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YUCCA auxin biosynthetic genes are required for Arabidopsis shade avoidance

Patricia Müller-Moulé [1], **Kazunari Nozue** [1], **Melissa L Pytlak** [1,2], **Christine M Palmer** [1,3], **Michael F Covington** [1,4], **Andreah D Wallace** [1,5], **Stacey L Harmer** [1], **Julin N Maloof** Corresp. [1]

[1] Department of Plant Biology, University of California, Davis, Davis, California, United States

[2] PASCO Scientific, Roseville, California, United State of America

[3] Natural Sciences Department, Castleton University, Castleton, Vermont, United States

[4] Amaryllis Nucleics, Berkeley, California, United States

[5] Clontech Laboratories, Mountain View, California, United States

Corresponding Author: Julin N Maloof
Email address: jnmaloof@ucdavis.edu

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.

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Patricia Müller-Moulé, Kazunari Nozue, Melissa L. Pytlak, Christine M. Palmer, Michael F. Covington, Andreah D. Wallace, Stacey L. Harmer, and Julin N. Maloof.

Department of Plant Biology, College of Biological Sciences, University of California, Davis, Davis, CA, 95616, USA.

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

Present Addresses:

Michael Covington: Amaryllis Nucleics, Berkeley, CA

Christine Palmer: Castleton University, Castleton, VT 05735

Melissa Pytlak: PASCO scientific, 10101 Foothills Blvd, Roseville, CA 95747

Andreah Wallace: Clontech Laboratories, Inc., 1290 Terra Bella Ave., Mountain View, CA 94043

Corresponding Author:
Julin N Maloof
jnmaloof@ucdavis.edu

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 monooxygenases 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

107 essential both for upregulation of the auxin reporter and for both EOD-FR and low R-FR shade-
108 induced increases in hypocotyl and petiole elongation. These results conclusively show that the
109 *YUCCA* genes are required for a normal EOD-FR and shade avoidance response.
110

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 *e*DR5*::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

157 the LED chambers using the same light and temperature conditions as for seedlings (short day 35
158 µmol m^{-2} s^{-1} red, 5 µmol m^{-2} s^{-1} blue light; 21˚ C.) and then pulsed as above.
159 For analysis of juvenile plants under high and low R:FR (Figure 4D), stratified seeds
160 were sown on soil and grown under long days in a Conviron walk-in chamber with cool white
161 bulbs and far-red LEDs (Orbitec) (16h light/8 h night; 100 µmol m^{-2} s^{-1} PAR, R:FR 1.8, 22˚ C).
162 Two week old plants were transferred to shelves in the same chamber with increased FR (100
163 µmol m^{-2} s^{-1} PAR, R:FR 0.5) to stimulate the shade avoidance response or kept under high R:FR
164 for ten days. Leaves were scanned and petiole length measured as described (Maloof et al.,
165 2013). Plants for Figure 6 were grown under these same high R:FR conditions but were not
166 transferred to low R:FR.
167 For *N*-1-naphthylphthalamic acid (NPA; Chem Service, PS-343,
168 http://www.chemservice.com) treatment of eDR5*::LUC* juvenile plants, seeds were sown and
169 grown as above. 24 hours and 1 hour prior to EOD-FR treatment each plate of plants was
170 sprayed with 1.5ml of DMSO containing 100 µM NPA or an equivalent volume of DMSO alone.
171 Powdered NPA was dissolved in DMSO and stored at -20˚ C.
172
173 **Quantitative RT-PCR**
174 Columbia and *athb-2* seedlings were grown as described above for seedling EOD-FR except that
175 they had 30 min EOD-FR pulses on days 3 through 7 and were harvested on day 7, one hour
176 after the end of the final EOD-FR pulse. RNA was prepared with Plant RNeasy (Qiagen) and
177 cDNA prepared with Superscript II (Invitrogen). Real-time qRT-PCR was performed using an
178 iCycler IQ™5 (Bio-Rad) in self-made buffer (final concentration: 40 mM Tris-HCL, pH 8.4, 100
179 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 $NaPO_4$, 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

