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The shoot regeneration capacity of excised Arabidopsis cotyledons is established during the initial hours after injury and is modulated by a complex genetic network of light signalling

BLAIR NAMETH, STEVEN J. DINKA, STEVEN P. CHATFIELD, ADAM MORRIS, JENNY ENGLISH, DORRETT LEWIS, ROSALINDA ORO & MANISH N. RAIZADA

Department of Plant Agriculture, University of Guelph, Guelph, Ontario, Canada N1G 2W1

ABSTRACT

Excised plant tissues (explants) can regenerate new shoot apical meristems *in vitro*, but regeneration rates can be inexplicably variable. Light affects rates of shoot regeneration, but the underlying mechanisms are poorly understood. Here, excised Arabidopsis cotyledons were dark–light shifted to define the timing of explant light sensitivity. Mutants and pharmacological agents were employed to uncover underlying physiological and genetic mechanisms. Unexpectedly, explants were most light sensitive during the initial hours post-excision with respect to shoot regeneration. Only ~100 μmol m^{-2} s^{-1} of fluorescent light was sufficient to induce reactive oxygen species (ROS) accumulation in new explants. By 48 h post-excision, induction of ROS, or quenching of ROS by xanthophylls, increased or decreased shoot regeneration, respectively. Phytochrome A-mediated signalling suppressed light inhibition of regeneration. Early exposure to blue/UV-A wavelengths inhibited regeneration, involving photoreceptor CRY1. Downstream transcription factor HY5 mediated explant photoprotection, perhaps by promoting anthocyanin accumulation, a pigment also induced by cytokinin. Surprisingly, early light inhibition of shoot regeneration was dependent on polar auxin transport. Early exposure to ethylene stimulated dark-treated explants to regenerate, but inhibited light-treated explants. We propose that variability in long-term shoot regeneration may arise within the initial hours post-excision, from inadvertent, variable exposure of explants to light, modulated by hormones.

Key-words: adventitious shoot; anthocyanin; callus; HY5; photoreceptor; polar auxin transport; reactive oxygen species; somatic organogenesis; stem cell; xanthophyll.

INTRODUCTION

Excised plant tissues (explants) that have lost an apical meristem have the ability to regenerate new root and/or shoot apical meristems in the absence of sexual fertilization (Steeves & Sussex 1989; Birnbaum & Alvarado 2008; Duclercq *et al*. 2011; Sugimoto, Gordon & Meyerowitz 2011). If an explant gives rise to a root- or shoot-forming apical meristem without an intervening embryo phase, the process is termed somatic organogenesis. Somatic organogenesis is critical to the survival of many species in nature, but can also be induced *in vitro* using exogenous phytohormones. Unfortunately, *in vitro* organ regeneration suffers from considerable experimental variability, for reasons that are not well understood and which have slowed down the progress of *in vitro* plant stem cell research (Steeves & Sussex 1989).

Organ regeneration that occurs *in vitro* typically involves an intervening callus phase (Skoog & Miller 1957). Callus is classically hypothesized to be a necessary de-differentiation step prior to regeneration (Christianson & Warnick 1983). In *Arabidopsis thaliana* (Arabidopsis), callus formation can be stimulated by an initial low dose of cytokinin to trigger cell division along with a high dose of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D; Zhao, Fisher & Auer 2002). This auxin appears to be a poor substrate for auxin efflux carriers and tends to accumulate in cells instead of participating in polar transport (Estelle 1998; Petrasek *et al*. 2006), contributing to disorganized growth referred to as callus (Rahman *et al*. 2007). To stimulate subsequent shoot organogenesis, high levels of cytokinin are used along with low levels of 1-naphthaleneacetic acid (NAA), a form of auxin that can be transported out of cells directionally (Christianson & Warnick 1983). The initial 2,4-D-containing media is called callus induction media (CIM) and the subsequent, high cytokinin-containing media is called shoot induction media (SIM; Zhao *et al*. 2002).

Recent studies in Arabidopsis have begun to elucidate the molecular genetic mechanisms underlying adventitious organ regeneration *in vitro* (Ozawa *et al*. 1998; Banno *et al*. 2001; Baldwin, Kessler & Halitschke 2002; Hibara, Takada & Tasaka 2003; Gallois *et al*. 2004; Zhang & Lemaux 2004; Nishimura *et al*. 2005; Che *et al*. 2006; Decook *et al*. 2006). These results have been summarized (Birnbaum & Alvarado 2008; Duclercq *et al*. 2011; Sugimoto *et al*. 2011).

