**YUCCA auxin biosynthetic genes are required for Arabidopsis shade avoidance**

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Plants respond to neighbor shade by increasing stem and petiole elongation. Shade, sensed by phytochrome photoreceptors, causes stabilization of **PHYTOCHROME INTERACTING FACTOR** proteins and subsequent induction of **YUCCA** auxin biosynthetic genes. To investigate the role of **YUCCA** genes in phytochrome-mediated elongation we examined auxin signaling kinetics after an end-of-day far-red (EOD-FR) light treatment, and found that an auxin responsive reporter is rapidly induced within 2 hours of far-red exposure. **YUCCA2, 5, 8, and 9** are all induced with similar kinetics suggesting that they could act redundantly to control shade-mediated elongation. To test this hypothesis we constructed a **yucca2,5,8,9** quadruple mutant and found that the hypocotyl and petiole EOD-FR and shade avoidance are completely disrupted. This work shows that **YUCCA** auxin biosynthetic genes are essential for detectable shade avoidance and that **YUCCA** genes are important for petiole shade avoidance.
YUCCA auxin biosynthetic genes are required for Arabidopsis shade avoidance

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Summary: A quadruple knock-out of auxin biosynthesis genes abolishes shade avoidance responses.

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ABSTRACT

Plants respond to neighbor shade by increasing stem and petiole elongation. Shade, sensed by phytochrome photoreceptors, causes stabilization of PHYTOCHROME INTERACTING FACTOR proteins and subsequent induction of YUCCA auxin biosynthetic genes. To investigate the role of YUCCA genes in phytochrome-mediated elongation we examined auxin signaling kinetics after an end-of-day far-red (EOD-FR) light treatment, and found that an auxin responsive reporter is rapidly induced within 2 hours of far-red exposure. YUCCA2, 5, 8, and 9 are all induced with similar kinetics suggesting that they could act redundantly to control shade-mediated elongation. To test this hypothesis we constructed a yucca2,5,8,9 quadruple mutant and found that the hypocotyl and petiole EOD-FR and shade avoidance are completely disrupted. This work shows that YUCCA auxin biosynthetic genes are essential for detectable shade avoidance and that YUCCA genes are important for petiole shade avoidance.
INTRODUCTION

Because plants are dependent on light for photosynthesis they have developed a complex system of photoreceptors and downstream responses enabling them to optimize growth to their light environment (Kami et al., 2010). One critical aspect of plant light responses is neighbor detection and shade avoidance (Casal, 2013; Gommers et al., 2013). Plants detect the presence of neighbors by changes in the light quality: since photosynthetic tissue absorbs more red light (R) than far-red light (FR), foliar shade uniquely lowers the R:FR ratio. Changes in the R:FR ratio are detected by phytochrome photoreceptors that exist in two photoconvertible forms, the red light absorbing form, Pr, and the far-red light absorbing form, Pfr. In high R:FR conditions, such as direct sunlight, type II phytochromes are converted from Pr to Pfr and translocated from the cytoplasm to the nucleus (Yamaguchi et al., 1999). Once in the nucleus phytochrome binds to and triggers the degradation of a family of bHLH transcription factors known as PHYTOCHROME INTERACTING FACTORS (PIFs), thereby inhibiting elongation and other phenotypes associated with foliar shade or darkness (Ni et al., 1998; Park et al., 2004).

The PIF proteins were originally identified as phytochrome binding factors but are now known to be regulated not only by light but also to integrate signals from the circadian clock, high temperature, and hormone signaling (Leivar and Monte, 2014). They have partially overlapping roles in regulating multiple aspects of development, including promotion of cell elongation and inhibition of both seed germination and chloroplast maturation.