306 plants. We found that EOD-FR induction of eDR5*::LUC2* expression was essentially abolished
307 in the *yucQd* mutant (juvenile plants; Figure 4 E). Thus, *YUCCA2, 5, 8,* and *9* are required for
308 increased auxin signaling in response to EOD-FR and shade for the subsequent induction of
309 hypocotyl and petiole elongation.
310
311 **CONCLUSIONS**
312 The phenotypic plasticity exhibited by plants in response to shade from other plants is visually
313 striking and is of agronomic importance. Accumulating evidence has led to a model whereby
314 inactivation of phytochromes in shade allows accumulation of PIF transcription factors that
315 upregulate *YUCCA* transcription and a concomitant increase in auxin biosynthesis. Given this
316 model it has been something of a conundrum that multiple *yucca* mutants retain a significant
317 (albeit reduced) shade avoidance response, leaving open the possibility of a parallel, *YUCCA*-
318 independent pathway. By creating a multiple mutant that removes all of the shade-inducible
319 *YUCCA* genes we demonstrate that *YUCCAs* are essential for measurable shade avoidance
320 responses in the hypocotyl and also the petiole.
321
322

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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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514

Figure 1(on next page)

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)).

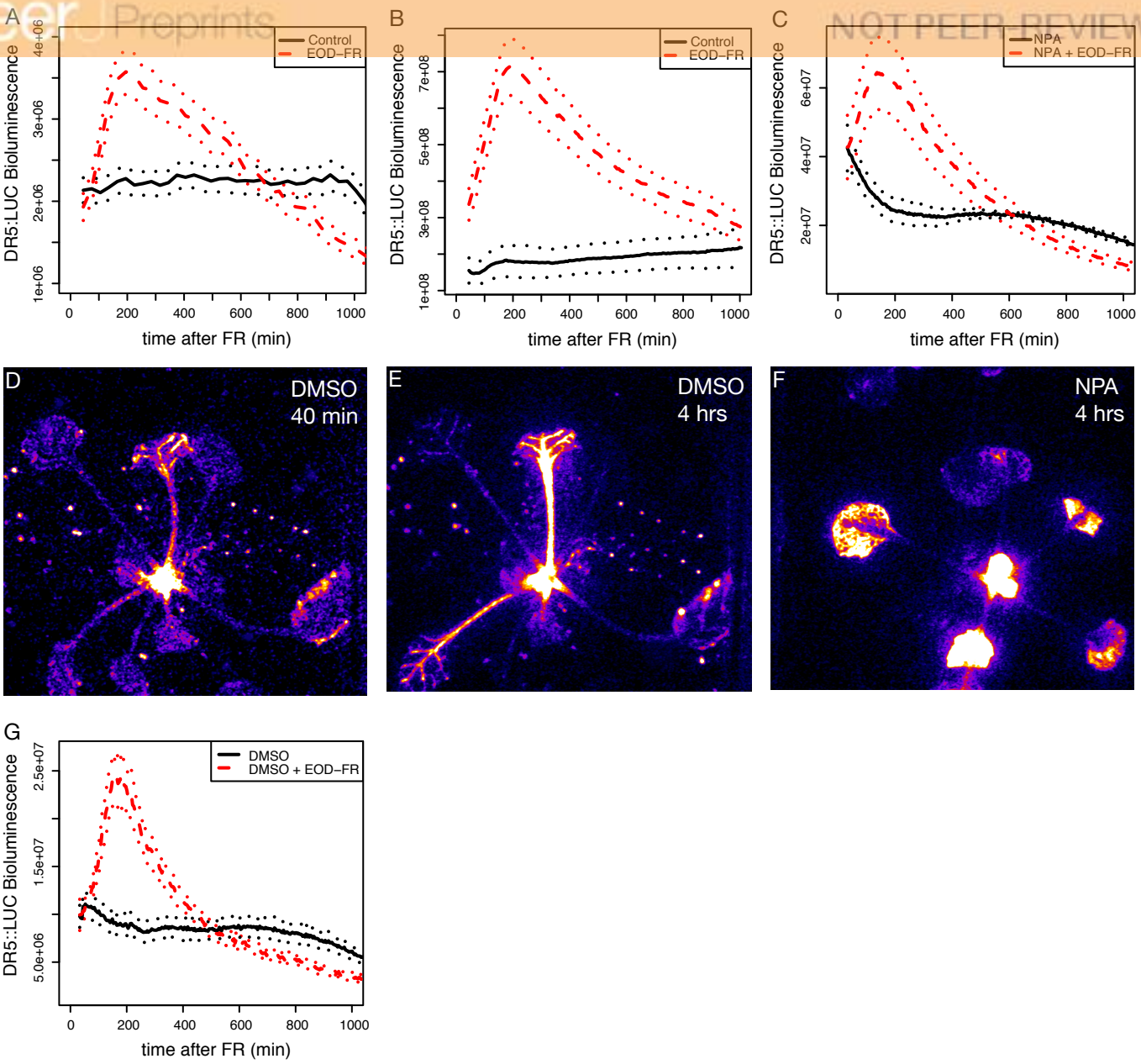

Figure 2(on next page)

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).