Correspondence: M. N. Raizada. Fax: +519 763 8933, e-mail: raizada@uoguelph.ca

Particularly interesting have been reports that callus, from which new shoots originate, is derived from root pericycle-like cells even in non-root organs such as cotyledons (Atta *et al*. 2009; Sugimoto, Jiao & Meyerowitz 2010). This discovery may explain some mechanistic aspects of the phytohormones commonly implicated in somatic organogenesis, auxin, cytokinin and ethylene (Skoog & Miller 1957; Chatfield & Raizada 2008). In intact seedlings, auxin triggers lateral root initiation, cytokinin inhibits lateral root formation, and ethylene has concentration-dependent positive and negative effects on lateral root production (Fukaki & Tasaka 2009).

The environment modulates shoot organogenesis. In particular, studies conducted in multiple plant species have demonstrated that week(s) of light or dark treatments after tissue excision can have profound effects on shoot regeneration (Dong *et al*. 2006). For example, Chaudhury & Signer (1989) reported that high light intensity reduced shoot regeneration from Arabidopsis explants *in vitro*. However, little is known about the mechanisms underlying light regulation of adventitious shoot meristem formation, though many potential mechanisms have been invoked, including light effects on signalling involving auxin (Jensen, Hangarter & Estelle 1998; Kimura & Kagawa 2006; Nishimura *et al*. 2006), cytokinin (Smets *et al*. 2005), ethylene (Saitou *et al*. 1993), red/far-red (R/FR) light photoactivation (Saitou *et al*. 1999, 2004; Saitou, Hashizume & Kamada 2000; Qin *et al*. 2005), blue/UV-A light photoactivation (Bertram & Lercari 2000; Hunter & Burritt 2004) and photo-oxidative damage (Dong *et al*. 2006).

In this study, we undertook light–dark shifting experiments to define when Arabidopsis cotyledon explants were most light-sensitive with respect to rates of shoot regeneration. We then used genetic mutants, pharmacological agents and indicator dyes to understand how light during the most sensitive time interval interacts with phytohormones, pigments, photoreceptors and photo-oxidative pathways, to regulate shoot regeneration. Unexpectedly, our survey suggests that an underlying cause of some of the variability between plant tissue culture experiments may be inadvertent light and/or dark exposure during the initial hours following tissue excision.

MATERIALS AND METHODS

Seed stocks

For natural variation and light-shifting assays (Figs 1, 2; Supporting Information Fig. S2), Arabidopsis wild-type ecotypes were obtained from Lehle Seeds (Round Rock, TX, USA): Col-0 (WT-2), DijG (WT-10), Est-1 (WT-6A), L*er*-0 (WT-4) and No-0 (WT-9). As controls for mutant and inhibitor studies (Figs 3–6; Supporting Information Figs S3–S5), wild-type seeds were obtained from the Arabidopsis Biological Resource Center (ABRC) Stock Center: L*er*-0 (CS20), Ws2 (CS2360/CS22659) and C24 (CS906). The homozygous CaMV35S-IPT161 cytokinin-overproducing line (pCYT::IPT, CS117; C24 background, CS906) was obtained from ABRC. The quadruple blue mutant [*cry1cry2phot1phot2,* line 210; mixed L*er*-0 (CS20)/Ws2 background (CS2360/CS22659)] was a gift from the Sakai Lab (Ohgishi *et al*. 2004). All other mutants were also obtained from ABRC and were in a L*er*-0 (CS20) background: *phyA-201* (CS6219), *phyA-203* (CS6221), *phyB-1* (CS6211), *hy1-1* (CS67), *cry1* (*hy4-1*, CS70), *hy5-1* (CS71), *tt4-1* (CS85) and *npq1-2* (CS3771). *phyA-203* was only used for the anthocyanin test; all other experiments used *phyA-201*.

Germination conditions

Seeds were surface sterilized as follows: 15% bleach for 15 min, rinsed in sterile water then 70% ethanol for 30 s, and finally rinsed five times in sterile water. Sterilized seeds were then cold treated at ~4 °C for 2–7 d (ecotype dependent) in sterile water for stratification. Seeds were then resuspended in 0.1% agar and plated onto germination media consisting of 2.2 g L^{-1} (half-strength) MS Basal Media with Gamborg's Vitamins (Sigma M0404; Sigma, St Louis, MO, USA), 0.97 g L^{-1} 2-(N-Morpholino) ethanesulfonic acid (MES) (Sigma MES2933), 10 g L^{-1} sucrose (1% final), at a pH of 5.7 with KOH, and 3 g L^{-1} Phytagel (Sigma, P8169) in 100 × 25 mm Petri dishes. Seeds were plated onto a grid consisting of 26 evenly spaced spots around a 100 mm diameter circle to create uniform growth environments for each explant. All plates were sealed with Micropore™ (3M Corp, St Paul, MN, USA) surgical tape (3M, 1530-1). The germination growth conditions were 24 h constant light (Cool White fluorescent lamps at 50–80 μmol m^{-2} s^{-1}) at 25 °C for 6–7 d under ambient indoor conditions in a room lacking natural sunlight.

