Cotyledon-Generated Auxin Is Required for Shade-Induced Hypocotyl Growth in *Brassica rapa*¹

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One-sentence summary:
*Shade light perceived in cotyledons induces de novo auxin biosynthesis and transport to hypocotyl cells leading to their elongation.*
Footnotes:

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ABSTRACT

Plant architecture is optimized for the local light environment. In response to foliar shade or neighbor proximity (low red to far-red light), some plant species exhibit shade-avoiding phenotypes, including increased stem and hypocotyl growth, which increases the likelihood of outgrowing competitor plants. If shade persists, early flowering and the reallocation of growth resources to stem elongation ultimately affects the yield of harvestable tissues in crop species. Previous studies have shown that hypocotyl growth in low red to far-red shade is largely dependent on the photoreceptor phyB and the phytohormone auxin. However, where shade is perceived in the plant and how auxin regulates growth spatially is less well understood. Using the oilseed and vegetable crop species Brassica rapa, we show that the perception of low red to far-red shade by the cotyledons triggers hypocotyl cell elongation and auxin target gene expression. Furthermore, we find that following shade perception, elevated auxin levels occur in a basipetal gradient away from the cotyledons and that this is coincident with a gradient of auxin target gene induction. These results show that cotyledon-generated auxin regulates hypocotyl elongation. In addition, we find in mature B. rapa plants that simulated shade does not affect seed oil composition but may affect seed yield. This suggests that in field settings where mutual shading between plants may occur, a balance between plant density and seed yield per plant needs to be achieved for maximum oil yield, while oil composition might remain constant.
INTRODUCTION

In close proximity to neighboring plants, shade-intolerant plants exhibit a suite of phenotypes collectively referred to as the shade avoidance syndrome (SAS). SAS includes hypocotyl and stem elongation, reduced root and leaf growth, and reduced defenses against herbivores and pathogens. This adaptive response can be viewed as a competitive strategy to allocate resources towards growth that alters plant architecture to enable better light harvesting. If shade persists, shade-intolerant plants typically transition to early flowering with reduced seed set, ensuring genetic survival, albeit at the cost of fecundity (Casal, 2013).

Shade from neighboring plants is perceived as a reduction in the ratio of red (R) to far-red (FR) light. Neighboring plants preferentially absorb R light for photosynthesis, while FR light is reflected (Casal, 2013). The major photosensor of low R:FR shade is phytochrome B (phyB) (Reed et al., 1993), although other phytochromes play additional roles (Robson et al., 1993; Franklin et al., 2003). phyB photoconverts between two states: an inactive, cytosolic R-absorbing form (Pr, $\lambda_{\text{max}} = 660$ nm), and a nuclear FR-absorbing form (Pfr, $\lambda_{\text{max}} = 730$ nm). Under shade conditions (R:FR < 1), Pr is the major form; while in sunlight (R:FR ~ 1.1), Pfr is the predominant form.

In Arabidopsis thaliana (Arabidopsis), the growth hormone auxin plays a major role in driving hypocotyl elongation in low R:FR shade. Auxin levels are rapidly elevated by shade, and plants carrying mutations in the auxin biosynthetic pathway have reduced hypocotyl growth (Tao et al., 2008). Auxin synthesis in shade is regulated by the interaction of phyB with specific members of the phytochrome interacting factors (PIFs) family of bHLH transcription factors. Under high R:FR light, nuclear Pfr phytochrome interacts directly with PIFs to induce PIF phosphorylation. This results either in PIF destruction via the ubiquitin-proteasome system (Al-Sady et al., 2006; Shen et al., 2007; Lorrain et al., 2008; Shen et al., 2008), or inhibition of PIF binding to its target gene promotors (Li et al., 2012). In low R:FR shade, the Pr form of phyB predominates and non-phosphorylated PIFs accumulate, resulting in upregulation of target gene expression. PIF targets include genes encoding the YUCCA family of flavin monooxygenases that
catalyze the rate-limiting step in auxin biosynthesis, and other growth promoting genes (Mashiguchi et al., 2011; Won et al., 2011; Hornitschek et al., 2012; Li et al., 2012).

Previous studies have established the importance of the leaves/cotyledons as sites of phytochrome-mediated perception of R/FR signals that modulate stem growth. For example, shielding the cotyledons of *Cucumis sativus* seedlings from light abrogates R light-mediated hypocotyl growth inhibition, whereas shielding the hypocotyl has no effect (Black and Shuttleworth, 1974). Other studies, however, have suggested that the opposite may also be the case: that the stem itself is the major site of R/FR perception. For example, debladed *Vigna sinensis* epicotyls show growth responses to end-of-day FR light, suggesting that the epicotyl itself is the primary light-sensing organ (Garcia-Martinez et al., 1987). These mechanisms need not be mutually exclusive, and the perception of R/FR light in both leaves/cotyledons and hypocotyl/stems may play additive roles in directing stem growth. In *Helianthus annuus*, FR light control of internode elongation requires irradiance almost equally on the internode as well as other aerial tissues of the plant (Garrison and Briggs, 1975). Similarly, in light-grown *Sinapis alba*, both internodes and other tissues perceive addition of supplemental FR light and contribute to internode growth, but with different kinetics (Morgan et al., 1980; Child and Smith, 1987; Casal and Smith, 1988). By contrast, end-of-day FR treatments induced internode growth in *S. alba* only when applied to the leaves and cotyledons (Casal and Smith, 1988, 1988). This suggests that a complicated interaction between signals from the leaf and stem may be taking place, and might be dependent on the specific light environment.

Experiments in Arabidopsis indicate that for this species the cotyledons are the major sites of R/FR perception and auxin synthesis regulating hypocotyl growth in response to changes in the R:FR ratio. Enhancer trap lines that express phyB-GFP specifically in the cotyledons of Arabidopsis seedlings can complement the elongated hypocotyl phenotype of *phyB*-deficient mutants (Endo et al., 2005), while spotlight FR irradiation localized to the cotyledons induces expression of an auxin-dependent gene in the hypocotyl (Tanaka et al., 2002). In addition, the auxin biosynthesis gene *SAV3/TAA1*, which is required for growth responses to shade, is predominately expressed in the margins and vasculature of cotyledons and emerging leaves (Tao et al., 2008). Treatment
of Arabidopsis seedlings with the auxin transport inhibitor NPA or mutations in the *PIN-FORMED 3 (PIN3)* auxin efflux transporter both result in reduced hypocotyl growth in shade, suggesting that auxin transport is necessary for responses to shade (Keuskamp et al., 2010). However, it is unknown if all shade-regulated genes in the Arabidopsis hypocotyl require cotyledon-derived signals, and an understanding of how low R:FR shade modifies gene expression spatially in the plant is lacking.

Mutual shading within densely-sown, shade-intolerant crop species might affect crop growth and yield (Ballare et al., 1997). Elongated plants are more susceptible to physical damage (Morinaka et al., 2006), and shade responses may diminish the allocation of photosynthate and other growth resources to harvested tissues (Ballare et al., 1997). *Brassica rapa* and other species of the *Brassica* genus are important vegetable and oilseed crops, which in low R:FR light display phyB-dependent SAS phenotypes (Devlin et al., 1992; Devlin et al., 1997). The recent sequencing of the *B. rapa* genome (Wang et al., 2011), its close relationship with the well-studied model plant Arabidopsis, economic value, large seedling size amenable to spatial studies and availability of mutants and rapid-cycling varieties (Williams and Hill, 1986; Stephenson et al., 2010) make this an attractive model system for the study of shade avoidance.

Here, using *B. rapa* seedlings, we show directly that low R:FR shade perceived by cotyledons regulates hypocotyl cell elongation, and that cotyledon-generated auxin drives a gradient of auxin-dependent gene induction down the hypocotyl in shade. However, not all shade-induced genes in the hypocotyl are auxin- or cotyledon-dependent. Interestingly, studies of soybean (*Glycine max*) grown under low blue light suggested that spectral quality may alter the oil composition of oilseed crops (Britz and Cavins, 1993). However, we show for *B. rapa* grown under simulated low R:FR shade that this is not the case, and that oil composition might be constant in field settings, even when plant density is high.

**RESULTS**

**Low R:FR Shade Induces Phenotypic and Transcriptional Changes in *B. rapa***
Previous work has shown that low R:FR light can induce shade avoidance phenotypes in the Wisconsin Fast Plants variety of rapid-cycling *B. rapa* (Devlin et al., 1992; Devlin et al., 1997). We find that this is a general attribute of *B. rapa* varieties: seedlings of an inbred line of *B. rapa* subsp. *trilocularis* (yellow sarson), R-o-18, also exhibit typical shade avoidance phenotypes when grown in simulated shade (Fig. 1A; here, simulated shade approximates neighbor proximity; see Materials and Methods and Tao et al., 2008). R-o-18 was the primary *B. rapa* strain employed in this study. It is a yellow sarson-type oilseed crop grown in Pakistan that is self-fertile with high fecundity, and is similar to other *Brassica* oilseed crops (Rana et al., 2004; Stephenson et al., 2010). As such, it is likely that findings derived using R-o-18 will be generally applicable to oilseed crop varieties.

We find that in response to simulated shade, R-o-18 *B. rapa* seedlings exhibited increased hypocotyl growth, reduced cotyledon expansion, and fewer lateral roots (Fig. 1, B-D). Hypocotyl growth was reduced in the presence of the auxin transport inhibitor NPA and the presence of the TIR1 auxin receptor antagonist auxinole (Hayashi et al., 2008; Hayashi et al., 2012) (Fig. 1E), suggesting that *B. rapa* hypocotyl growth in response to shade requires auxin transport and signaling.