Auxin has long been thought to play a role in shade avoidance (Morelli and Ruberti, 2002; Tanaka et al., 2002). As predicted by Morelli and Ruberti, phytochromes were shown to regulate auxin transport through the shoot (Salisbury et al., 2007) and shade treatment was demonstrated to alter localization of the PIN3 auxin transporter (Keuskamp et al., 2010). Shade also increases endogenous auxin levels (Kurepin et al., 2007; Tao et al., 2008) and auxin signaling (Bou-Torrent et al., 2014; Carabelli et al., 2007; Hersch et al., 2014). Disruption of auxin synthesis by mutation of the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1) gene reduced both shade-induced increases in auxin and shade avoidance elongation responses (Tao et al., 2008; Won et al., 2011). Treatment of leaves with an end-of-day far-red pulse (EOD-FR) will convert type II phytochromes from Pfr to Pr and has been found to increase stem elongation (Gorton and Briggs, 1980), similar to low R:FR. Also similar to low R:FR,
EOD-FR induces many auxin-responsive genes, while disruption of auxin signaling via the
big/doc1 mutant prevents EOD-FR promotion of petiole elongation (Kozuka et al., 2010). These
studies strongly implicate auxin in growth responses to shade and EOD-FR.

PIF proteins were first suggested to promote increases in auxin production and sensitivity
based on microarray and dose-response studies of plants with perturbed PIF4 and PIF5
expression (Nozue et al., 2011). More conclusive evidence came when it was shown that PIF4
regulates auxin biosynthesis in response to high temperature by promoting transcription of auxin
biosynthesis genes (Franklin et al., 2011). More recently it has been demonstrated that PIF4, 5,
and 7 are required for normal shade avoidance and function by promoting transcription of the
YUCCA family of auxin biosynthesis genes and potentiating auxin responsiveness (Hersch et al.,
2014; Hornitschek et al., 2012; Li et al., 2012; de Wit et al., 2014).

The YUCCA family consists of eleven genes encoding flavin monoxygenases that
function in tryptophan-dependent auxin biosynthesis (Cheng et al., 2006; Mashiguchi et al.,
2011; Won et al., 2011; Zhao et al., 2001). They are expressed in developmentally interesting
spatiotemporal patterns (Cheng et al., 2006, 2007). These genes are partially redundant: single
knockouts often have no obvious phenotypes but double and higher-order combinations have
defects in many aspects of development (Cheng et al., 2006, 2007).

Although the phytochrome/PIF/YUCCA/auxin connection seems clear, most yucca
mutant combinations that have been examined to date (yucca1,4 or yucca3,5,7,8,9) only show
minimal to moderate shade avoidance phenotypes (Li et al., 2012; Tao et al., 2008; Won et al.,
2011). More recently, as part of a large phenotypic profiling experiment we reported that the
yucca2,5,8,9 quadruple mutant has a strong shade avoidance phenotype (Nozue et al., 2015).
Because of the centrality of YUCCA genes to the current shade avoidance model, here we
analyze that mutant strain in more detail, beginning with why we decided to make the yucca2, 5,
8, 9 quadruple in the first place.

To better understand the role of the YUCCA genes in shade avoidance and EOD-FR
response we used live imaging of an auxin reporter (eDR5::Luciferase) and found a rapid
increase in auxin response following an end-of-day far-red (EOD-FR) pulse. We found that the
kinetics of the eDR5 reporter response to EOD-FR were similar to the kinetics of YUCCA2,5,8,
and 9 upregulation, suggesting that these genes are the critical YUCCAs for response to EOD-FR.
We tested this idea by generating a yucca2,5,8,9 quadruple mutant and found that these genes are
essential both for upregulation of the auxin reporter and for both EOD-FR and low R-FR shade-
induced increases in hypocotyl and petiole elongation. These results conclusively show that the

*YUCCA* genes are required for a normal EOD-FR and shade avoidance response.
MATERIALS & METHODS

Plasmids

eDR5::LUC+ is described in (Covington and Harmer, 2007). The pZP-eDR5::LUC2 plasmid was constructed in two steps. First, the luciferase+ gene in the eDR5::LUC plasmid (Covington and Harmer, 2007) was replaced with the luciferase2 (luc2) gene (from pGL4.10, Promega, Madison, WI) using the HindIII and XbaI sites in the two plasmids. Second, the eDR5::LUC2 cassette was removed from the resulting plasmid using the BamHI and PstI sites and cloned into the BamHI and PstI sites of pPZPXomegaLUC+ (a derivative of pPZP221 (Hajdukiewicz et al., 1994) that contains the RbcS E9 polyadenylation region). The resulting plasmid confers resistance to spectinomycin in bacteria and gentamycin in plants.