Regeneration assay and analysis

Using fine forceps, cotyledons were severed at the base of the blade (excluding petiole) at 6 d post-germination unless otherwise noted. Detached cotyledons were placed first on to CIM (CIM pretreatment, default) or if noted, directly on to SIM, in 100 × 25 mm Petri dishes using the grid procedure noted earlier. SIM media (Zhao *et al*. 2002) consisted of 20 g L^{-1} glucose (2% final), 0.5 g L^{-1} MES (Sigma MES2933) with normal strength Gamborg's B5 Basal Media with Minimal Organics (Sigma, G5893) and 3 g L^{-1} Phytagel (Sigma, P8169; pH 5.8 with KOH), in addition to 4.4 μM N^6-(Δ^2-isopentenyl)adenine (2-iP; Sigma, D7674) and 0.5 μM 1-naphthaleneacetic acid (NAA; Sigma, N0640). The hormones in CIM media were substituted with 0.1 mg L^{-1} Kinetin (Sigma, K1885) and 0.5 mg L^{-1} 2,4-D (Sigma, D6679). The hormones were added after the media had been autoclaved and cooled to 55 °C. Unless otherwise noted, the default regeneration conditions were 5 d on CIM, SIM for 10 d and then fresh SIM for the remaining 3 weeks. Post-excision regeneration employed continuous fluorescent light (Sylvania CW/VHO; Osram Sylvania Ltd, Mississauga, ON, Canada) at the light levels and durations