Transcriptional analysis of Arabidopsis seedlings shifted to low R:FR conditions has identified numerous genes whose expression is induced by shade (Devlin et al., 2003). We analyzed the *B. rapa* whole genome sequence (accession Chiifu-401-42; Wang et al., 2011), using a combination of methods based on homology and syntenic relationships to identify presumptively orthologous loci that might also show increased expression in *B. rapa*. Indeed, we found by using quantitative PCR assays (qPCR; Figs. 2A and 3, A and B; and data not shown) that *B. rapa* orthologs of Arabidopsis genes *ATHB2*, *HFR1* and *PIL1* (hereafter termed *BrATHB2* [*BraA.ATHB2.a*], *BrHFR1* [*BraA.HFR1.a*] and *BrPIL1* [*BraA.PIL1.a*]), are up-regulated following 2 h exposure to low R:FR shade. In Arabidopsis, the promoters of these genes are directly bound by PIF transcription factors (Hornitschek et al., 2012). In addition, we identified five other shade-induced genes representing possible orthologs to other known shade targets: the cell wall modifying enzyme *BrXTH33* (*BraA.XTH33.a*), and the likely auxin-dependent genes *BrlAA29* (*BraA.IAA29.a*), *BrlAA19* (*Br.IAA19.a*), *BrGH3-5* (*BraA.GH3-5.a*) and
BrIAA2 (BraIAA2.a). Our results suggest that many aspects of the transcriptional response to shade are conserved between Arabidopsis and Brassica rapa.

**B. rapa** Responses to Shade Are phyB-Dependent

_Elongated Internode (ein)_ mutants of the rapid-cycling Wisconsin Fast Plants variety of _B. rapa_ lack detectable expression of phyB protein and display constitutive shade-avoiding hypocotyl growth in white (W) light (Devlin et al., 1992) (Supplemental Fig. S1, A and B). An additional _B. rapa_ phyB mutant, _ein194_, was recovered in an EMS mutant screen of M2 families derived from the FPsc (for “Fast Plants, self-compatible”) variety. FPsc is a highly inbred and rapid-cycling analog of the self-incompatible Wisconsin Fast Plants variety (wild-type FPsc and mutant _ein194_ seeds were provided by Scott Woody and Rick Amasino). While the rapid growth of FPsc is unlike _B. rapa_ oilseed crop varieties, we have employed the strain when it was desirable to compare wild-type seedlings to phyB mutants. We also took advantage of the rapid growth of FPsc and its small stature when measuring adult traits of plants grown in simulated shade chambers (see below). Like the R-o-18 variety, FPsc seedlings exhibited a typical hypocotyl SAS response to low R:FR light; however, due to greater variation amongst the seedlings the response was less (Supplemental Fig. S2).

We sequenced the _PHYB_ alleles of wild-type and _ein194_ FPsc plants and verified that a premature stop mutation exists in the _ein194_ background (Supplemental Fig. S3; and see below). The predicted _B. rapa_ FPsc phyB protein (Bra022192) is > 90% identical to that encoded by _A. thaliana_ locus AT2G18790, with most divergence in the N-terminal region (~ 60% identity over the first 55 amino acids). Previous work has shown that deleting amino acids 1-57 has little effect on the function of Arabidopsis phyB, suggesting that this less-conserved region is nonessential (Wagner et al., 1996). We find that _ein194_ seedlings have reduced induction of shade-regulated genes in low R:FR light (Fig. 2B). For many of these genes, this may be because _ein194_ mutants have a higher basal level of expression (Supplemental Fig. S4), an observation previously reported for some shade-induced transcripts in Arabidopsis phyB mutants (Devlin et al., 2003). However, we do not expect all shade-induced genes to have higher basal
expression in phyB mutants, as compensatory mechanisms might occur when plants lack phyB signaling for their entire lifespan.

In addition, we find that over-expression of the highly-conserved Arabidopsis PHYB sequence (AtPHYB) in transgenic B. rapa FPsc plants resulted in a stereotypical PHYB over-expression phenotype (Wagner et al., 1996), with short hypocotyls in W light (Supplemental Fig. S5; a single line was generated, likely with two insertions of the transgene). These results confirm that phyB is a major photoreceptor mediating low R:FR shade responses in B. rapa.

**Transcriptional Responses to Shade Are Both Auxin-Dependent and Independent, and Spatially Distinct**

To examine spatial patterns of changes in gene expression induced by shade, we performed qPCR analysis of mRNAs isolated separately from cotyledon, hypocotyl and root tissues after 2 h low R:FR shade treatment of R-o-18 seedlings. Cotyledons and hypocotyl segments showed a largely similar pattern of gene induction in response to shade (Fig. 3, A and B). One notable exception was that of BrXTH33, a cell-wall modifying enzyme with a role in cell expansion (Sasidharan et al., 2010) that was uniquely up-regulated in the hypocotyl cell types treated under these simulated shade conditions. This observation may explain why little induction of BrXTH33 was observed in whole plant FPsc gene expression assays (Fig. 2A), when hypocotyl RNA is diluted amongst the RNA of other organs. Alternatively, the low induction of BrXTH33 in FPsc seedlings may be due to a lower response of this variety to shade.

Perhaps surprisingly, none of the marker genes we examined were induced in root tissue (Fig. 3C), even though shade affects root growth and the seedlings were grown on agar and thus all organs equally exposed to light. This lack of a response was not due to a delay in induction kinetics, since even after 24 h of low R:FR treatment no change in expression of these marker genes was observed (Supplemental Fig. S6). Neither was the lack of induction in the root due to an absence of gene expression compared to the other organs. While some of the genes were expressed only at low levels in the root compared to the hypocotyl in W light-grown seedlings (Supplemental Fig. S7; e.g. BrATHB2 and
BrHFRI), other shade-regulated genes were expressed at similar levels but failed to be induced. These findings suggest that transcriptional responses to shade are distinct within different organs and tissue-types of the plant.

To determine which of the shade-regulated loci in B. rapa might be dependent on auxin signaling, we treated whole seedlings with exogenous indole-3-acetic acid (IAA), the main auxin found in plants, and examined gene expression in hypocotyl tissues. We found that of the eight marker genes, BrIAA29, BrIAA19, BrGH3-5 and BrIAA2 were significantly up-regulated in a dose-dependent manner by IAA, while no or only marginal effects were observed on the other genes tested (Fig. 3D). Unlike IAA treatment, exogenous brassinosteroid (BL) or gibberellin (GA3) did not cause changes in expression, suggesting auxin is primarily responsible for the responses observed (Supplemental Fig. S8). To verify that up-regulation of these genes in response to shade is auxin-dependent, we measured transcript levels of the related sequences in Arabidopsis sav3-1 mutants, which are defective in auxin synthesis (Tao et al., 2008). For three of the four genes whose upregulation in shade was shown to be auxin-dependent in B. rapa (IAA29, IAA19 and IAA2), we observed a loss of induction in the sav3 mutant background in response to shade (Fig. 3E). The GH3-5 marker gene followed a similar trend, although the change was not significant (Student’s t test). By contrast, induction of expression at loci presumed to be auxin-independent (ATHB2, HFRI, PIL1 and XTH33) was essentially unaffected in the sav3-1 mutant background; in fact, shade-mediated induction of PIL1 and HFRI expression exceeded that observed in wild-type seedlings. As such, we propose that shade-induced changes in transcription in B. rapa in response to perturbation of the R:FR light environment proceed through both auxin-dependent and auxin-independent pathways.

Cotyledon Perception of R/FR Light Regulates Hypocotyl Growth and Auxin Gene Expression

De-etiolated Arabidopsis seedlings carrying a PHYBpro::GUS transcriptional reporter transgene display GUS expression in cotyledons, hypocotyl and roots (Somers and Quail, 1995). In our hands, GUS expression in the hypocotyl was strongest in the
vasculature, while in the root the strongest staining was observed at the root tip (Supplemental Fig. S1C). Similarly, we found that phyB protein was expressed in all three organs of W-light grown *B. rapa* seedlings (Supplemental Fig. S1D). Thus, all three organs might have the potential to elaborate responses to changes in R:FR light ratios. However, as demonstrated above, root cells have no change in transcription for the shade-induced genes that we examined here, suggesting that the root may have limited responses to light.

To assess the role of cotyledonary and hypocotyl cell types in mediating the *B. rapa* response to shade, we removed the cotyledons and petioles of *B. rapa* R-o-18 seedlings immediately prior to simulated shade treatment (Fig. 4A). To compensate for loss of photosynthate derived from the cotyledons, the plants were grown on medium supplemented with 1% sucrose. 4 d-old operated seedlings grown for an additional 2 d in shade showed no measurable change in hypocotyl length compared to operated seedlings maintained in W light (Fig. 4B). This is unlikely due to a general lack of growth in the absence of the cotyledons, as the hypocotyls were marginally longer than they were at the time of operation (Fig. 4B). After an additional day of growth, shade-treated operated seedlings did exhibit a small but significant increase in hypocotyl length over W light-treated controls; however, the emergence of developing true leaves at this later time point confounds interpretation of these results (data not shown).

The removal of cotyledons might eliminate not only signals induced by shade, but also other growth signals generated by the organ. Therefore, to assess the role of the cotyledons in shade perception in intact plants, we grew *B. rapa* R-o-18 seedlings in a split light chamber. The cotyledons and upper petioles were exposed to either W light (high R:FR) or simulated shade (low R:FR; W light supplemented with a FR LED bulb, LumiGrow ECC-FR). The hypocotyl and root were exposed separately to W light or shade (Fig. 5A and Supplemental Fig. S9, A-F). We found that when the hypocotyl alone experienced simulated shade there was only a minor, non-significant increase in hypocotyl length (Fig. 5B; Student’s *t* test). This was not due to an inability of the chamber conditions in which the hypocotyl was exposed to induce growth, as hypocotyls elongated when the cotyledons were exposed to the same chamber conditions (Supplemental Fig. S9G). By contrast, exposure of the cotyledons to shade induced
hypocotyl growth, even under circumstances in which the hypocotyl simultaneously experienced high R:FR light (Fig. 5B). Furthermore, the response observed with cotyledon-only shade-treated seedlings was not statistically different than that seen when both cotyledons and the hypocotyl were simultaneously exposed to a low R:FR environment (Student’s *t* test; however, *P* < 0.05 in a two-way analysis of variance when light treatment of the hypocotyl was compared against all cotyledon treatments).

Together, our results show that in *B. rapa* the cotyledons (and/or upper petioles) are the primary sites for the perception of low R:FR light and thus the source of a signal that drives hypocotyl elongation.