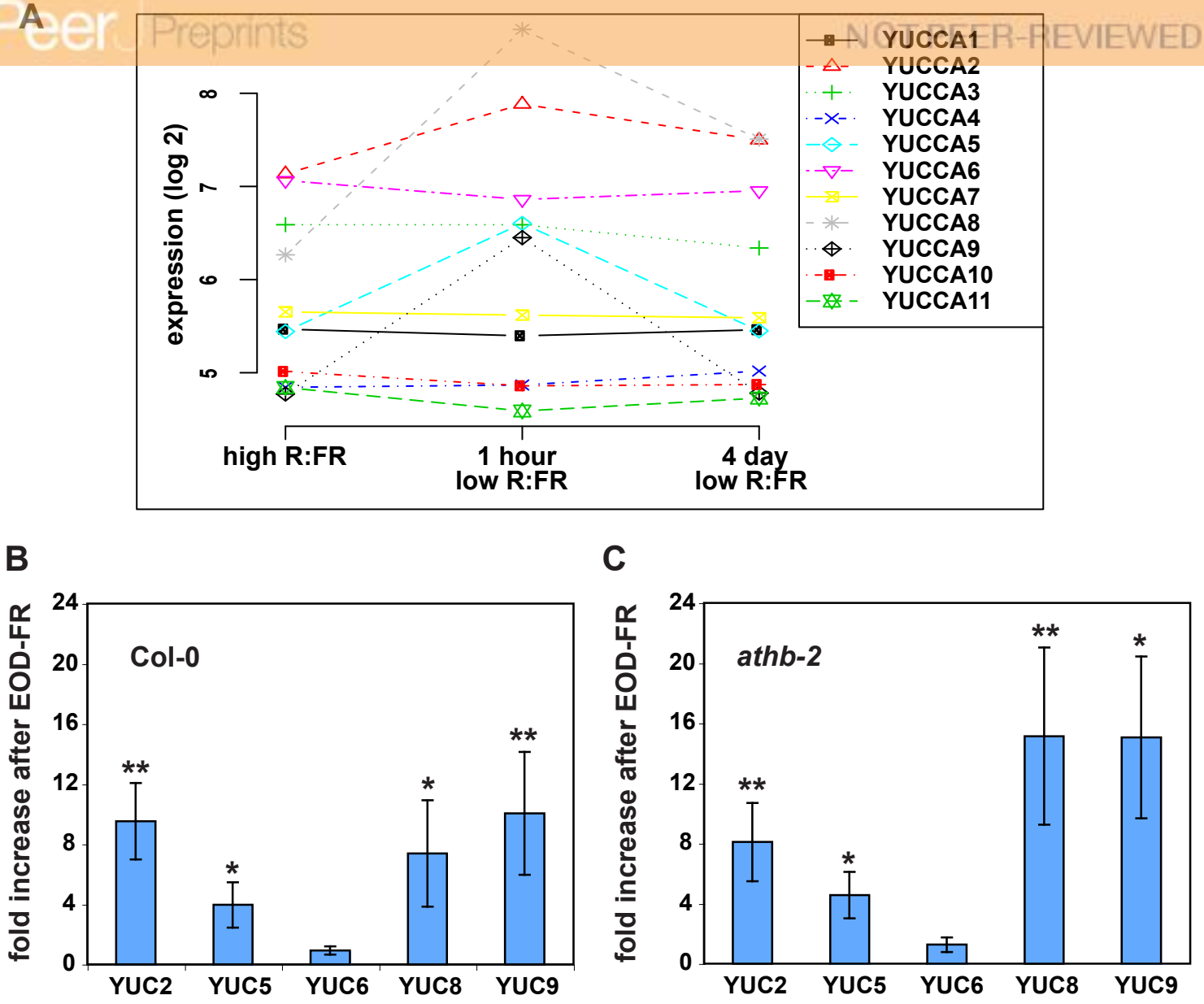

Figure 3(on next page)

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.