Plant materials and growth conditions

Plant transformations were performed by floral dip as previously described (Clough and Bent, 1998). eDR5::LUC2 transformants were selected on gentamycin-containing growth media. The T-DNA and transposon insertion lines were obtained from the Arabidopsis Biological Resource Center (ABRC), the Cold Spring Harbor Lab (CSHL) or GABI-Kat. Mutant yucca lines and plants carrying YUCCA promoter-GUS constructs were obtained from Yunde Zhao and have been previously described (Chen et al., 2014; Cheng et al., 2006). Multiple mutant combinations were obtained by repeated crossing and PCR genotyping using described primers (Chen et al., 2014; Cheng et al., 2006). Homozygous athb-2 mutants were obtained from SALK line_106790 (Alonso et al., 2003; O’Malley and Ecker, 2010). Homozygotes were identified by PCR genotyping using standard techniques and the primers listed in Table 1. A reverse-transcription PCR assay was used to confirm that no wild-type message was made.
For seedling stage EOD-FR analysis, seeds were surface sterilized with 70% ethanol, 0.1% TritonX-100 for 5 minutes, stratified for four days at 4°C, then sown on medium containing 1/2X MS with minimal organics (Sigma M6899) and 0.7% agar (Sigma A1296). Seeds were grown in custom chambers outfitted with Quantum Devices Snaplite LEDs under short-day (8 hour day/16 hour night) conditions with 35 μmol m⁻² s⁻¹ “red” (peak wavelength 670nm, half power spectral bandwidth 655-685nm) and 5 μmol m⁻² s⁻¹ “blue” (peak wavelength 470nm, half power spectral bandwidth 455-485nm). EOD-FR treatment consisted of a 30 minute, 14 μmol m⁻² s⁻¹ “far-red” (peak wavelength 730nm, half power spectral bandwidth 715-745nm) pulse given nightly for 1 (Figure 1A) or 4 (Figures 4A, 4C) nights before measurement. LED chamber temperature was 21˚C.

For seedling stage high and low R:FR analysis (Figure 5), seedlings were grown in the same custom chambers as described above for seedling EOD-FR analysis. Light conditions were continuous illumination with 35 μmol m⁻² s⁻¹ “red” (peak wavelength 670nm, half power spectral bandwidth 655-685nm) and 5 μmol m⁻² s⁻¹ “blue” (peak wavelength 470nm, half power spectral bandwidth 455-485nm). After 24 hours, “far-red” (peak wavelength 730nm, half power spectral bandwidth 715-745nm) illumination was added to bring the red-to-far-red ratio (R:FR) to 2. After an additional 48 hours the R:FR ratio in one chamber was lowered to 0.5 and plants were grown for an additional 4 days. The chambers assigned to high and low R:FR were swapped for each trial.

For analysis of juvenile plants under EOD-FR (Figures 1B-1G, 4B, 4E) seeds were sown as above but plants were grown under 12/12 or short day (8 hr light:16 hr dark) conditions at 22˚C in a Conviron E7 chamber for approximately 18 days with cool white and incandescent lights (75μmol m⁻² s⁻¹ PAR, R:FR 1.4). Two days prior to the EOD-FR pulse, plants were transferred to
the LED chambers using the same light and temperature conditions as for seedlings (short day 35 μmol m$^{-2}$ s$^{-1}$ red, 5 μmol m$^{-2}$ s$^{-1}$ blue light; 21° C.) and then pulsed as above.

For analysis of juvenile plants under high and low R:FR (Figure 4D), stratified seeds were sown on soil and grown under long days in a Conviron walk-in chamber with cool white bulbs and far-red LEDs (Orbitec) (16h light/8 h night; 100 μmol m$^{-2}$ s$^{-1}$ PAR, R:FR 1.8, 22° C).

Two week old plants were transferred to shelves in the same chamber with increased FR (100 μmol m$^{-2}$ s$^{-1}$ PAR, R:FR 0.5) to stimulate the shade avoidance response or kept under high R:FR for ten days. Leaves were scanned and petiole length measured as described (Maloof et al., 2013). Plants for Figure 6 were grown under these same high R:FR conditions but were not transferred to low R:FR.

For N-1-naphthylphthalamic acid (NPA; Chem Service, PS-343, http://www.chemservice.com) treatment of eDR5::LUC juvenile plants, seeds were sown and grown as above. 24 hours and 1 hour prior to EOD-FR treatment each plate of plants was sprayed with 1.5ml of DMSO containing 100 μM NPA or an equivalent volume of DMSO alone. Powdered NPA was dissolved in DMSO and stored at -20° C.