To test if the signal from the cotyledons driving hypocotyl growth might be auxin-related, we removed the cotyledons and petioles of *B. rapa* seedlings immediately prior to a 2 h low R:FR shade treatment and tested the effect on gene expression in the hypocotyl (the region of the hypocotyl immediately above the root-hypocotyl junction and below the petioles). We found that the auxin-dependent shade response genes were not induced in dissected seedlings under low R:FR conditions as compared to dissected seedlings maintained in W light (Fig. 4, C and D). By contrast, most presumptively auxin-independent loci were still up-regulated to approximately wild-type levels. Intriguingly, *B. rapa* genes whose expression were unaffected by removal of the cotyledons (*BrATHB2, BrHFR1* and *BrPIL1*) are thought to be direct targets of the PIF gene family in Arabidopsis (Hornitschek et al., 2012). Only the auxin-independent gene *BrXTH33*, which might be directly involved in elongation growth (Sasidharan et al., 2010), also failed to be induced in the absence of the cotyledons.

To verify that auxin-dependent shade-regulated genes required signals from the cotyledons in intact plants, we measured gene expression changes in the cotyledons and hypocotyls of seedlings grown in our split light chamber setup. In the cotyledons, we observed that some of the genes representing both auxin-dependent and independent target genes were induced when the cotyledons were exposed to simulated shade, irrespective of the light environment in which the hypocotyl was placed (Fig. 5C). By contrast, in the hypocotyl, the auxin-independent target gene *BrPIL1* was dependent on the light quality perceived directly by the hypocotyl and not that experienced by the cotyledons (Fig. 5D). *BrATHB2* and *BrHFR1* followed a similar pattern; however, their
levels were only marginally and, in the case of \textit{BrHFRI}, non-significantly increased by the shade conditions used in this experiment. Consistent with our gene expression data of seedlings in which we had removed the cotyledons, we found that hypocotyl expression of the auxin target genes \textit{BrIAA19}, \textit{BrIAA2}, \textit{BrIAA29} and \textit{BrGH3-5}, as well as the presumptive auxin-independent gene \textit{BrXTH33}, are instead dependent on the light quality perceived by the cotyledons (Fig. 5D). Low R:FR treatment of the hypocotyl had little to no effect on the expression of these genes.

Together, our results suggest that auxin-mediated transcription in the hypocotyl is dependent on a mobile signal(s) produced in the cotyledons, likely auxin, while most of the auxin-independent shade-induced genes do not require signals derived from the cotyledons.

**Shade Results in a Gradient of Auxin-Induced Gene Expression extending Downward in the Hypocotyl**

The median speed of auxin movement along the eudicot hypocotyl is 7 mm/h (Kramer et al., 2011). If we assume this speed to be true for the \textit{Brassica} genus, it would take greater than 2 h for newly synthesized apical auxin to move to the base of a typical 7 d-old \textit{B. rapa} hypocotyl. As such, if the cotyledons are the sites of increased auxin production in shade, it is likely that a gradient of increased free auxin and auxin-independent transcriptional responses would occur downward along the hypocotyl and away from the cotyledons during the early shade response. This may be particularly apparent in species such as \textit{B. rapa}, whose seedlings have a relatively large hypocotyl compared to Arabidopsis.

To test this hypothesis, hypocotyls of \textit{B. rapa} R-o-18 seedlings were dissected into three segments following low R:FR shade treatment (Fig. 6A). This treatment was associated with an increase in free auxin levels in the cotyledons (Fig. 6B). In the hypocotyl, we did not observe a detectable increase in free IAA levels after 2 h low R:FR shade (data not shown), although robust gene expression changes are already apparent at this time (Fig. 3B). This may be due to a lack of sensitivity in our measurements of free IAA levels. However, after 6 h of exposure we did observe increased levels of IAA in
the hypocotyl, and those increases were manifest as a basipetal gradient extending downward (high IAA to low IAA) with respect to the cotyledons (Fig. 6C). Consistent with this finding, we observed that rapid changes in auxin-dependent gene expression occurred in a basipetal gradient along the hypocotyl axis. After 2 h low R:FR shade, BrGH3-5, BrIAA19, and BrIAA2 were all induced in shade-treated hypocotyl segments as compared to equivalent segments from non-shade treated controls, with greatest induction in the apical segment (Fig. 6D). BrIAA29 followed a similar pattern, although the effect of hypocotyl segment on gene induction was not significant. By 6 h low R:FR light, the effect of shade on transcript levels is greater still, although a clear basipetal pattern of induction is less apparent and not statistically significant (Fig. 6E). By contrast, the presumptive auxin-independent target genes BrATHB2, BrHFR1, and BrPIL1, as well as BrXTH33, did not exhibit any clear collective pattern of gene expression along the hypocotyl in response to shade (Supplemental Fig. S10). Taken together, our data suggests that auxin is synthesized in the cotyledons and is transported or diffuses into the hypocotyl, thereupon triggering up-regulation of genes associated with elaboration of the shade avoidance response.

**Shade-Induced Hypocotyl Growth Is Correlated With Epidermal Cell Elongation**

To determine the regions of the *B. rapa* hypocotyl that are responsible for shade-induced elongation, we marked hypocotyls of 4 d-old W-light grown R-o-18 seedlings to delineate three segments of approximately equal length. The length of each segment was then measured after 3 d of additional growth in either W light or shade (Fig. 7A). Growth was greatest in apical segments under both light conditions, while shade-treated plants had significantly more growth across all three segments than W-light grown controls.

Using scanning electron microscopy (SEM), we measured the lengths of epidermal cells along the hypocotyls of W-light grown plants and found a similar pattern: epidermal cell length decreased in a basipetal gradient, and hypocotyl cell elongation after 7 d growth was greatest in apical regions compared to 4 d-old plants (Fig 7, B and D-F). Following shade, epidermal cells were longer in both apical and basal regions of
the hypocotyl, with most growth at the apex (Fig. 7, C and G-I). This growth pattern may reflect a gradient of auxin and/or other growth signals along the apical-basal axis from the shoot apical meristem and/or cotyledons, consistent with our findings above. Alternatively, it might result from decreased malleability of basal cells, or a combination of these two mechanisms.

**Low R:FR Shade Does Not Affect Seed Oil Composition of Mature *B. rapa* Plants**

Stem elongation phenotypes similar to those studied here may be of agronomic importance. For example, increased stem length can make plants more susceptible to physical damage (Morinaka et al., 2006), while elongation growth may reallocate photosynthate and growth resources away from harvested tissues (Ballare et al., 1997; Casal, 2013). In addition, the reallocation of resources towards growth may make plants more susceptible to herbivory and infection (Ballare, 2009). Many *Brassica* varieties are important oilseed crops and, as such, we sought to further explore how shade signals might affect oilseed-related traits. Specifically, in addition to our studies above, we sought to test how shade might affect *B. rapa* seed oil quality and yield in mature plants.

To test how low R:FR light might affect *Brassica* seed oil quality, FPsc plants were grown in long-day conditions (16 h light, 8 h dark) under W light, in the absence or presence of supplemental FR during daylight hours (high and low R:FR, respectively; see Materials and Methods). The FPsc variety was chosen for these experiments due to the availability of a *phyB* mutant, as well as the plant’s smaller stature compared to mature R-o-18 plants, which facilitated growth in the limited physical space of our shade light chambers. Under these conditions, we observed little difference in the height of 60 d-old wild-type plants grown in W light or W light supplemented with FR (Fig. 8, A and E) or flowering time (31 +/- 1 d until first bud opening under W light, versus 30 +/- 1 d in low R:FR; Student’s *t* test, not significant; it should be noted that more rapid flowering times [~18 d] are observed among FPsc plants grown in constant light at higher temperature, Scott Woody, personal communication). By contrast, Arabidopsis plants grown under these same conditions had a marked reduction in flowering time (6.9 +/- 0.1 rosette leaves at time of bolting under W light, versus 2.9 +/- 0.1 in low R:FR; Student’s *t* test, *P*
< 0.005; Ler accession). Notwithstanding this apparent exception of *B. rapa* FPsc flowering time on elaboration of certain aspects of SAS, leaf chlorophyll content of *B. rapa* plants was strongly reduced in the shade environment (Fig. 8F), and siliques were noticeably paler and longer, produced fewer mature seeds, and those seeds were smaller by weight (Fig. 8, D and G-I). We were unable to assess total spontaneous seed yield per FPsc plant as we had intervened to facilitate self-crossing in order to maximize seed yield for oil analysis. However, Arabidopsis plants grown under the same conditions had significantly reduced seed yield per plant in shade (14.1 +/- 1.5 mg total seed weight per plant in W light, compared to 7.1 +/- 0.6 mg in low R:FR light; Student’s *t* test, *P* < 0.005; Ler accession). In addition, previous field experiments using *B. rapa* have shown that silique production (and therefore likely seed yield) is reduced when plants are grown at high density where mutual shading occurs (Dechaine et al., 2007).

To test which of these defects might be phyB-dependent, we grew *ein194* mutant plants expressing a truncated form of the phyB protein (Supplemental Fig. S3; and Fig. 8, B and C). In W light conditions, *ein194* mutants displayed dramatically elongated stature, reduced chlorophyll content, and reduced seed number per silique compared to wild-type plants, suggesting that some of these phenotypes are in part determined by phyB signaling (Fig. 8, A and E-I). phyB activity did not appear to play a significant role in the determination of other phenotypes examined (silique length and seed weight); however, it is possible that these traits are regulated by other phytochromes encoded in the *B. rapa* genome, or that this observation is a result of the taller *ein194* plants receiving more light from the overhead lighting than the wild-type strain.

Seed oil composition was determined by gas chromatography-mass spectrometry analysis (GC-MS). In initial experiments, five major peaks representing different fatty acids were separated (Supplemental Table S1; and Table 1). No difference in oil content was observed between seeds harvested from siliques of different plants in W light, or between siliques growing at different heights on the same plant (Supplemental Table S1). This shows that inter- and intra-plant variation in seed oil composition is minimal. When seeds were compared from both wild-type and *ein194* mutant plants growing across the two light environments, we observed no change in the relative amounts of the five fatty acid groups (Table 1).
It had previously been reported that low blue light altered the saturation of 18 carbon-chain fatty acids in soybean (Britz and Cavins, 1993). In the experiments described above, we were unable to adequately separate peaks for linoleic acid (18:2) from monounsaturated 18:1 fatty acids. Therefore, we tested additional diluted oil samples from wild-type plants (Fig. 8, J and K) and found no difference between the ratio of 18:2 to 18:1 fatty acids from seeds of plants grown in either W light or W light supplemented with FR, nor a significant change in relative oil content per seed weight. As such, we conclude that seed oil composition of *B. rapa* FPsc plants is stable across changing ratios of R:FR light, even though photosynthesis and total seed yield per plant is affected. This suggests that fatty acid synthesis is a highly regulated process, even in non-optimal light environments.