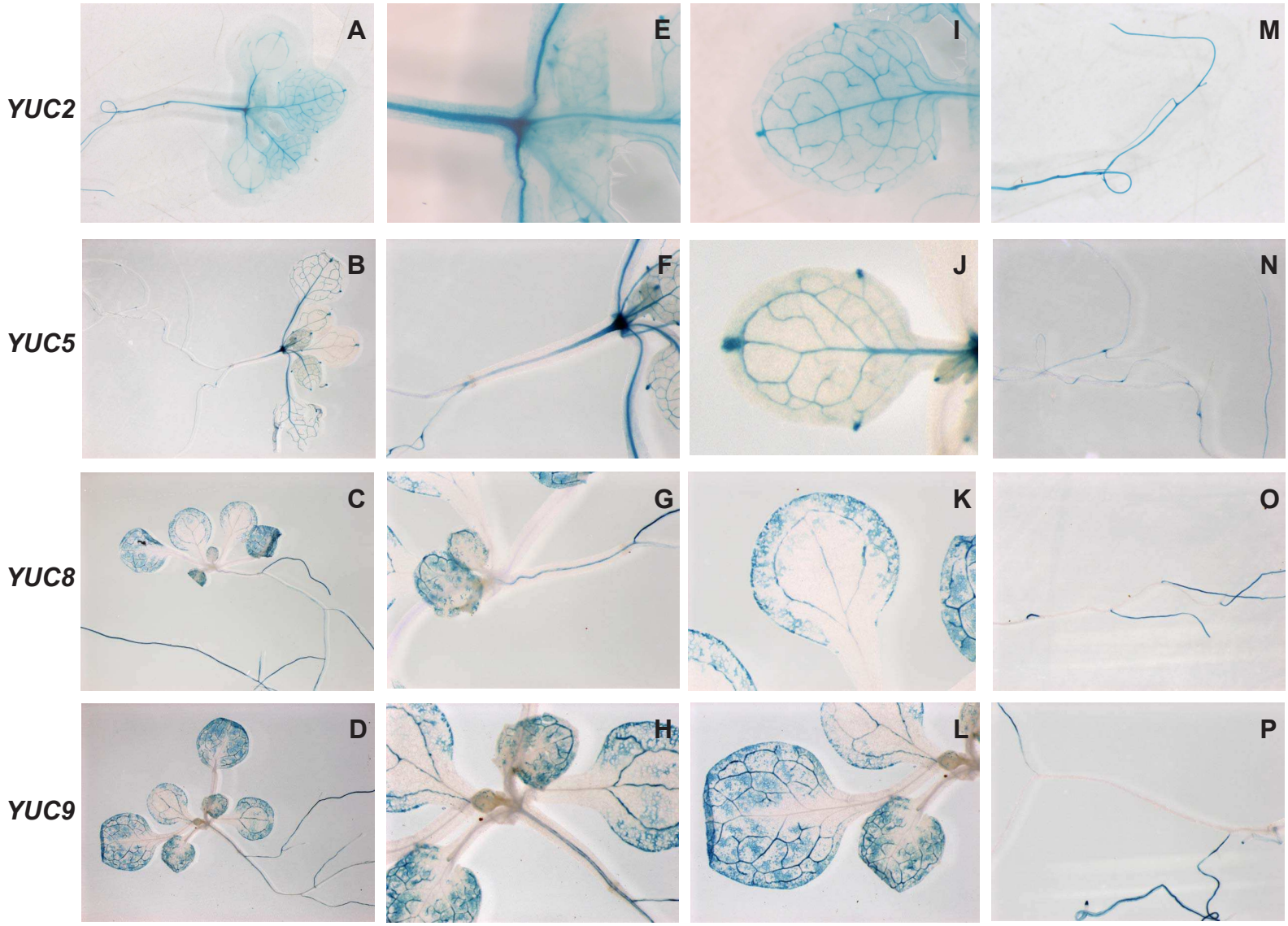

Figure 4(on next page)

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.