Quantitative RT-PCR

Columbia and athb-2 seedlings were grown as described above for seedling EOD-FR except that they had 30 min EOD-FR pulses on days 3 through 7 and were harvested on day 7, one hour after the end of the final EOD-FR pulse. RNA was prepared with Plant RNeasy (Qiagen) and cDNA prepared with Superscript II (Invitrogen). Real-time qRT-PCR was performed using an iCycler IQ™5 (Bio-Rad) in self-made buffer (final concentration: 40 mM Tris-HCL, pH 8.4, 100 mM KCl, 6 mM MgCl2, 8% glycerol, 20 nM fluorescein, 0.4x SYBR Green I (Molecular
Probes), 1x bovine serum albumin (New England Biolabs), and 1.6 mM dNTPs) using primers
described in Table 1, 10 ng of RNA-equivalent cDNA and Taq polymerase. Each of five to six
independent cDNA preparations was assayed two times for each transcript analyzed. Data
presented are normalized to the expression level of the control gene **PP2a** (At1g13320;
(Czechowski et al., 2005). Transcript abundance was calculated using the relative expression
software tool (REST-MCS; (Pfaffl et al., 2002)).

**GUS staining**

Columbia, YUCCA5::**GUS**, YUCCA8::**GUS** and YUCCA9::**GUS** seeds were grown as described
for juvenile plants above. On day 2 in the LED chamber half of the plants were treated with an
EOD-FR pulse. Two hours after the pulse plants were taken for GUS analysis. Plants were
harvested in 80% acetone on ice and kept in acetone for 30 minutes. They were then washed
twice with pre-staining solution (100 mM NaPO₄, pH 7.0, 0.1% (v/v) Triton X-100, 2 mM
potassium ferrocyanide, 2 mM potassium ferricyanide, 1 mM EDTA), after which they were
vacuum-infiltrated for 10 minutes with GUS-infiltration buffer (pre-staining solution + 1 mM X-
gluc). Images were taken with a Zeiss Discovery-V12 stereo microscope and AxioCam MRC
(Zeiss).

**Imaging and Analysis**

For hypocotyl length measurements, whole seedlings were placed on transparency film and
scanned with a flatbed scanner (Microtek ScanMaker 8700, http://www.microtek.com). For
luminescence measurements, 24 hours prior to luciferase imaging each plant plate was sprayed
with 1.5 ml of 3 mM D-luciferin (Biosynth AG) in 0.1% Triton X-100. Bioluminescence was
captured with an XR/Mega-10Z ICCD camera (Stanford Photonics) and Piper Imaging software
(Stanford Photonics) (Figure 1) or an iKon M-934 CCD camera (Andor) controlled by LabView
software (National Instruments) (Figure 4). Photo analysis software ImageJ (Rasband, 1997) was
used to measure both hypocotyl lengths and bioluminescence. Subsequent data analysis was
performed in R (R Core Team, 2016) using base packages and the add-on packages ggplot2
(Wickham, 2009), reshape2 (Wickham, 2007), lme4 (Bates et al., 2014), lmerTest (Kuznetsova
et al., 2014), and arm (Gelman and Su, 2014).

Data and Scripts

The raw data and scripts to recreate plots are available on github at
https://github.com/MaloofLab/Mueller-Moule-PeerJ-2016
RESULTS AND DISCUSSION

End-of-day far-red treatment rapidly increases auxin responses.

It is clear that changes in auxin biosynthesis and sensitivity are critical to shade avoidance responses (Bou-Torrent et al., 2014; Hornitschek et al., 2012; Li et al., 2012; de Wit et al., 2014).

To examine phytochrome/auxin pathway interactions in real-time we used an enhanced version of the synthetic auxin responsive promoter DR5 (Ulmasov et al., 1997) to drive the expression of firefly luciferase ($LUC$; (Welsh and Kay, 2005), eDR5::$LUC$ (Covington and Harmer, 2007). We initially used an end-of-day far-red (EOD-FR) pulse that, like low R:FR, will reduce the amount of active type II phytochromes, increases expression of auxin responsive genes (Kozuka et al., 2010), and increases stem elongation (Gorton and Briggs, 1980). Plants treated with EOD-FR displayed a strong increase in eDR5::$LUC$ bioluminescence peaking two to three hours after the treatment, consistent with prior reports on eDR5::$GUS$ (Carabelli et al., 2007). This response is found in both seedling stage (Figure 1A) and juvenile (Figure 1B) plants and occurred in cotyledons, hypocotyls, petioles, the shoot apex, and developing leaves (Figure 1D,E).