**DISCUSSION**

**The Cotyledons Regulate Shade-Induced Hypocotyl Growth in *B. rapa***

Studies using Arabidopsis have indicated that the cotyledons are the most likely source of increased auxin synthesis in low R:FR shade (Tanaka et al., 2002; Tao et al., 2008), and that phyB functions specifically in cotyledons to modulate hypocotyl growth (Endo et al., 2005). By contrast, experiments using other systems have suggested that the direct perception of R and/or FR light by the stem can also affect stem growth (see Introduction). For example, it has been reported that R/FR light regulates the growth of excised hypocotyl segments of *Phaseolus vulgaris*, and spectral information perceived by one region of the hypocotyl can be transmitted to another, either through the movement of a phytohormone or light funneling (Gotô and Suzuki, 1980). In *Sinapis alba*, a species of the Brassicaceae family closely related to *B. rapa*, addition of supplemental FR light is perceived by both internodes and other tissues to induce internode growth, while end-of-day FR treatment induced elongation growth only when perceived by the leaves and cotyledons (Morgan et al., 1980; Child and Smith, 1987; Casal and Smith, 1988, 1988). This suggests that specific light treatments may also alter the interplay of light-sensing organs.
The cause of the discrepancy in these studies is unclear. Differences in the species examined, light conditions and tissues (internode, epicotyl or hypocotyl) might all provide some explanation for the different contributions observed of cotyledons/leaves and hypocotyls/stems in perceiving changes in R:FR light that drive stem growth. In addition, it is possible that the cotyledons of large plant species are unable to synthesize and transport enough auxin or another growth-promoting signal into the hypocotyl to cause robust changes in elongation. However, our results with *B. rapa* suggest that this need not be the case. Instead, we favor a simple model, even in large plants, where perception by the cotyledons of light quality indicative of neighbor proximity increases cotyledonary auxin synthesis, which in turn is transported or diffuses into the hypocotyl to trigger growth. In support of this model we have first shown directly that the perception of shade light by the cotyledons drives hypocotyl growth, while in seedlings where the cotyledons have been removed the hypocotyl fails to elongate in response to shade. Second, we have shown that following a shift to shade a gradient of free auxin and auxin-dependent gene induction occurs in a basipetal gradient along the hypocotyl, consistent with an apical auxin maxima. Third, in the absence of the cotyledons or absence of shade perception by the cotyledons, auxin target genes fail to be induced by shade.

While the R:FR ratio perceived by the cotyledons largely determined hypocotyl length in our split light chamber experiments, we did observe an additional slight increase in hypocotyl growth when the hypocotyl was also irradiated with supplemental FR light (Fig. 5B). This increase in hypocotyl growth was significant by two-way analysis of variance (*P* < 0.05). This may indicate that a minor contribution of shade perception in the hypocotyl to growth does exist. In addition, our studies do not preclude the possibility that shade perception by the hypocotyl alters the dynamics of the growth response (for example, see (Morgan et al., 1980)). Alternatively, the slight increase in growth might be a result of light piping from the hypocotyl to the cotyledons. However, the observation that hypocotyl length is determined largely by the light quality perceived by the cotyledons, irrespective of hypocotyl treatment, suggests that the effects of light piping from the hypocotyl to cotyledons are minimal. Neither do we observe strong effects of light piping from the cotyledons to the hypocotyl; for example, induction of
*BrPIL1* and *BrATHB2* expression in the hypocotyl is only evident when the hypocotyl is exposed to shade, irrespective of the light quality perceived by the cotyledons.

Although we cannot rule out the possibility that a non-auxin signal moves from the cotyledons to direct auxin synthesis in the hypocotyl, this is unlikely in light of previous studies using Arabidopsis. For example, the SAV3 aminotransferase required for auxin synthesis in shade is expressed in cotyledons (Tao et al., 2008), and the expression of an auxin reporter transgene increases in cotyledons and decreases in hypocotyls when auxin movement is blocked by NPA treatment, consistent with cotyledonary auxin synthesis and accumulation (Tao et al., 2008). Interestingly, however, Tanaka et al. (2002) showed that transcriptional reporters that display increased expression in the Arabidopsis hypocotyl in response to end-of-day FR treatment can be either auxin-dependent or independent. FR irradiation of cotyledons could induce expression of both reporter types, suggesting that the cotyledons may also be the source of a non-auxin signal. In our studies with *B. rapa*, we have shown that the removal of the cotyledons prior to simulated shade resulted in a loss of auxin-dependent gene induction. In addition, we saw a loss of induction of the cell wall-modifying enzyme *BrXTH33*, even though this gene was not responsive to exogenous auxin (Figs. 3D and 4, C and D). As such, the expression of *BrXTH33* could require the unknown cotyledon-derived signal proposed by Tanaka et al. (2002), perhaps in addition to auxin. This may be a photoassimilate or other phytohormone.

Consistent with our observation that the cotyledons direct hypocotyl elongation in shade, *wag1 wag2 pid* AGC kinase triple mutants of Arabidopsis, which fail to develop cotyledons (Cheng et al., 2008), display no hypocotyl growth response to low R:FR shade (Supplemental Fig. S11). However, these mutants have defects in the localization of auxin efflux transporters, and as such we cannot rule out the possibility that the lack of hypocotyl growth is not rather due to a general defect in auxin movement in these plants (Friml et al., 2004).

In addition, our findings are consistent with the previously reported observation that phyB-GFP expression specifically in the cotyledons can complement the elongated hypocotyl phenotype of *phyB*-deficient Arabidopsis mutants (Endo et al., 2005). We
hypothesize that phyB is likely functioning within the cells of the cotyledon that are responsible for auxin synthesis, in turn regulating hypocotyl growth.

The fact that the cotyledons perceive R/FR light signals that direct hypocotyl growth might be advantageous in a natural setting. The hypocotyl of young seedlings might be embedded almost entirely underground when the cotyledons first perceive light at the soil surface, or might be self-shaded by the cotyledons in older seedlings. As such, we argue that the cotyledons are best poised to transmit signals about the seedling’s light environment.

**Not All Shade-Regulated Genes in Hypocotyl Cells Require Cotyledon-Derived Signals**

Surprisingly, not all shade-regulated genes in cells of the *B. rapa* hypocotyl required the cotyledons for their induction (Figs. 4, C and D, and 5D). These genes (*BrHFR1, BrPIL1* and *BrATHB2*) are all likely orthologs of genes in Arabidopsis that are bound directly by PIF transcription factors (Hornitschek et al., 2012). This suggests that the hypocotyl can respond to shade independently of the cotyledons and auxin. However, this response is neither necessary nor sufficient for stimulated growth in our experiments (Figs. 4B and 5B). In addition, expression of at least one of these genes, *BrPIL1*, displayed an acropetal pattern of induction along the hypocotyl in response to shade (Supplemental Fig. S10), opposite of hypocotyl growth. This provides additional support that the expression of these genes, at least in the hypocotyl, does not directly correlate with growth. Interestingly, a study of Arabidopsis mutants defective in hypocotyl elongation in shade previously noted that HFR1 and PIL1 expression in whole seedlings does not always correlate with hypocotyl length (Cifuentes-Esquivel et al., 2013). However, this study analyzed gene expression in whole-seedlings rather than just the growing organ (the hypocotyl) or the site of shade perception (the cotyledons). Indeed, we hypothesize that the expression of these genes might correlate with growth, but only in the auxin-producing cells of the cotyledons, where the genes might function to alter auxin production, as has been postulated for HFR1 (Hersch et al., 2014).
If the cotyledon-independent transcriptional response to shade is neither necessary nor sufficient for stimulated growth as our studies would suggest, what, therefore, is the role of this response? One possibility is that this response regulates other aspects of the shade avoidance phenotype; for example, the down-regulation of defense genes in the hypocotyl (Cerrudo et al., 2012), or the reduction of stem photosynthetic capacity (Cagnola et al., 2012). Alternatively, the cotyledon-independent response might potentiate the response of hypocotyl cells to cotyledon-derived growth signals. For example, in Sinapis alba it has been shown that R light irradiation of the internode inhibits growth-promoting signals from leaf tissue, perhaps by altering auxin transport (Casal and Smith, 1988). Similarly, in Arabidopsis, low R:FR shade leads to PIF-dependent up-regulation of the AFB1 auxin receptor gene in hypocotyl tissue, suggesting that there is increased sensitivity of the hypocotyl to auxin in shade (Hersch et al., 2014). Another possibility is that light perception by the hypocotyl is important only under certain environmental conditions, and might be a means for integrating multiple push-and-pull growth signals. Such a mechanism may allow the hypocotyl to integrate information about the R:FR light environment with other environmental and/or developmental cues that are perceived directly by the hypocotyl.

We also find that the transcriptional response of the root to low R:FR shade is very different from the cotyledons and hypocotyl. These results are consistent with previous observations that light- and dark-grown Arabidopsis seedlings have distinct changes in gene expression in root, hypocotyl and cotyledon, even though all three organs likely share the same phyB-dependent photoperception machinery (Ma et al., 2005; Bou-Torrent et al., 2008). In addition, our observation here that auxin target genes are not induced in the root following shade perception may indicate that the shoot is a closed system of auxin signaling and transport in shade. How then phytochrome-dependent signals from the shoot regulate lateral root formation in shade remains unclear (Salisbury et al., 2007).

**B. rapa** Hypocotyl Growth Occurs in a Basipetal Gradient
Generally, plant elongation occurs in a gradient along the stem, with most growth occurring at the apex (for example, see (Gotô and Suzuki, 1980; Martinez-Garcia and Garcia-Martinez, 1992; Shinkle et al., 1992)). These results are consistent with our observation that hypocotyl growth in *B. rapa* occurs in a basipetal gradient. This may be explained by a gradient of a growth signal/s from the apex, which may include auxin or photoassimilates, or, rather, a gradient in cellular growth potential and amenability along the hypocotyl. In *Vigna sinensis*, elongation in response to end-of-day FR treatment has been observed in derooted and debladed epicotyl explants (Martinez-Garcia and Garcia-Martinez, 1992). The region of the epicotyl that displayed the greatest change in growth was localized 5-20 mm below the apex (the entire epicotyl was 40-80 mm long).