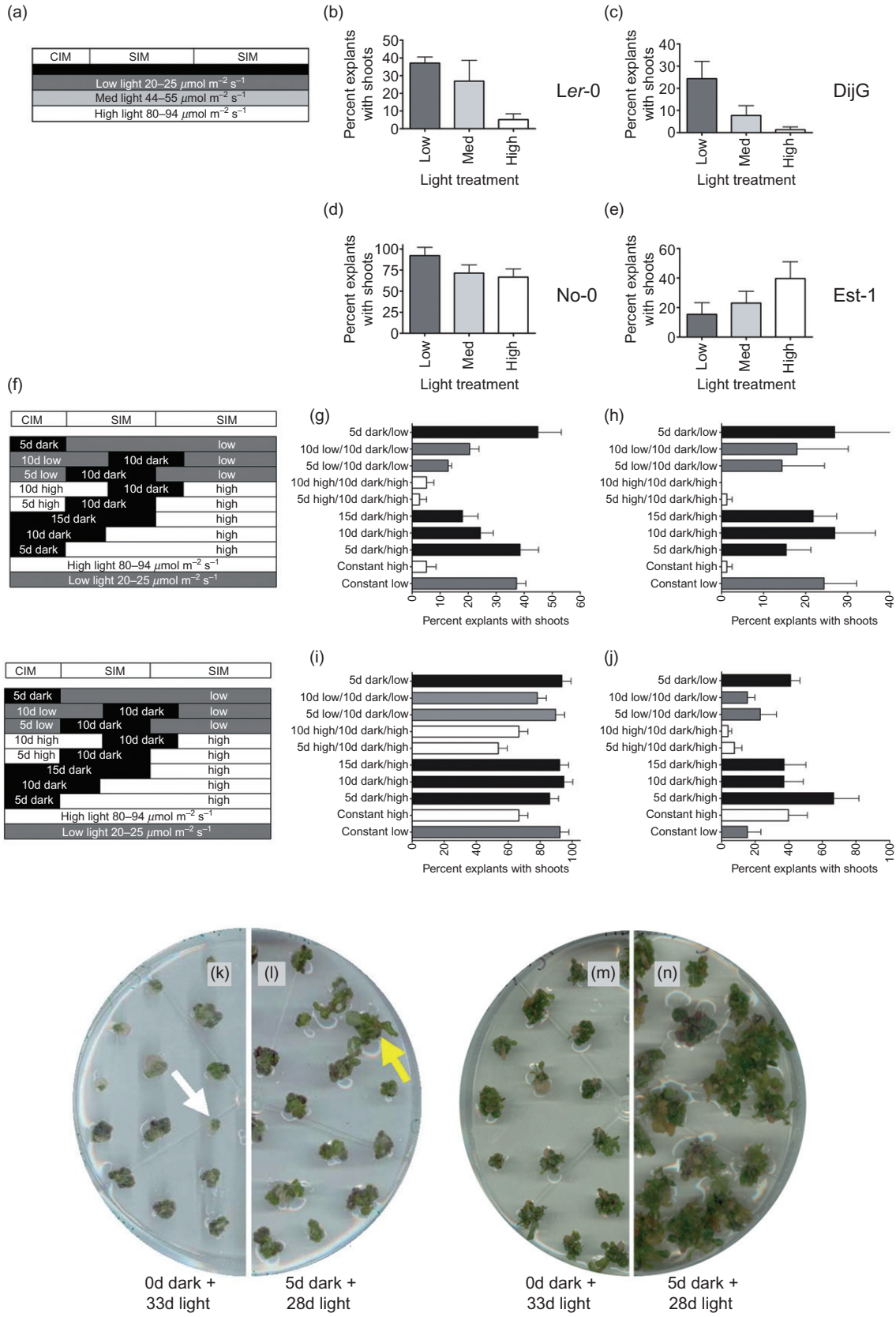

(a)
CIM
SIM
SIM
Low light 20–25 μmol m−2 s−1
Med light 44–55 μmol m−2 s−1
High light 80–94 μmol m−2 s−1
(b)
Percent explants with shoots
Low
Med
High
Light treatment
Ler-0
(c)
DijG
(d)
No-0
(e)
Est-1
(f)
5d dark
low
10d low
10d dark
5d high
15d dark
high
(g)
5d dark/low
10d low/10d dark/low
5d low/10d dark/low
10d high/10d dark/high
5d high/10d dark/high
15d dark/high
10d dark/high
5d dark/high
Constant high
Constant low
Percent explants with shoots
(h)
(i)
(j)
(k)
(l)
(m)
(n)
0d dark + 33d light
5d dark + 28d light

Figure 1. The light intensity experienced by Arabidopsis cotyledon explants during the first 5 d after excision determines the long-term frequency of shoot regeneration. (a, f) Shown are the media [callus induction media (CIM); shoot induction media (SIM)] and light treatment schemes. For all results shown, explants (6–7 d post-germination) were placed on CIM plates for 5 d, then SIM for 10 d followed by fresh SIM for the remaining ~3 weeks. (a–e) Excised cotyledons were placed under constant light intensities as shown in panel (a) for ~4 weeks. Shown are the percentage of cotyledon explants that regenerated shoots 3–5 weeks after excision for (b) L*er*-0, (c) DijG, (d) No-0 and (e) Est-1. (f–n) In a subsequent experiment, explants were exposed to complex light treatments as indicated in panel (f). Shown are the percentage of cotyledon explants that regenerated shoots 4 weeks after excision for (g) L*er*-0, (h) DijG, (i) No-0 and (j) Est-1. For (g–j), the histograms are shaded according to the light level experienced during the first 5 d following excision. Explants for (k,l) L*er*-0 and No-0 (m,n) treated with (k,m) 5 d of high light or (l,n) 5 d of darkness, both followed by continuous high light. The white arrow points to an explant that has regenerated poor callus under early post-excision high light. The yellow arrow shows an example of proliferative shoot regeneration under early post-excision darkness. The ecotypes shown are from Lehle Seeds: DijG (WT-10), Est-1 (WT-6A), L*er*-0 (WT-4) and No-0 (WT-9). See Supporting Information Table S1 for all related statistical information. The error bar represents the standard error of the mean (SEM). Each histogram is the mean of three replicates ($n = 26$ per replicate; experimental $n = 4992$ cotyledons).

indicated for each experiment at a constant temperature of 23 °C in a Conviron growth chamber (Conviron, Winnipeg, MB, Canada) with 50% relative humidity. Plates were sealed with Micropore™ surgical tape (3M, 1530-1).

Uniformly sized cotyledons were selected for excision. Precociously regenerating shoots, if observed 1 week post-excision, were removed from the experiment as these were presumed to contain residual shoot apical meristem cells. Petri dishes were continuously randomized. All studies were conducted in a laboratory lacking windows and care was taken never to expose tissues to sunlight during transport. Hormone-containing plates were stored at 4 °C for no more than 30 d before use.

Regeneration was scored 4–5 weeks post-injury for the number of explants with at least one shoot (minimum of two leaves), total number of shoots per plate and fresh weight (with roots dissected away, then washed in water). Statistical significance between genotypes or treatments was determined using an unpaired *t*-test (for initial light treatments) or the Mann–Whitney test for all subsequent mutant/filter/pharmacological treatments (InStat 3.0, GraphPad Software, La Jolla, CA, USA; Supporting Information Tables S1–S2).

Light treatments and measurements

Light output was measured at plate level using a photosynthetic photon flux meter in the photosynthetically active radiation (PAR) range of 400–700 nm (Apogee Instruments, Logan, UT, USA, Lehle BQM-01). All experiments used cool white fluorescent lamps (F72T12CW/VHO, Sylvania, USA). Unless otherwise indicated, excised cotyledons were exposed to 24 or 48 h of darkness, respectively, or continuous high light (90–120 μmol $m^{-2} s^{-1}$) and then 4–5 weeks of continuous high light.