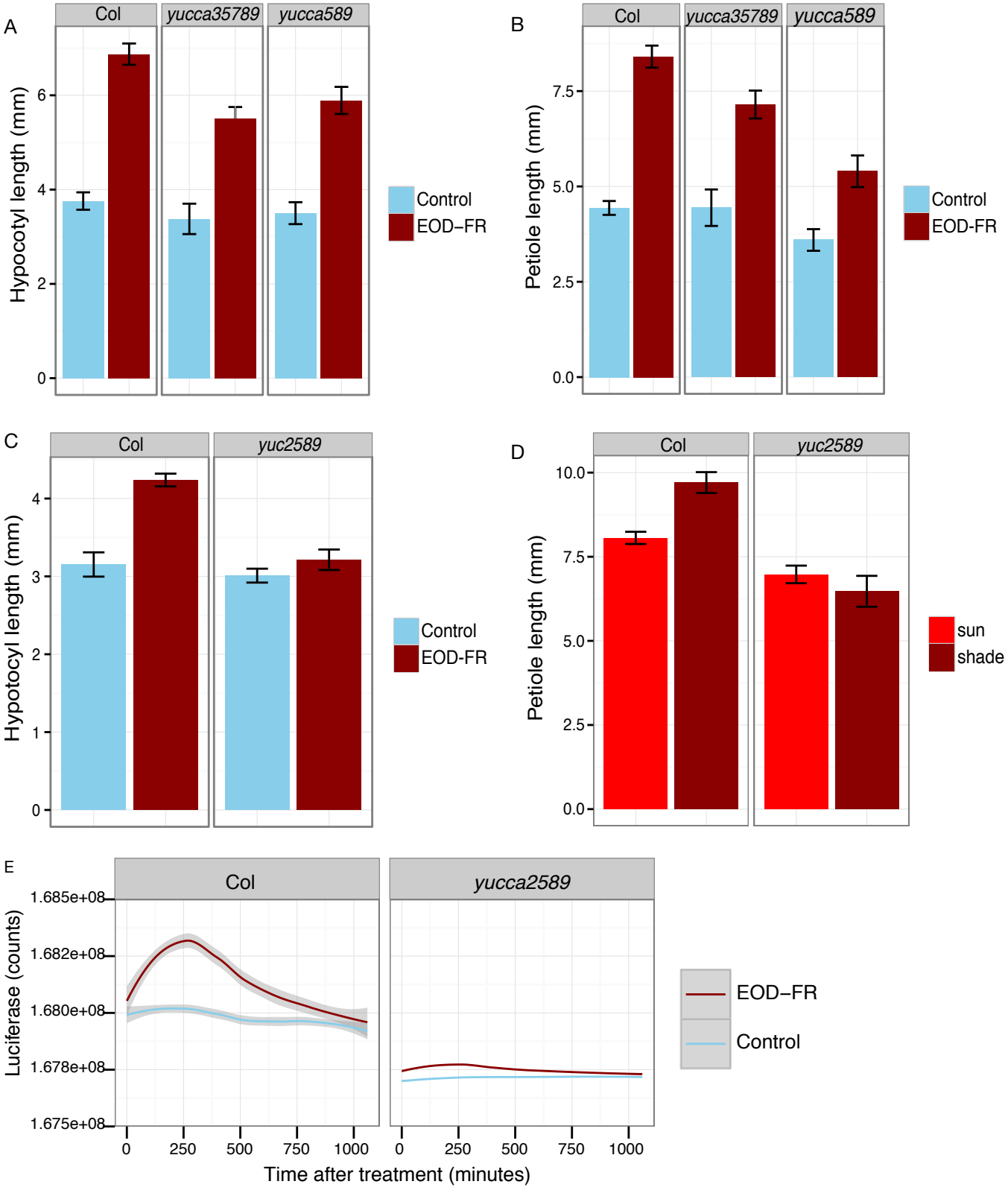

Figure 5(on next page)

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.