Similarly, *Phaseolus vulgaris* hypocotyl explants have been shown to respond to FR light, and the region that showed the greatest growth response was localized away from the apex, 5-15 mm from the top of the shank (the total length of the shank was 40 mm) (Gotô and Suzuki, 1980). These findings are not entirely inconsistent with our own; generally, the region near the apex of the growing organ elongates the most. However, the localization of the growing region specifically to 5 mm or more below the apex in *Vigna sinensis* epicotyl and in *Phaseolus vulgaris* hypocotyl may be due either to species-specificity, a lack of resolution in defining the hypocotyl regions most sensitive to growth in our own studies, or a difference in using excised epicotyl/hypocotyl explants that lack additional growth signals from the leaves/cotyledons. In our studies using *B. rapa*, we only observe shade-induced hypocotyl growth in intact plants, wherein the major source of signals regulating elongation in response to shade is the apical-localized cotyledons.

Stem growth is either a result of cell elongation, or cell elongation and cell division, depending on the plant species. In the case of *B. rapa*, our measurements indicate that most growth resulting in hypocotyl elongation is likely due to cell elongation rather than division (with the caveat that we only measure epidermal cell length, and not other cell types of the hypocotyl). This was true whether seedlings were grown under white light or simulated shade conditions. However, we have not examined directly the possibility that increased rates of cell division also contribute to hypocotyl elongation.
Low R:FR Shade May Contribute to Loss of Oil Yield in Agriculture

*Brassica* species are important oilseed crops, and breeding efforts have developed varieties that have fatty acid compositions optimized for human benefit. Stem elongation phenotypes may affect traits of agronomic importance in some plant species by reallocating resources towards stem growth and increasing the likelihood of mechanical damage (Morinaka et al., 2006; Casal, 2013). Here, we sought to explore how shade signals might also affect *Brassica* seed oil quality and yield. For example, the effect of environmental temperature on oil composition has been well studied, but little is known about how a plant’s spectral environment might also play a role (Izquierdo et al., 2009). The study of changing oil composition in different light environments may be particularly relevant for the low R:FR mutual shading found in densely planted crops (Casal, 2013).

We took advantage of the FPsc strain and specifically the availability of phyB mutants in this background to test the effects of low R:FR shade on *B. rapa* seed oil composition. FPsc is a self-compatible and highly inbred analog of the self-incompatible Wisconsin Fast Plants (WFP) variety (Williams and Hill, 1986). Like WFPs, the FPsc variety was developed primarily to serve as a model system for hands-on student exploration of plant biology and genetics. In such settings, rapid progression through the plant life cycle is useful, and that consideration was a high priority in the selective breeding program that produced the FPsc *B. rapa* accession. The fact that we did not observe a detectable advance in flowering time among FPsc plants exposed to low R:FR light may simply be due to the fact that FPsc flowers as rapidly as possible under all circumstances. We report in this paper the first transformant and transformation protocol of the new FPsc educational model system (Supplemental Fig. S5).

Here, we find that low R:FR simulated shade causes a reduction in chlorophyll content of mature FPsc plants, reduced seed number per siliqua, and reduced seed weight. In Arabidopsis, we also observed that seed yield per plant was reduced. Previous work has shown that many plant species exhibit reduced chlorophyll content in shade and that this response is in part phyB-dependent (McLaren and Smith, 1978; Reed et al., 1993), while field studies of *B. rapa* reported fewer siliques per plant when grown at high
density (Dechaine et al., 2007). However, we observed no change in *B. rapa* seed oil composition either in shade light or in the *ein194 phyB* mutant. This suggests that fatty acid synthesis in *B. rapa* is a highly compartmentalized process, as the balance of molecular species is unchanged even when chlorophyll content/photosynthesis is perturbed. It may be that reduced fecundity in response to shade is an evolved and adaptive trait: by reducing the total number of progeny seeds produced, the available energy resources can be delivered to the fewer seeds, but each would be equipped with energy stores best able to promote successful germination and growth in the succeeding generation.

Natural environments are complex, and plant density can affect not only light quality (R:FR ratio) but also light quantity and other aspects of the environment above and below ground. Determination of the optimal balance between planting density, seed quality and seed yield is a challenge faced by all growers. If our results are generalizable to *B. rapa* oilseed crop varieties growing in such complex field conditions, then the proper balance may be relatively straightforward in this species as seed yield may be optimized independent of seed oil quality.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

*B. rapa* strains used were R-o-18 (Stephenson et al., 2010); Wisconsin Fast Plants (standard) (Williams and Hill, 1986) and the *ein* mutant (Williams and Hill, 1986); and FPsc and *ein194* mutants (gift of Scott Woody and Rick Amasino). Arabidopsis strains used were Col-0, Ler, *sav3-1* (Tao et al., 2008), and *wag1 wag2 pid* triple mutants (Cheng et al., 2008). Seeds were sterilized, stratified and germinated on solid agar plates with ½ Linsmaier and Skoog salts and vitamins, and without sucrose (1% sucrose was added in the experiment described in Fig. 4B). After stratification, plates were incubated in either constant W light or simulated low R:FR shade chambers, using the light conditions described in (Tao et al., 2008) unless otherwise indicated. Temperature was 22°C.
The mature FPsc plants used for fatty acid analysis were grown on soil under long day conditions (16 h light, 8 h dark) at 22°C. Photosynthetically active radiation (PAR; 400-700 nm) of W light was 75-90 μE x m⁻² x sec⁻¹ at a height of 10 cm above soil level, with a R:FR ratio of ~10. Lighting was provided by Cool White and Gro-Lux fluorescent bulbs. Low R:FR conditions were W light supplemented with FR LED bulbs (LumiGrow ECC-FR); PAR 70-85 μE x m⁻² x sec⁻¹, R:FR ratio ~ 0.75. A consequence of the chamber setups used in this study is that the ratios of R to FR light in our W light conditions were typically greater than that which is encountered within natural environments (we have used R:FR ratios of > 10, while natural sunlight is ~1.1 (Casal, 2013)). However, our shade conditions have ratios < 0.8, which are typical of those experienced by plants under natural foliar shade or in the proximity of neighboring plants (Casal, 2013).

**Determination of Hypocotyl Length**

Quantitative measurements of hypocotyl length and cotyledon area were performed using ImageJ software analysis of scanned images of seedlings. At least 10 plants were scored for each data point. To measure the hypocotyl length of FPsc plants carrying UBQ10pro::AtPHYB and their controls, only seeds that had germinated within 24 h after stratification and transfer to W light were scored.

**Transformation of B. rapa FPsc**

The UBQ10pro::AtPHYB plasmid (pCP12.22) was made using the multisite Gateway system (Invitrogen). Donor vectors pDONR-P4P1R-UBQ10pro (Jaillais et al., 2011), pDONR221-AtPHYB (gift of Kazu Nito) and pDONR-P2RP3-6xHis3xFlag tag were recombined with destination vector pK7m34GW. FPsc plants for transformation were grown on sterile agar plates, and young cotyledon explants were transformed with Agrobacterium GV3101 carrying the UBQ10pro::AtPHYB construct. Hormone conditions used to generate callus and regenerate shoots were as described by (Kunvshinov et al., 1999). We were unable to regenerate roots under a variety of
conditions tested; however, when we transferred shoots to soil one of these regenerated a root to provide transgenic seeds.

**Western Blot Analysis and GUS Staining**

Western blots and GUS staining were performed using standard protocols. Primary antibodies used were α-Flag (Sigma-Aldrich), α-actin (MP Biomedicals), α-UGPase (α-UDP-glucose pyrophosphorylase; Agrisera), and α-phyB (gift of Akira Nagatani).

**qPCR and Statistical Analysis**

qPCR was performed using standard techniques, using three biological replicates. Fold changes in gene expression were calculated using the ΔΔCt method. At present, no consistent statistical analysis for qPCR results is presented in the literature. Here, we have assumed that the ΔCt values fit a normal distribution, and have tested ΔCt values using *t* test or the appropriate analysis of variance (ANOVA) test (Yuan et al., 2006). Primers for Arabidopsis gene expression studies using cDNA template were: *IPP2* (reference gene; GTATGAGTTGCTTCTCCAGCAAG and GAGATGGCTGCAACAAAGTGT), *PIL1* (TGGACTAATTCCAAACACTCTATCTT and CACACGAAGGCACCACGA), *IAA29* (ACGGTTGCTCRTACAGG and AATGAGGGATAGTCTTGAAAGG), *IAA2* (ACCTACGACCTCTATATCTT and GACATAAGCCCAACATCTCTG), *XTH33* (TACTAGAAACGGGAAGAGAGAG and AATGAGGGATAGTCTTGAAAGG), *IAA2* (ACCAAGCGTCTATTTGAGG and CTACGAGGAAGATCTCTG), *GH3-5* (TTGCACCAACTACACAAGCC and GGAGTTATCACCATCTTCGT), *ATHB2* (GAGTCTCAAGCCTCACAGG and GGAGTTATCACCATCTTCGT), and *HFR1* (TCCCACATTGCATCTCTC and GGAAATAAGGAACCACCGTG). Orthologous gene sequences in *B. rapa* were found using the *Brassica* Database (http://brassicadb.org/brad). qPCR primers for gene expression studies were designed
based on the *Brassica* Database sequences, and were: *BrGAPDH* (reference gene; *BraA.GAPDH*/*Bra016729*, CACCACCGAGTACATGACGTACA and TGCCCGTGAAACCTGTGTA), *BrPIL1* (*BraA.PIL1*/*Bra004489*, TTACGCTTCTCATCTAGTCTTC and TCTCCACACTTCCTCTTTTG), *BrIAA29* (*BraA.IAA29*/*Bra011332*, GCCAGTCAGACAAGGAAGAT and CCTCGTGTCGTCACAAGGAAGAT and *CCTCGTGTCGTCACAAGGAAGAT*), *BrXTH33* (*BraA.XTH33*/*Bra01843*, TGGTGACAAAGAACACGTATCAT and TTGACAAATAGAAAGCAACCACA), *BrIAA2/Bra001899*, GTGAATACAGCGAAAGGAAGAT and ACCAACCAACATCCAATCTCC), *BrGH3-5* (*BraA.GH3-5*/*Bra019060*, GACCCCTTACACGGCTACTAC and CATAGGCCACAAAGCATCTGAG), *BrIAA19* (*Br.IAA19*/*Bra001598*, TCTCGATAAGCTCTCAGT and CAGTCTCCATCTTTGCTTCTTG), *BrATHB2* (*BraA.ATHB2*/*Bra040094*, CGCAAGGCTAAGGAGGTATCA and TCCTCGAGTTATACCGTCTG), and *BrHFR1* (*BraA.HFR1*/*Bra032610*, GCAACAACCACCTTTTGAGGCG and GCCGGTTTAGGCCGTG). qPCR primers used for determining *UBQ10pro::AtPHYB* copy number from genomic DNA template were: control (Bra011332, CTTGATCTTCTCTTTTCACCATC and CCTCGTGTCGTCACAAGGAAGAT) and *UBQ10pro::AtPHYB* transgene (TGTGCGATCGAATTTTGTG and CCGTTATTAGGAGTGACTTGA).