Blue/UV-A-deficient light filter (BDF) experiment

The blue/UV-A-deficient filter (BDF) experiment used a yellow acetate filter (LEE 101, LEE Filters, Burbank, CA, USA) placed under fluorescent bulbs (F72T12CW/VHO, Sylvania, USA) to remove nearly all light below 450 nm, with the greatest reduction from 450 to 530 nm. Control tissues were placed under unfiltered fluorescent bulbs. Following 5 d of blue/UV-A-deficient or control light, all tissues were exposed to fluorescent light for the remaining 4 weeks. Light output was normalized to 60 μmol $m^{-2} s^{-1}$ or 100 μmol $m^{-2} s^{-1}$ (as indicated) measured at plate level using a photosynthetic photon flux meter in the range of 400–700 nm (Apogee Instruments USA, Lehle BQM-01).

Chemical inhibitor/elicitor treatments

The final concentration and respective buffer used for each inhibitor or elicitor were as follows: 12.5–50 μM 1-N-naphthylphthalamic acid (NPA, Chem Service, PS-343), West Chester, PA, USA in dimethyl sulfoxide (DMSO); 1 μM 1-aminocyclopropane-1-carboxylic acid (ACC, Research Organics, 1373A, Cleveland, OH, USA) in ddH_2O; 75 μM norflurazon (NF, Syngenta Crop Protection Inc., SAN9789A, Greensboro, NC, USA) in ethanol; 0.5 mM dithiothreitol (DTT, Fisher, BPI 172-5, Waltham, MA, USA) in ddH_2O; 20 μM $AgNO_3$ (Sigma, S-6506) in ddH_2O; and 50 nM Paraquat (active ingredient methyl viologen; Sigma, M-2254) in ddH_2O. Inhibitors were filter-sterilized and added to CIM media after autoclaving. Unless otherwise noted, 6-day-old post-germination cotyledons were detached and placed on to CIM media containing either the chemical inhibitor/elicitor or appropriate buffer for 24 or 48 h (as noted) accompanied with either 24 or 48 h, respectively, of darkness or continuous fluorescent high light (90–120 μmol $m^{-2} s^{-1}$). Tissues were transferred on to CIM (lacking chemicals) for 3 d, then to SIM for 10 d and then on to fresh SIM for the remaining 3 weeks, all under continuous fluorescent light (90–120 μmol $m^{-2} s^{-1}$, unless otherwise noted).

Nitroblue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB) staining

Seven-day-old cotyledons were excised using a razor blade and then stained as previously described (Fryer *et al*. 2002). The cotyledons were pre-soaked in 6 mM NBT (Biosynth, N-8100, Itasca, IL, USA), 5 mM DAB (Aldrich, D12384,