To investigate the importance of auxin transport in eDR5::$LUC$ activation we examined the effect of the auxin transport inhibitor $N$-$1$-naphthylphthalamic acid (NPA) on eDR5::$LUC$ expression. Plants grown on NPA still responded with a peak of luminescence following an EDO-FR treatment (Figure 1C), but in this case the increased bioluminescence was limited to the apex and young leaves (Figure 1F). The magnitude of induction was somewhat lower on NPA because of higher basal luminescence, however the peak strongly resembles the response of the control plants without NPA (Figure 1G) and occurs within a similar time-frame. These results suggest that auxin transport is not required to generate the peak of auxin reporter expression
following EOD-FR treatment but that transport is required for increased auxin signaling in the petiole. Alternatively, it is possible that the lack of signal in the EOD-FR, NPA treated petioles is due to increased IAA conjugation that can occur in the presence of NPA.

**Shade treatment induces expression of four YUCCA auxin biosynthetic genes**

Shade treatment is known to lead to increased expression of some YUCCA auxin biosynthetic genes (Hornitschek et al., 2012; Li et al., 2012; Tao et al., 2008), so it seemed possible that the induction of eDR5 could be due to increased YUCCA expression. However, most studied of *yucca* mutants have not found strong shade avoidance phenotypes. One explanation for the observed weak shade phenotypes might be redundancy within the YUCCA gene family. To determine if this could be the case we asked which YUCCA genes were induced by EOD-FR or shade treatments. We first analyzed a published microarray data set (Sessa et al., 2005) and found that three members of this family, YUCCA5, 8, and 9, were all significantly and rapidly induced by low R:FR (P < 0.002; Figure 2A), suggesting that they would be interesting targets for further analyses. A fourth member, YUCCA2, was marginally induced (P > 0.02). All YUCCA genes returned to pre-induction levels after four days, indicating that they are involved in early response to shade conditions. We used quantitative real-time reverse transcription PCR (qRT-PCR) to confirm that YUCCA2, 5, 8, and 9 are induced after a series of EOD-FR treatments. One hour after the last EOD-FR treatment all four genes were significantly induced with mRNA levels up to 10 times higher than in control plants (Figure 2B), consistent with previous microarray studies (Li et al., 2012; Tao et al., 2008).

**YUCCA genes 2, 5, 8, and 9 are expressed in organs responsive to shade-treatment.**

To determine whether these genes were expressed in tissues relevant to shade avoidance, we examined staining in YUCCA2, 5, 8, or 9 promoter::GUS fusions (Figure 3). All four genes were
expressed in the hypocotyls and leaf veins (Figure 3E-L). *YUCCA2* was also expressed strongly in the primary root, whereas the other three expressed more weakly in primary roots (Figure 3M-P). The *YUCCA2* and 5 genes were expressed in the shoot apical meristem (Figure 3E,F) and in very defined locations in the leaf. In the leaf they were highly expressed in the veins, petioles, and hydathodes (Figure 3G). In the roots *YUCCA5* was highly expressed at the branching points between primary and secondary roots (Figure 3N), similar to reported patterns of eDR5::LUC (Moreno-Risueno et al., 2010) suggesting that it may play a role in defining these patterns. The *YUCCA8* and 9 genes were expressed in a more diffuse pattern in the leaves starting from the leaf margins (Figure 3K and L), similar to previously reported patterns of eDR5::GUS and *Ptaa1::TAA1::GUS* (Tao et al., 2008). They were also expressed in secondary roots (Figure 3O and P) but not in the petioles or the shoot apical meristem. In summary, these genes are expressed in the main organs where shade induction of eDR5::LUC expression is observed: all four are expressed in leaves and *YUCCA2* and 5 also in the shoot apex.