**Free IAA Measurements**

Samples containing 10-20 mg plant material (fresh weight) were analyzed by gas chromatography-tandem mass spectrometry (GC-MS/MS) as previously described (Andersen et al., 2008) with minor modifications. 500 pg $^{13}$C$_6$-IAA internal standard was added to each sample before purification, and five biological replicates were analyzed per tissue type and treatment.

**Scanning Electron Microscopy (SEM)**
Whole plants were carefully removed from the agar by the roots and placed into ice cold ethanol for 10 minutes before being loaded and dried in an automated critical point drier (Leica EM CPD300, Leica, Vienna) which was set to perform 25 exchange cycles of CO$_2$ at medium speed and 40% stirring. The fill, heating, and venting steps were performed at medium speed. After drying, the cotyledons were carefully removed with forceps and the sample was adhered to a glass slide with carbon adhesive. The mounted samples were then sputter coated (Leica SCD500, Leica, Vienna) with approximately 7nm of platinum while being rotated and imaged on an environmental SEM (EVO HD, Zeiss Ltd., Cambridge, UK). 3 plants from each light condition were imaged, and for each plant the lengths of 25 epidermal cells in each segment of the hypocotyl being analysed were measured. Only the large, ‘bulbous’ epidermal cells were measured.

Alternatively, still hydrated samples were removed from the agar and suspended by the roots and cotyledons between a set of split toothpicks. The samples were then imaged in the variable pressure mode of a FE-SEM (Sigma VP, Zeiss Ltd., Cambridge, UK) at 5Pa of nitrogen with the variable pressure SE detector.

**Fatty Acid Measurements**

Samples for fatty acid extraction and GC-MS analysis were collected from individual siliques. For Table 1, three biological replicates were sampled. For Fig. 8, J and K, five biological replicates were sampled. Flowers were self-pollinated by hand to increase yield for analysis. Cross-pollination between FPsc plants was very occasionally observed. To ensure that the seeds sampled were not cross-progeny between plants of different genotypes, some seeds from each silique used for GC-MS were sequenced.

The fatty acids were extracted and analyzed according to previously published methods (Ngaki et al., 2012). Briefly, frozen seeds were homogenized by agitation with ball bearings and then dissolved in 0.5 mL of 10% (w/v) barium hydroxide, adding nonadecanoic acid as an internal standard (20 μg/mL final concentration). Mixture was transferred to a glass tube, 500 μL of 1,4-dioxane added and vortexed. Saponification was carried out over 24 h at 105°C. Cooled mixture was acidified using 6 M aqueous
HCl and fatty acids extracted into 4 mL of n-hexane. This organic phase was evaporated and remaining fatty acids derivatized by addition of 2 mL HCl:MeOH (1:5.25 v/v) at 80°C for 1 h. The cooled mixture was extracted with 2 mL n-hexanes after the addition of 2mL 0.9% (w/v) NaCl. Organic phase was then transferred to a glass vial, evaporated and 500 μL acetonitrile and 35 μL N,O-Bis(trimethylsilyl) trifluoroacetamide were added to the residue and heated for 20 min at 60°C. After evaporation of the mixture, the derivatized fatty acids were dissolved in chloroform in a volume that scaled based on the original weights of the seeds (approx. 200 μL).

GC-MS analyses were performed on an Agilent 6890N GC coupled with an Agilent 5973 inert MSD detector. Samples were injected (1 μL) on an HP-5MS fused silica column (30 m x 250 μm; 0.25 μm film thickness). Initial temperatures of the injector and MSD interface were set at 275°C and 280°C, respectively. Metabolites were separated at a flow rate of 1.2 mL/min using He as the carrier gas, and ramping temperature from 80°C (2 min) to 260°C at 4°C/min (held for 5 min) then to 320°C at 5°C/min. Data were acquired and analyzed in MSD Chemstation (Agilent Technologies). Fatty acids were identified via mass fragmentation pattern alignment with standards from the National Institute of Standards and Technology Mass Spectral Search Program. Peak areas determined from total ion count (TIC).

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FIGURE LEGENDS

**Figure 1.** *B. rapa* seedlings display auxin-dependent shade avoidance phenotypes. A, Representative image of 7 d-old *B. rapa* R-o-18 seedlings grown in constant white light (Wc) or 4 d Wc followed by 3 d low R:FR light (↓R:FR). Scale bar, 1 cm. B-D, Hypocotyl length (B), cotyledon area (C), and lateral root formation (D) of 7 d-old seedlings treated as in (A). E, Hypocotyl length of 7 d-old seedlings grown for 4 d Wc then 3 d liquid culture in Wc or low R:FR light, in the presence of DMSO (vehicle), 10 μM auxinole, or 10 μM NPA. Data show mean +/- SEM. Student’s *t* test, **P < 0.005, *P < 0.05.

**Figure 2.** qPCR expression analysis of low R:FR shade-induced marker genes. 4 d-old W light-grown wild-type (A; FPsc strain) and *phyB* mutant (B; *ein194*) seedlings treated for 2 h W light (Wc) or 2 h low R:FR light (low R:FR). For each gene, expression is shown relative to the 2 h Wc treatment, +/- SEM. Student’s *t* test, **P < 0.005, *P < 0.05, ns = not significant.

**Figure 3.** Shade-induced genes are expressed in distinct organs and can be auxin-dependent or independent. A-C, qPCR expression analysis of shade-induced marker genes in the cotyledons (A), hypocotyl (B), and roots (C) of 7 d-old W-light grown *B. rapa* R-o-18 seedlings, treated for 2 h white (Wc) or low R:FR light. For each gene, expression is shown relative to the 2 h Wc treatment. D, Gene expression in the hypocotyl of 7 d-old R-o-18 seedlings treated for 150 min in liquid culture with the indicated concentration of IAA. Normalized fold induction for each gene is shown relative to the vehicle control (0 nM; ethanol). E, Expression of orthologous genes in whole seedlings of wild-type (Col-0) and *sav3-1* mutants of Arabidopsis. 7 d-old W-light grown seedlings were treated for 1 h with W (Wc) or low R:FR light. Expression is shown relative to the Wc-treated wild-type plants, +/- SEM. Asterisks indicate significant difference for (A-C and E) Student’s *t* test, and (D) one-way analysis of variance. **P < 0.005, *P < 0.05, ns = not significant. In (C), all comparisons are ns. In (E), *sav3* mutants are compared to wild-type seedlings grown in equivalent conditions.
**Figure 4.** The cotyledons regulate hypocotyl growth and gene expression. A, Experimental setup of (B-D). The cotyledons [C] of W-light grown R-o-18 *B. rapa* seedlings were removed immediately prior to light treatment. Light treatments consisted of continued constant W light (Wc) or simulated shade (low R:FR). The cotyledons of operated seedlings were removed at the base of the petioles, while the hypocotyl [H] and root [R] were left intact. B, Hypocotyl length of 7 d-old seedlings grown for 4 d Wc then an additional 3 d Wc or low R:FR light. In operated seedlings, the cotyledons of 4 d-old seedlings were removed as described in (A). Data shows mean +/- SEM. C and D, Gene expression in the hypocotyls of 7 d-old W-light grown R-o-18 seedlings treated with either 2 h W light (Wc) or low R:FR light, of intact seedlings (C) or seedlings where the cotyledons were removed immediately prior to the 2 h light treatment (D). For each gene, expression is shown relative to the 2 h Wc treatment, as measured by qPCR analysis, +/- SEM. In (B-D), Student’s t test, **P < 0.005, *P < 0.05, ns = not significant.

**Figure 5.** The cotyledons regulate hypocotyl growth and gene expression in intact seedlings. A, Experimental setup of (B-D). W-light grown R-o-18 *B. rapa* seedlings were positioned in a split light chamber. The cotyledons [C], or the hypocotyl [H] and root [R], were exposed to either high R:FR (W light; R:FR > 15) or low R:FR light (W light supplemented with FR; R:FR < 0.8). The cotyledons were fixed in position, while the roots were suspended in liquid growth medium to allow vertical growth of the hypocotyl down. See Supplemental Fig. S9 for a complete description of the light conditions used. B, Hypocotyl length of R-o-18 seedlings grown as described in (A). 4 d-old seedlings were transferred to the split light chamber and grown for an additional 3 d. Data shows mean +/- SEM. C and D, Gene expression in the cotyledons (C) and hypocotyls (D) of seedlings grown in the split light chamber. W-light grown plants were transferred to the split light chamber at 4 d and allowed to equilibrate to W-light conditions (high R:FR). At 6 and a half d, the ratio of R:FR light was adjusted as described in (B), and gene expression levels measured by qPCR 12 h later. The light treatments of each column (white, gray, diagonal striped and black) are as indicated in (B). For each gene, expression is shown relative to seedlings where both the cotyledons and hypocotyl plus
root were exposed to high R:FR light, +/- SEM. Asterisks indicate significant difference for (B) Student’s t test, and (C and D) one-way analysis of variance. **P < 0.005, *P < 0.05, ns = not significant.