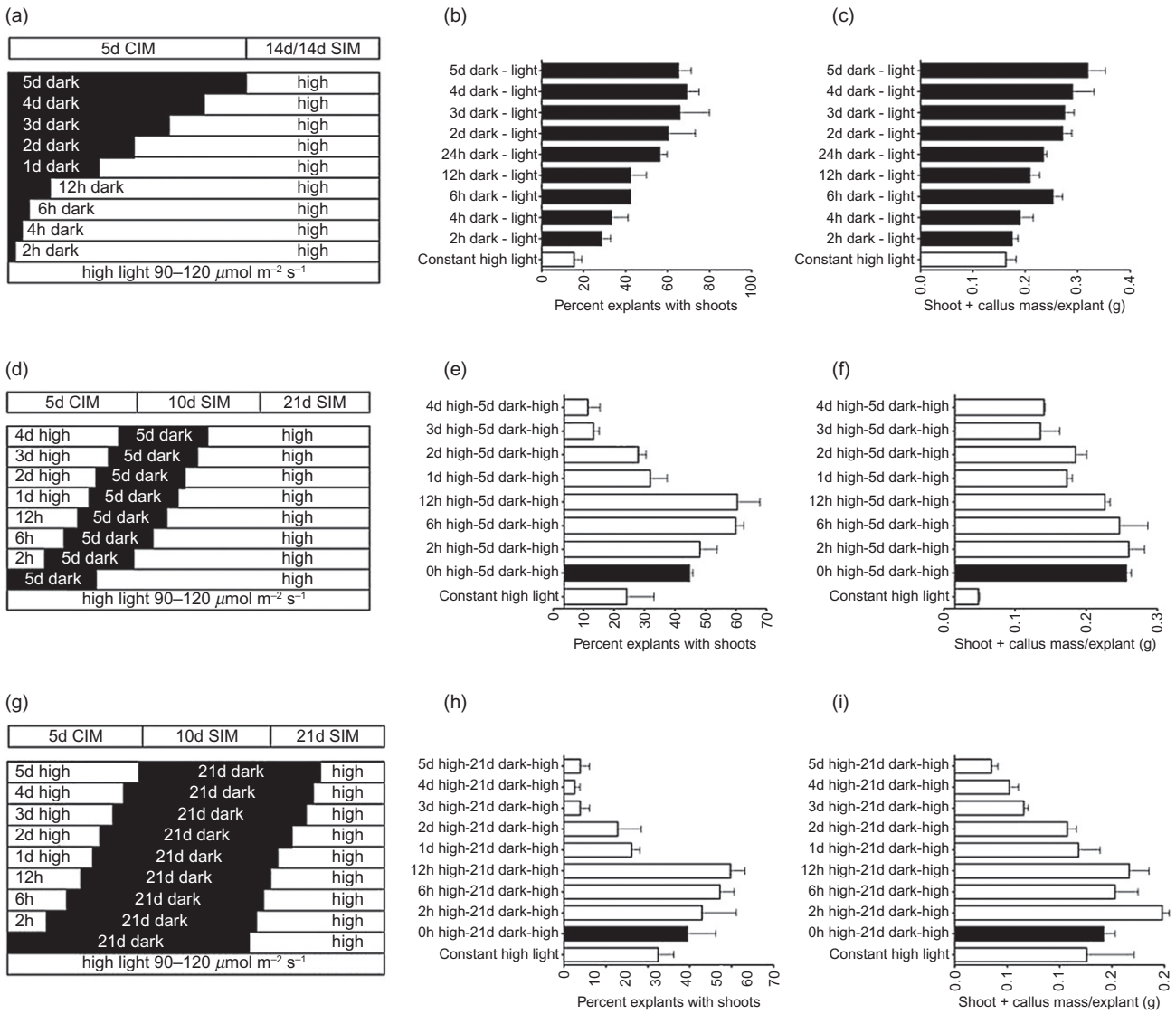


Figure 2. Exposure of cotyledon explants (ecotype L*er*-0) to darkness or light during the first 24 h after excision is critical for long-term shoot regeneration. (a,d,g) Shown are the media type [callus induction media (CIM); shoot induction media (SIM)] and light–dark shifting treatment schemes. For all experiments shown, explants (6–7 d post-germination) were placed on CIM plates for 5 d, then SIM for 10 d followed by fresh SIM for the remaining ~3 weeks. (b,e,h) Shown are the corresponding percentages of cotyledon explants that regenerated shoots 4 weeks after excision, and (c,f,i) the corresponding dissected fresh weight of callus and regenerated shoots (no roots). L*er*-0 seed was from Lehle Seeds (WT-4). See Supporting Information Table S1 for all related statistical information. The error bar represents the standard error of the mean (SEM). Each histogram is the mean of three replicates ($n = 26$ per replicate; experimental $n = 1492$ cotyledons).

Sigma-Aldrich, St Louis, MO, USA) for 1 h under darkness and then placed on CIM under continuous fluorescent light (100 μmol m^{-2} s^{-1}) or darkness for 0 h, 1 h, 2 h, 6 h, 24 h or 2 d. Tissues were then fixed by infiltrating with lacto-glycerol-ethanol (1:1:4 per volume), cleared with 95% ethanol, and imaged using a Sony DXC-905P camera (Sony Corp, Tokyo, Japan) mounted on a Zeiss Axiophot ID/CZC-M0213 microscope (Carl Zeiss AG, Oberkochen, Germany) using Northern Eclipse software (Empix Imaging Inc, Mississauga, ON, Canada).

Evans Blue staining

Seven-day-old cotyledons were excised using a razor blade and stained as previously described (Liu *et al*. 2008). The cotyledons were placed on to CIM under continuous fluorescent light (100 μmol m^{-2} s^{-1}) or darkness for 0 h, 1 h, 2 h, 6 h, 24 h or 5 d and then stained with 0.1% Evans Blue (Aldrich, 206334) w/v for 15 min. Tissues were rinsed with ddH_2O, cleared with 95% ethanol and imaged using a Sony DXC-905P camera mounted on a Leica MZ8