**AtHB-2 is not required for YUCCA induction.**

The HD-zip transcription factor *AtHB-2* is strongly induced by shade and affects both shade-avoidance traits and auxin-responsive processes (Carabelli et al., 1993, 1996; Morelli and Ruberti, 2002; Steindler et al., 1999). We were therefore curious if *athb-2* mutations would affect *YUCCA* induction. However, we found full induction of *YUCCA2*, 5, 8, and 9 in *athb-2* mutants (Figure 2C). Although not statistically significant the induction appears higher in *athb-2* than in wild type, perhaps hinting at a compensatory feedback loop. *AtHB-2* may primarily affect auxin transport, as previously proposed (Morelli and Ruberti, 2002) but is not required for *YUCCA* expression.
YUCCA genes 2, 5, 8, and 9 are required for EOD-FR and low R:FR stimulation of auxin signaling and cell elongation.

To determine the relative importance of YUCCA genes for EOD-FR or shade-mediated increases in auxin signaling and subsequent hypocotyl and petiole elongation, we constructed a quadruple mutant with insertions disrupting YUCCA2, 5, 8, and 9 (yucQd) and compared this to yucca5, 8, 9 (yucT) and yucca3, 5, 7, 8, 9 (yucQt) mutant strains. The yucT and yucQt strains behaved similarly, partially reducing hypocotyl and petiole EOD-FR responses (Fig 4A and B), similar to previous studies of yucca1, 4 or yucQt lines (Li et al., 2012; Tao et al., 2008; Won et al., 2011).

In contrast, the quadruple mutant line completely disrupted EOD-FR in hypocotyls (Figure 4V) and low R:FR growth responses in petioles (Figure 4D). In separate experiments we also compared hypocotyl low R:FR response in the yucQd strain to yucca2, yucca5, yucca8, and yucca9 single mutants, a yucca1, 4 double mutant strain, and a yucca2,5,9 triple mutant strain (Figure 5). In this assay all strains were shade responsive except for yucQd (Figure 5). Across these different experiments the only consistent non-responder to low R:FR and EOD-FR is the yucQd. The difference between the yucQd mutant and the yucT and yucQt combinations is that the yucQd mutant is the only line missing the function of all four of the EOD-FR / low R:FR inducible YUCCA genes. Therefore, this result shows that YUCCA2, 5, 8, and 9 act additively and together are required for the shade avoidance response. In growing the mutant lines for these studies we did not observe any severe morphological defects, although yucQd had reduced fertility (Figure 6).

The failure of the yucQd mutant to show a morphological shade avoidance response suggested that induction of eDR5::LUC2 by EOD-FR was likely also diminished. To investigate this possibility, the eDR5::LUC2 construct was transformed into the yucQd strain and wild-type
plants. We found that EOD-FR induction of eDR5::LUC2 expression was essentially abolished in the *yucQd* mutant (juvenile plants; Figure 4 E). Thus, *YUCCA2, 5, 8, and 9* are required for increased auxin signaling in response to EOD-FR and shade for the subsequent induction of hypocotyl and petiole elongation.

CONCLUSIONS

The phenotypic plasticity exhibited by plants in response to shade from other plants is visually striking and is of agronomic importance. Accumulating evidence has led to a model whereby inactivation of phytochromes in shade allows accumulation of PIF transcription factors that upregulate *YUCCA* transcription and a concomitant increase in auxin biosynthesis. Given this model it has been something of a conundrum that multiple *yucca* mutants retain a significant (albeit reduced) shade avoidance response, leaving open the possibility of a parallel, *YUCCA*-independent pathway. By creating a multiple mutant that removes all of the shade-inducible *YUCCA* genes we demonstrate that *YUCCAs* are essential for measurable shade avoidance responses in the hypocotyl and also the petiole.
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FIGURE LEGENDS

Figure 1. EOD-FR induction of eDR5::LUC luminescence. (A-C) Mean luminescence of 5-day-old seedlings (A), 3-week-old juveniles (B), or 3-week-old juveniles in the presence of NPA (C) moved to darkness (solid black line) or treated with a 30 minute EOD-FR pulse prior to transfer to darkness (dashed red line). Dotted lines indicate SEM. Time 0 indicates the beginning of the EOD-FR treatment. n = 4-11 plants for each treatment. Representative plots for one of three independent experiments are shown. (D-F) False-color images of eDR5::LUC plants. Representative DMSO treated plant 40 (D) or 240 (E) minutes after EOD-FR pulse showing increase in petiole luminescence after treatment. (F) NPA treated plants 240 minutes after EOD-FR do not have observable petiole luminescence but show increased luminescence in the leaves and apices. (G) Mean luminescence of 3-week-old juveniles treated with DMSO (compare with (C)).