**Figure 6.** Low R:FR shade induces a basipetal gradient of auxin-dependent gene expression. A, Experimental setup of (C-E). The hypocotyl of 7 d-old W-light grown seedlings (R-o-18) was sectioned after the indicated light treatments into apical, middle, and basal fragments. [C], cotyledons; [H], hypocotyl; [R], root. B, Free IAA levels in the cotyledons following 6 h W or low R:FR light treatment. C, Free IAA levels in apical, middle, and basal segments of the hypocotyl following 6 h W or low R:FR light treatment. In (B and C), data show mean +/- SEM, n = 5. Student’s t test, **P < 0.005, *P < 0.05, ns = not significant. D and E, qPCR expression analysis of auxin-dependent genes in the hypocotyl following 2 h (D) or 6 h (E) low R:FR light treatment. Expression is shown for low R:FR-treated hypocotyl segments relative to the Wc-treated control segments, +/- SEM. In (D), asterisks indicate a significant interaction between light treatment and hypocotyl segment using a two-factor analysis of variance with repeated measures on one factor (hypocotyl segment), **P < 0.005, ns = not significant. By 6h, no significant interaction is observed for any of the genes.

**Figure 7.** *B. rapa* hypocotyl growth occurs in a basipetal gradient. A, R-o-18 seedlings were grown for 4 d in constant white (Wc) light and the hypocotyl marked into three divisions of equal size (apex, middle, and base). After a further 3 d growth in either Wc light or low R:FR shade, the length of each segment was measured. B, Length of hypocotyl epidermal cells of 4 and 7 d-old W-light grown plants, as determined by scanning electron microscopy (SEM). The hypocotyls of imaged plants were divided into 5 equal segments (1-5) for analysis. C, Epidermal cell lengths at the hypocotyl apex (0-2 mm below the hypocotyl-petioles junction) and base (1-3 mm from hypocotyl-root junction). 4 d-old W-light grown seedlings were treated with W (Wc) or low R:FR light for 3 d. In (A-C), data show mean +/- SEM. Student’s t test, **P < 0.005, *P < 0.05, ns = not significant. D and I, Overlaid SEM images of full-length hypocotyls of Wc (D) and low R:FR (I) seedlings grown under the conditions described in (C). Transparent boxes
mark equivalent positions of the images shown in (E-H). E-H, Representative SEM images of epidermal cells from the apex (E and G) and base (F and H) of the hypocotyl of Wc-light grown (E and F) and low R:FR-light grown (G and H) seedlings. White outlines show examples of the large, ‘bulbous’ epidermal cells measured in (B and C). In all images, apex is up. Scale bar (D and I), 0.5 cm. Scale bar (E-H), 500 μm.

**Figure 8.** Mature *B. rapa* plants show defects in seed number and weight in low R:FR light, but not in oil content. A, Representative image of 60 d-old wild-type (FPsc) and *phyB* mutant (*ein194*) plants grown in long days (16 h light, 8 h dark), in W light (W) or W light supplemented with FR (↓R:FR). Scale bar, 10 cm. B and C, Schematic of PHYB apoprotein (B), showing the position of the *ein194* mutation. Domains are represented by boxes. *ein194* codes for a truncated form of the protein, seen by western blot (C). D, Representative image of dissected siliques 14 d post-pollination from wild-type plants grown in W light or low R:FR shade. Scale bar, 1 cm. E-K, Plant height at 60 d (E), leaf chlorophyll content (F), silique length (G), number of seeds per silique (H), seed weight (I), oil weight per seed weight (J), and the ratio of C18:2 to C18:1 fatty acids (K), of wild type and *ein194* plants grown under W or low R:FR light. Data show mean +/- SEM. Student’s *t* test, **P < 0.005, *P < 0.05, ns = not significant.
SUPPLEMENTAL FIGURE & TABLE LEGENDS

Table S1. Seed oil composition does not change between siliques grown in the same environment.

Figure S1. B. rapa hypocotyl elongation is phyB-dependent. A, Representative image of 4 d-old wild-type Wisconsin Fast Plants (standard) and the phyB mutant ein. 2 d-old W-light grown seedlings were shifted to constant white (Wc) or low R:FR shade (↓R:FR) for 2 d before imaging. Scale bar, 1 cm. B, Hypocotyl length of plants grown under the conditions described in (A). Data show mean +/- SEM. Student’s t test, **P < 0.005, ns = not significant. C, Representative image of a 7 d-old Arabidopsis seedling (Col-0 background) carrying a AtPHYBpro::GUS transcriptional reporter. Note staining in cotyledons, hypocotyl and root. Scale bar, 1 mm. D, Western blot of phyB protein using tissue harvested from cotyledons [C], hypocotyl [H] and root [R] of 7 d-old W-light grown R-o-18 B. rapa seedlings.

Figure S2. B. rapa FPsc seedlings respond to simulated shade. Hypocotyl lengths of 7 d-old FPsc seedlings grown in constant white light (Wc) or 4 d Wc followed by 3 d low R:FR light. Data show mean +/- SEM. Student’s t test, **P < 0.005.

Figure S3. PHYB gene (Bra022192) and predicted protein sequence from B. rapa FPsc plants. Uppercase, exons; lowercase, introns. The location of the ein194 mutation is shown.

Figure S4. qPCR expression analysis of low R:FR shade-induced marker genes in phyB mutants (ein194). Data shown is the same as in Fig. 2, except expression values for each gene are shown relative to Wc-treated wild-type FPsc plants, +/- SEM; Student’s t test relative to Wc-treated wild-type, **P < 0.005, *P < 0.05, ns = not significant.

Figure S5. Over-expression of Arabidopsis phyB causes short hypocotyls in B. rapa. A, A UBQ10pro::AtPHYB (6xHis3xFlag-tag)-transformed FPsc plant, most likely carrying
two insertions of the transgene, sired progeny that had 0, 1, 2, 3 or 4 relative copies of the transgene by qPCR. Plants carrying 4 relative copies are likely homozygous for both insertions of the transgene. Results for example progeny plants numbered 2, 18, 14, 29, and 3 are shown. B, Flag-tagged Arabidopsis phyB protein levels increase with transgene copy number. Shown is a western blot using leaf tissue from the same plants described in (A). Arabidopsis phyB runs as two bands (arrow heads). The lower band corresponds to monomeric protein (~130 kDa), while the upper band is likely a dimer. C, Example of 6 d-old, W-light grown seedlings carrying no copy (plant 2) or 4 relative copies (plant 3) of the UBQ10pro::AtPHYB transgene. Note the change in leaf angle and shape, and short hypocotyl. D and E, Representative images (D) and measured hypocotyl lengths (E) of 6 d-old, non-transformed (control) seedlings, and seedlings carrying at least one copy of the UBQ10pro::AtPHYB transgene. Scale bars, 1 cm. Data show mean +/- SEM. Student’s t test, **P < 0.005.

Figure S6. qPCR time course showing shade-induced gene expression in cotyledons (left), hypocotyl (center) and roots (right) of B. rapa R-o-18 plants. 7 d-old W-light grown seedlings were shifted to low R:FR shade for the indicated period of time. Fold induction is shown relative to time 0, +/- SEM.

Figure S7. Shade-induced genes are expressed in distinct organs of B. rapa. Gene expression was assayed by qPCR in the cotyledons, hypocotyl and roots of 7 d-old W-light grown R-o-18 seedlings, treated for 2 h white (Wc) or low R:FR light. Data shown is the same as in Fig. 3, A-C, except expression values for each gene are shown relative to that of the hypocotyl of Wc-treated plants, +/- SEM. Results are shown for genes BrATHB2 (A), BrHFR1 (B), BrPIL1 (C), BrXTH33 (D), BrIAA29 (E), BrIAA19 (F), BrGH3-5 (G) and BrIAA2 (H). Student’s t test, **P < 0.005, *P < 0.05, ns = not significant.

Figure S8. Effect of exogenous gibberellin (GA3; A) and brassinosteroid (BL; B) treatment on hypocotyl gene expression of B. rapa R-o-18 seedlings by qPCR. Conditions are as described in Fig. 3D. Vehicle controls are ethanol and DMSO.
respectively. Expression changes within any given gene set are not significant (one-way analysis of variance).

**Figure S9.** Experimental setup of the split light chamber used in Fig. 5. A and B, 4 d-old W-light grown R-o-18 *B. rapa* seedlings were positioned with their cotyledons [C], or the hypocotyl [H] and root [R] exposed to either high R:FR (W light) or low R:FR light (W light supplemented with FR). W light was provided by cool white (CW) fluorescent bulbs, while supplemental FR was provided by an FR light emitting diode (LED; LumiGrow ECC-FR). The cotyledons were fixed in position using adhesive putty, while the roots were suspended in liquid growth medium (½ Linsmaier and Skoog salts and vitamins) to allow vertical growth of the hypocotyl down. C-F, Spectral energy distribution of light in the top chamber without (C) and with (D) supplemental FR light, and in the lower chamber (E and F). Photosynthetically active radiation (PAR) was calculated over 400-700 nm, R light over 600-700 nm, and FR light over 700-800 nm. Spectra are reported for the central point where the seedlings were placed. G, Supplemental FR light in the lower chamber can induce hypocotyl growth when the entire seedling is exposed to the chamber conditions. 4 d-old W-light grown *B. rapa* R-o-18 seedlings were transferred to the lower chamber and exposed to either high (W) or low R:FR light (+ supplemental FR) for an additional 3 d using the conditions described in (E) and (F). Note that these conditions are sufficient to stimulate hypocotyl growth. Data shows mean +/- SEM. Student’s *t* test, **P < 0.005.