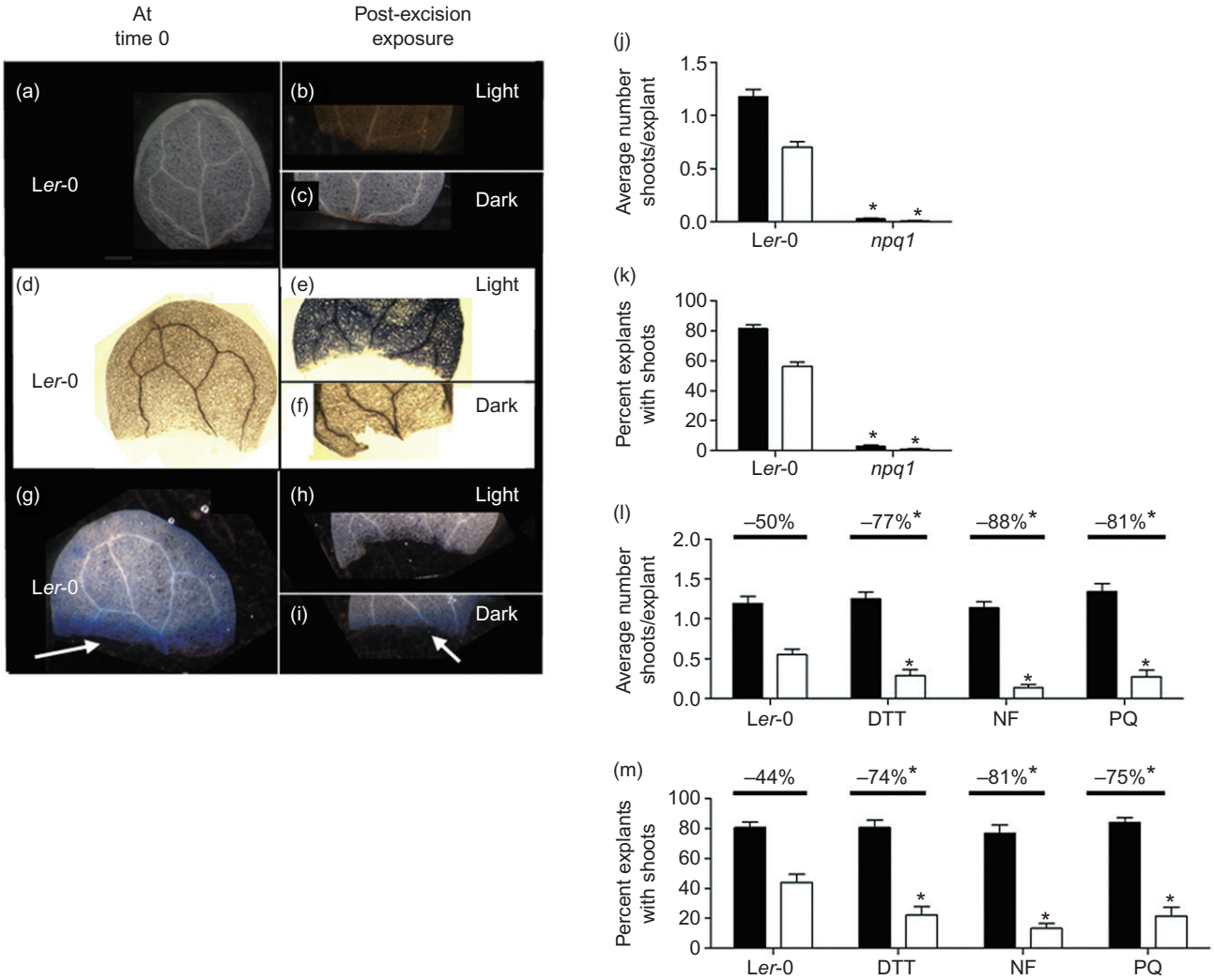


Figure 3. In cotyledon explants of ecotype L*er*-0, 100 μmol m^{-2} s^{-1} of fluorescent light exposure immediately after excision causes rapid reactive oxygen species (ROS) accumulation, while shoot regeneration is reduced by loss of photoprotective chloroplast xanthophyll pigments and increased accumulation of ROS. (a–i) The effects of ~100 μmol m^{-2} s^{-1} of fluorescent light on post-excised L*er*-0 cotyledons (CS20) were determined using indicator dyes. (a–c) 3,3-diaminobenzidine (DAB) staining for hydrogen peroxide indicated by brown pigment: tissue was assayed at (a) time of excision, (b) after 6 h light, (c) after 6 h darkness. (d–f) Nitroblue tetrazolium (NBT) staining for superoxide indicated by blue pigment: tissue was assayed at (d) time of excision, (e) after 6 h light, (f) after 6 h darkness. (g–i) Evans Blue staining for cell death, indicated by blue pigment. L*er*-0 was assayed at (g) time of excision, (h) after 5 d light, (i) after 5 d darkness. (j, k) Compared to wild-type L*er*-0 (CS20), the effect of a mutant, *non-photochemical quenching 1* (*npq1-2*, CS4771, L*er*-0 background) that lacks chloroplast zeaxanthin (carotenoid xanthophyll) pigment on (j) average number of regenerated shoots/explant and (k) percent explants with shoots, scored 4 weeks following excision. Cotyledons were exposed to either darkness (black bars) or high light (~100 μmol m^{-2} s^{-1}, white bars) for 24 h after excision, then both treated for another 4 weeks with continuous high light. (l,m) Effects of chemical inhibitors or elicitors of photo-oxidative stress pathways on (l) the average number of regenerated shoots/explant and (m) the percent explants with shoots in ecotype L*er*-0 (CS20). The inhibitors were dithiothreitol (DTT, 0.5 mM), which blocks NPQ1 and hence has reduced chloroplast carotenoid zeaxanthin pigment; norflurazon (NF, 75 μM), which blocks phytoene desaturase and hence has reduced carotenoid pigments; and paraquat (PQ, 50 mM), which causes an increase in chloroplast reactive oxygen species (ROS). The chemicals in (l,m) were applied for a duration of 48 h immediately after cotyledon excision with simultaneous exposure to either darkness (black bars) or high light (white bars) for 48 h after excision; the explants were then treated for 4 weeks with continuous high light without chemicals. For (j–m), explants (6–7 d post-germination) were placed on callus induction media (CIM) plates for 5 d, then shoot induction media (SIM) for 10 d followed by fresh SIM for the remaining ~3 weeks. For (j–m) panels, an asterisk above a histogram bar indicates that the rate of shoot regeneration of mutant or chemically treated explants is statistically different compared to the control, of the corresponding early light or dark treatment ($P < 0.05$; see Supporting Information Table S2). For (l,m) panels, the value shown above each horizontal line indicates the percent decline in shoot regeneration of explants exposed to light early after excision, compared to explants initially exposed to darkness; an asterisk besides the percentage value indicates that the decline is significantly different ($P < 0.05$) compared to the control (L*er*-0). See Supporting Information Table S2 for statistical analysis. The error bar represents the standard error of the mean (SEM). Each histogram is the mean of 5–18 replicates (26 cotyledons per replicate). Total experimental $n = 4446$ cotyledons.