Figure 2. Shade and EOD-FR induction of YUCCA genes. (A) Expression levels of YUCCA genes from a published shade-induction microarray experiment (Sessa et al., 2005). (B) mRNA levels in EOD-FR treated wild-type plants. (C) mRNA levels in EOD-FR treated athb-2 mutant plants. For (B and C) plants were treated for five days with EOD-FR, and samples were taken 1 hour after the last EOD-FR treatment. mRNA levels shown are normalized to untreated plants.
Results shown are averages of n=5-6 ± SEM. Asterisks mark statistical significance of induction (* p-value ≤ 0.05, ** p-value ≤ 0.005) calculated by the REST-program (Pfaffl et al. 2002).

Figure 3. Histochemical localization of GUS in transgenic *Arabidopsis thaliana* plants containing the YUCCA2::GUS, YUCCA5::GUS, YUCCA8::GUS or YUCCA9::GUS constructs. (A-D) Whole plants. (E-H) Hypocotyls and shoot-apical meristems. (I-L) Leaves. (M-P) Roots. Plants were grown in at 22°C, 75μmol m⁻² s⁻¹ PAR; R:FR 1.4.

Figure 4. YUCCA genes are required for shade avoidance. (A-C) Hypocotyl (A,C) or petiole (B) measurements of short day grown plants with (dark red) or without (blue) EOD-FR pulses. Means of n = 17-137 plants +/- SEM are shown. Representative data from one of three experiments is shown. (D) Petiole lengths of plants grown in long day high (red, simulated sun) or low (dark red, simulated shade) R:FR conditions. Means of n=48-116 petioles +/- SEM are shown. (E) Induction of eDR5::LUC2 expression in 15 day-old wild type and *yucca2,5,8,9* mutants moved from short day (8L:16D) conditions to darkness (blue line) or treated with a 30 minute EOD-FR pulse (dark red line). Shading indicates 95% confidence interval. Time 0 indicates the beginning of the EOD-FR treatment. Fourteen Col and 10 *yucca2589* plants were measured.

Figure 5. Hypocotyl length of additional lines in simulated sun and shade. Four independent experiments were performed with a total of 35-150 plants per treatment/genotype combination.
Figure 6. Adult *yucca* plants. The mutant lines did not show severe morphological defects, although some showed reduced fertility.

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EOD-FR induction of eDR5::LUC luminescence

(A-C) Mean luminescence of 5-day-old seedlings (A), 3-week-old juveniles (B), or 3-week-old juveniles in the presence of NPA (C) moved to darkness (solid black line) or treated with a 30 minute EOD-FR pulse prior to transfer to darkness (dashed red line). Dotted lines indicate SEM. Time 0 indicates the beginning of the EOD-FR treatment. n = 4-11 plants for each treatment. Representative plots for one of three independent experiments are shown. (D-F) False-color images of eDR5::LUC plants. Representative DMSO treated plant 40 (D) or 240 (E) minutes after EOD-FR pulse showing increase in petiole luminescence after treatment. (F) NPA treated plants 240 minutes after EOD-FR do not have observable petiole luminescence but show increased luminescence in the leaves and apices. (G) Mean luminescence of 3-week-old juveniles treated with DMSO (compare with (C)).
Shade and EOD-FR induction of *YUCCA* genes

(A) Expression levels of *YUCCA* genes from a published shade-induction microarray experiment (Sessa et al., 2005). (B) mRNA levels in EOD-FR treated wild-type plants. (C) mRNA levels in EOD-FR treated *athb-2* mutant plants. For (B and C) plants were treated for five days with EOD-FR, and samples were taken 1 hour after the last EOD-FR treatment. mRNA levels shown are normalized to untreated plants. Results shown are averages of n=5-6 ± SEM. Asterisks mark statistical significance of induction (* p-value ≤ 0.05, ** p-value ≤ 0.005) calculated by the REST-program (Pfaffl et al. 2002).
B