**Figure S10.** Induction of presumptive auxin-independent shade-regulated genes in the hypocotyl. qPCR expression analysis of *BrATHB2*, *BrHFR1*, *BrPIL1* and *BrXTH33* in the hypocotyl of R-o-18 seedlings following 2 h (A) or 6 h (B) low R:FR light treatment. The hypocotyl was divided into apical, middle and basal segments as described in Fig. 6. Expression is shown for low R:FR-treated hypocotyl segments relative to the Wc-treated control segments, +/- SEM. Asterisks indicate a significant interaction between light treatment and hypocotyl segment using a two-factor analysis of variance with repeated measures on one factor (hypocotyl segment), **P < 0.005, *P < 0.05, ns = not significant.
Figure S11. The hypocotyls of Arabidopsis wag1 wag2 pid mutants do not elongate in low R:FR shade. A, Representative image of 9 d-old seedlings, grown for 5 d under W-light and then transferred to W (left) or low R:FR shade (right) for 4 d. Shown are wild-type (Col-0), wag1 wag2 mutants (wild-type or heterozygous for the pid mutation), and wag1 wag2 pid triple mutants, which lack cotyledons and true leaves. Scale bar, 5 mm. B, Hypocotyl length of seedlings grown as in (A). Data shows mean +/- SEM. Student’s t test, **P < 0.005.
Table 1. Seed oil composition of B. rapa (FPsc) is not altered by low R:FR light.

| genotype  | condition | % total ion chromatogram (TIC) of major fatty acid peaks |
|-----------|-----------|--------------------------------------------------------|
|           |           | 16:0 | 18:2 and 18:1 | 18:0 | 20:1<sup>a</sup> | 22:1<sup>b</sup> |
| wild type | W light   | 6.3 +/- 3.1 | 60 +/- 2.3 | 4.9 +/- 0.5 | 11.6 +/- 1.1 | 16.8 +/- 0.4 |
| wild type | low R:FR  | 10.1 +/- 1.1 | 60.3 +/- 1.2 | 5.2 +/- 0.5 | 9.6 +/- 0.9 | 14.7 +/- 1.6 |
| ein194    | W light   | 9.1 +/- 0.2 | 58.6 +/- 0.1 | 3.8 +/- 0.2 | 10.5 +/- 0.1 | 18.0 +/- 0.7 |
| ein194    | low R:FR  | 10.3 +/- 0.6 | 58.1 +/- 0.2 | 4.6 +/- 0.4 | 8.9 +/- 1.4 | 18.1 +/- 0.2 |

<sup>a</sup> Eicosenoic acid monounsaturated at carbon 9, 11 or 13.

<sup>b</sup> Docosenoic acid monounsaturated at carbon 11 or 13.
Figure 1. *B. rapa* seedlings display auxin-dependent shade avoidance phenotypes. 

A. Representative image of 7 d-old *B. rapa* R-o-18 seedlings grown in constant white light (Wc) or 4 d Wc followed by 3 d low R:FR light (↓R:FR). Scale bar, 1 cm. 

B-D, Hypocotyl length (B), cotyledon area (C), and lateral root formation (D) of 7 d-old seedlings treated as in (A). 

E, Hypocotyl length of 7 d-old seedlings grown for 4 d Wc then 3 d liquid culture in Wc or low R:FR light, in the presence of DMSO (vehicle), 10 μM auxinole, or 10 μM NPA. Data show mean ± SEM. Student’s t test, **P < 0.005, *P < 0.05.
Figure 2. qPCR expression analysis of low R:FR shade-induced marker genes. 4 d-old W light-grown wild-type (A; FPsc strain) and phyB mutant (B; ein194) seedlings treated for 2 h W light (Wc) or 2 h low R:FR light (low R:FR). For each gene, expression is shown relative to the 2 h Wc treatment, +/- SEM. Student’s t test, **P < 0.005, *P < 0.05, ns = not significant.
Figure 3. Shade-induced genes are expressed in distinct organs and can be auxin-dependent or independent. A-C, qPCR expression analysis of shade-induced marker genes in the cotyledons (A), hypocotyl (B), and roots (C) of 7 d-old W-light grown *Brassica rapa* R-o-18 seedlings, treated for 2 h white (Wc) or low R:FR light. For each gene, expression is shown relative to the 2 h Wc treatment. D, Gene expression in the hypocotyl of 7 d-old R-o-18 seedlings treated for 150 min in liquid culture with the indicated concentration of IAA. Normalized fold induction for each gene is shown relative to the vehicle control (0 nM; ethanol). E, Expression of orthologous genes in whole seedlings of wild-type (Col-0) and sav3-1 mutants of Arabidopsis. 7 d-old W-light grown seedlings were treated for 1 h with W (Wc) or low R:FR light. Expression is shown relative to the Wc-treated wild-type plants. **P < 0.005, *P < 0.05, ns = not significant. In (C), all comparisons are ns. In (E), sav3 mutants are compared to wild-type seedlings grown in equivalent conditions.
Figure 4. The cotyledons regulate hypocotyl growth and gene expression. A, Experimental setup of (B-D). The cotyledons (C) of W-light grown R-o-18 B. rapa seedlings were removed immediately prior to light treatment. Light treatments consisted of continued constant W light (Wc) or simulated shade (low R:FR). The cotyledons of operated seedlings were removed at the base of the petioles, while the hypocotyl (H) and root (R) were left intact. B, Hypocotyl length of 7 d-old seedlings grown for 4 d Wc then an additional 3 d Wc or low R:FR light. In operated seedlings, the cotyledons of 4 d-old seedlings were removed as described in (A). Data shows mean ± SEM. C and D, Gene expression in the hypocotyl of 7 d-old W-light grown R-o-18 seedlings treated with either 2 h W light (Wc) or low R:FR light, of intact seedlings (C) or seedlings where the cotyledons were removed immediately prior to the 2 h light treatment (D). For each gene, expression is shown relative to the 2 h Wc treatment, as measured by qPCR analysis, ± SEM. In (B-D), Student’s t test, * P < 0.05, ** P < 0.005, ns = not significant.
Figure 5. The cotyledons regulate hypocotyl growth and gene expression in intact seedlings. A, Experimental setup of (B-D). W-light grown R-o-18 B. rapa seedlings were positioned in a split light chamber. The cotyledons [C], or the hypocotyl [H] and root [R], were exposed to either high R:FR (W light; R:FR > 15) or low R:FR light (W light supplemented with FR; R:FR < 0.8). The cotyledons were fixed in position, while the roots were suspended in liquid growth medium to allow vertical growth of the hypocotyl down. See Supplemental Fig. S9 for a complete description of the light conditions used. B, Hypocotyl length of R-o-18 seedlings grown as described in (A). 4 d-old seedlings were transferred to the split light chamber and grown for an additional 3 d. Data shows mean +/- SEM. C and D, Gene expression in the cotyledons (C) and hypocotyls (D) of seedlings grown in the split light chamber. W-light grown plants were transferred to the split light chamber at 4 d and allowed to equilibrate to W-light conditions (high R:FR). At 6 and a half d, the ratio of R:FR light was adjusted as described in (B), and gene expression levels measured by qPCR 12 h later. The light treatments of each column (white, gray, diagonal striped and black) are as indicated in (B). For each gene, expression is shown relative to seedlings where both the cotyledons and hypocotyl plus root were exposed to high R:FR light, +/- SEM. Asterisks indicate significant difference for (B) Student’s t test, and (C and D) one-way analysis of variance. **P < 0.005, *P < 0.05, ns = not significant.
Figure 6. Low R:FR shade induces a basipetal gradient of auxin-dependent gene expression. A, Experimental setup of (C-E). The hypocotyl of 7 d-old W-light grown seedlings (R-o-18) was sectioned after the indicated light treatments into apical, middle, and basal fragments. [C], cotyledons; [H], hypocotyl; [R], root. B, Free IAA levels in the cotyledons following 6 h W or low R:FR light treatment. C, Free IAA levels in apical, middle, and basal segments of the hypocotyl following 6 h W or low R:FR light treatment. In (B and C), data show mean +/- SEM, n = 5. Student’s t test, **P < 0.005, *P < 0.05, ns = not significant. D and E, qPCR expression analysis of auxin-dependent genes in the hypocotyl following 2 h (D) or 6 h (E) low R:FR light treatment. Expression is shown for low R:FR-treated hypocotyl segments relative to the Wc-treated control segments, +/- SEM. In (D), asterisks indicate a significant interaction between light treatment and hypocotyl segment using a two-factor analysis of variance with repeated measures on one factor (hypocotyl segment). **P < 0.005, ns = not significant. By 6h, no significant interaction is observed for any of the genes.
Figure 7. *B. rapa* hypocotyl growth occurs in a basipetal gradient. A, R-o-18 seedlings were grown for 4 d in constant white (Wc) light and the hypocotyl marked into three divisions of equal size (apex, middle, and base). After a further 3 d growth in either Wc light or low R:FR shade, the length of each segment was measured. B, Length of hypocotyl epidermal cells of 4 and 7 d-old W-light grown plants, as determined by scanning electron microscopy (SEM). The hypocotyls of imaged plants were divided into 5 equal segments (1-5) for analysis. C, Epidermal cell lengths at the hypocotyl apex (0-2 mm below the hypocotyl-petioles junction) and base (1-3 mm from hypocotyl-root junction). 4 d-old W-light grown seedlings were treated with W (Wc) or low R:FR light for 3 d. In (A-C), data show mean +/- SEM. Student's t test, **P < 0.005, *P < 0.05, ns = not significant. D and I, Overlaid SEM images of full-length hypocotyls of Wc (D) and low R:FR (I) seedlings grown under the conditions described in (C). Transparent boxes mark equivalent positions of the images shown in (E-H). E-H, Representative SEM images of epidermal cells from the apex (E and G) and base (F and H) of the hypocotyl of Wc-light grown (E and F) and low R:FR-light grown (G and H) seedlings. White outlines show examples of the large, ‘bulbous’ epidermal cells measured in (B and C). In all images, apex is up. Scale bar (D and I), 0.5 cm. Scale bar (E-H), 500 μm.
Figure 8. Mature *B. rapa* plants show defects in seed number and weight in low R:FR light, but not in oil content. A, Representative image of 60 d-old wild-type (FPsc) and phyB mutant (*ein194*) plants grown in long days (16 h light, 8 h dark), in W light (W) or W light supplemented with FR (WR:FR). Scale bar, 10 cm. B and C, Schematic of PHYB apoprotein (B), showing the position of the *ein194* mutation. Domains are represented by boxes. *ein194* codes for a truncated form of the protein, seen by western blot (C). D, Representative image of dissected silique 14 d post-pollination from wild-type plants grown in W light or low R:FR shade. Scale bar, 1 cm. E-K, Plant height at 60 d (E), leaf chlorophyll content (F), silique length (G), number of seeds per silique (H), seed weight (I), oil weight per seed weight (J), and the ratio of C18:2 to C18:1 fatty acids (K), of wild type and *ein194* plants grown under W or low R:FR light. Data show mean +/- SEM. Student's t-test, **P < 0.005, *P < 0.05, ns = not significant.