studies.

Supplemental Figure 10. Light spectra of the treatments used in this study.

Supplemental Table 1. Primer list.

Acknowledgements

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Author contributions

CMMG, RP and LACJV conceived the project. CMMG, RP and DHK designed the experiments. CMMG, DHK, SB, ER and ITK performed the experiments.
CMMG and HvV analysed the RNAseq data. CMMG and RP wrote the manuscript.

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Figure legends

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