Col-0

C

athb-2

fold increase after EOD-FR

fold increase after EOD-FR

YUC2 YUC5 YUC6 YUC8 YUC9

YUC2 YUC5 YUC6 YUC8 YUC9

** * ** *

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Figure 3 (on next page)

Histochemical localization of GUS in transgenic Arabidopsis thaliana plants containing the \textit{YUCCA2::GUS}, \textit{YUCCA5::GUS}, \textit{YUCCA8::GUS} or \textit{YUCCA9::GUS} constructs

(A-D) Whole plants. (E-H) Hypocotyls and shoot-apical meristems. (I-L) Leaves. (M-P) Roots.
**Figure 4** (on next page)

*YUCCA* genes are required for shade avoidance

(A-C) Hypocotyl (A,C) or petiole (B) measurements of short day grown plants with (dark red) or without (blue) EOD-FR pulses. Means of n = 17-137 plants +/- SEM are shown. Representative data from one of three experiments is shown. (D) Petiole lengths of plants grown in long day high (red, simulated sun) or low (dark red, simulated shade) R:FR conditions. Means of n=48-116 petioles +/- SEM are shown. (E) Induction of eDR5::LUC2 expression in 15 day-old wild type and *yucca2,5,8,9* mutants moved from short day (8L:16D) conditions to darkness (blue line) or treated with a 30 minute EOD-FR pulse (dark red line). Shading indicates 95% confidence interval. Time 0 indicates the beginning of the EOD-FR treatment. Fourteen Col and 10 *yucca2589* plants were measured.
Figure 5 (on next page)

Hypocotyl length of additional lines in simulated sun and shade

Four independent experiments were performed with a total of 35-150 plants per treatment/genotype combination.
**Figure 6** (on next page)

Adult wild-type and *yucca* mutant lines

The mutant lines did not show severe morphological defects, although some showed reduced fertility.
Table 1. PCR Primers

| Primer | DNA Region | PCR Product (kb) |
|--------|------------|-----------------|
| Primer A | Primer B |  |
Table 1 PCR primers

| Gene | Primer Type | Sequence                      | Final Concentration |
|------|-------------|-------------------------------|---------------------|
| AtHB-2 | LBb1 | GCGTGACGGCTGTGCTGCAACT          | 500 nM              |
| AtHB-2 | LP   | TGGTTGAAATAAAAAGAAAGTG         | 500 nM              |
| AtHB-2 | RP   | CGTCACTGATTCCTCTGTGAGC         | 500 nM              |
| AtHB-2 | qPCR  | ACATGAGCCCACCCACTAC           | 200 nM              |
| AtHB-2 | qPCR  | GAAGAGCGTCAAAGGTCAAGC         | 200 nM              |
| PP2a  | qPCR   | TAACGTGGCCAAAATGATGC          | 200 nM              |
| PP2a  | qPCR   | GTTCTCCACACCGCTTTGT          | 200 nM              |
| YUC2   | qPCR  | ACCATGTTGGCTAAAGGGAGTG        | 900 nM              |
| YUC2   | qPCR  | AATCCAAAGCTTTGTGAACCGACTG     | 900 nM              |
| YUC3   | qPCR  | CGTCCTCATGGCTTTAAAGCAACCAAC  | 900 nM              |
| YUC3   | qPCR  | GACGCAAAACAATCTCTTCTCTCG      | 50 nM               |
| YUC5   | qPCR  | ATGATGTTGATGAAGGTGGTCTCTCTCTG | 300 nM              |
| YUC5   | qPCR  | ATCGCCATGCAAGAATCAGTAGAATC    | 300 nM              |
| YUC6   | qPCR  | GAGACGCTGTGCACGCTCTA          | 300 nM              |
| YUC6   | qPCR  | ATGATCCTCCCGAGGTGAACC         | 300 nM              |
| YUC8   | qPCR  | ATCAACCTAAAGTCTACGAGTG        | 50 nM               |
| YUC8   | qPCR  | CTCCGTAGCCACCACAAG           | 300 nM              |
| YUC9   | qPCR  | TCTCTTGATCTTGCTACCCACAATGC   | 300 nM              |
| YUC9   | qPCR  | CCACCTCATCTCATCTCAGATTCC      | 50 nM               |