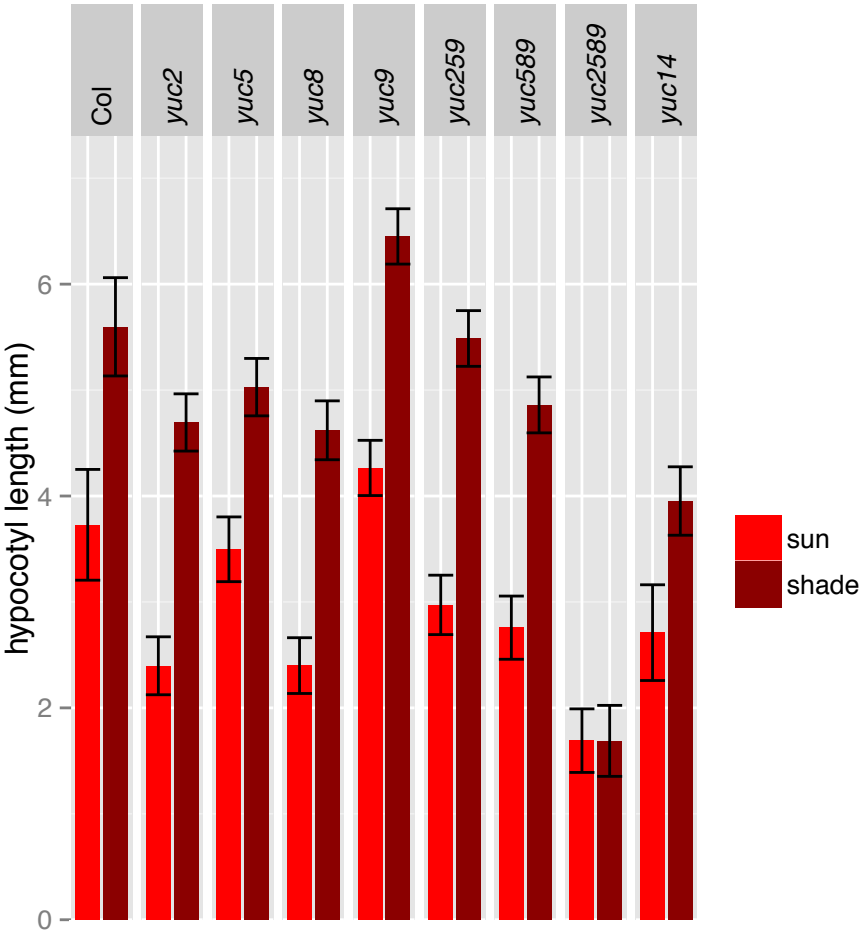

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Figure 6(on next page)

Adult wild-type and *yucca* mutant lines

The mutant lines did not show severe morphological defects, although some showed reduced fertility

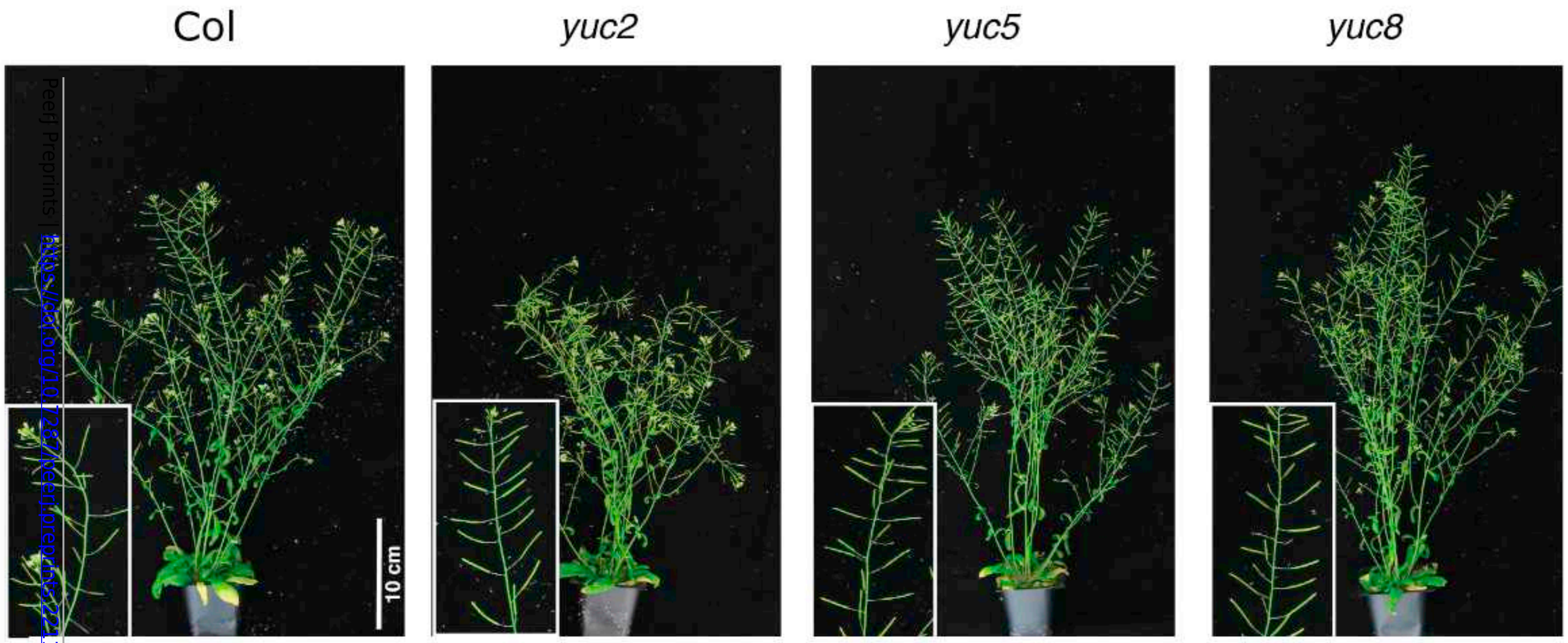

Table 1(on next page)

Table 1. PCR Primers

Table 1 PCR primers

Gene	Primer Type	Sequence	Final Concentration
AtHB-2	LBb1	GCGTGGACCGCTTGCTGCAACT	500 nM
AtHB-2	LP	TTGGTTGAAATAAAACGAAAAGTG	500 nM
AtHB-2	RP	CGTCACTGATTCCTCTTGAGC	500 nM
AtHB-2	qPCR	ACATGAGCCCACCCACTAC	200 nM
AtHB-2	qPCR	GAAGAGCGTCAAAAGTCAAGC	200 nM
PP2a	qPCR	TAACGTGGCCAAAATGATGC	200 nM
PP2a	qPCR	GTTCTCCACAACCGCTTGGT	200 nM
YUC2	qPCR	ACCCATGTGGCTAAAGGGAGTGA	900 nM
YUC2	qPCR	AATCCAAGCTTTGTGAAACCGACTG	300 nM
YUC3	qPCR	CGTCCCTTCATGGCTTAAGGACAAC	900 nM
YUC3	qPCR	GACGCACCAAACAATCCTTTTCTCG	50 nM
YUC5	qPCR	ATGATGTTGATGAAGTGGTTTCCTCTG	300 nM
YUC5	qPCR	ATCAGCCATGCAAGAATCAGTAGAATC	300 nM
YUC6	qPCR	GAGACGCTGTGCACGTCCTA	300 nM
YUC6	qPCR	AGTATCCCCGAGGATGAACC	300 nM
YUC8	qPCR	ATCAACCCTAAGTTCAACGAGTG	50 nM
YUC8	qPCR	CTCCCGTAGCCACCACAAG	300 nM
YUC9	qPCR	TCTCTTGATCTTGCTAACCACAATGC	300 nM
YUC9	qPCR	CCACTTCATCATCATCACTGAGATTCC	50 nM