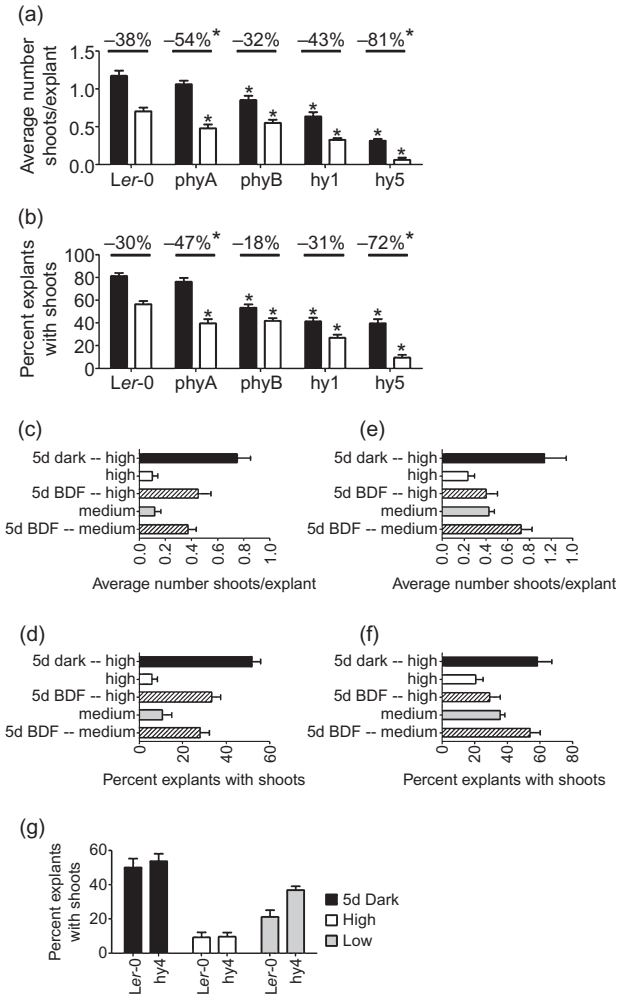


Figure 4. The effect of light quality during the initial days post-excision on long-term rates of shoot regeneration. (a,b) Shown are shoot regeneration rates for wild-type L*er*-0 (CS20) compared to phytochrome mutants [*phyA-201* (CS6219), *phyB-1* (CS6211), *hy1-1* (CS67), *hy5-1* (CS71)] for (a) the average number of regenerated shoots/explant, and (b) the percent explants with shoots. Explants were exposed to either darkness (black bars) or high light (~100 μmol m^{-2} s^{-1}, white bars) for 24 h after excision, then treated for another 4 weeks with continuous high light. Shoot regeneration was scored ~4 weeks after excision. (c–f) Effects of removing blue/UV-A light using a blue deficient acetate filter (BDF) for the first 5 d after excision on: (c,d) wild-type ecotype Ws2 (CS2360/CS22659) and (e,f) wild-type ecotype L*er*-0 (CS20). For (c–f), explants were exposed to one of five different light treatments during the first 5 d post-excision, followed by continuous high light (100 μmol m^{-2} s^{-1}) for 4 weeks. The initial post-excision 5 d treatments were darkness (black), 100 μmol m^{-2} s^{-1} continuous blue-deficient light (white with diagonal lines), 60 μmol m^{-2} s^{-1} continuous blue-deficient light (grey with diagonal lines), 100 μmol m^{-2} s^{-1} high light (white) and 60 μmol m^{-2} s^{-1} light (grey). (g) Effect of post-excision light exposure on excised *cry1* (*hy4-1*, CS70) cotyledons versus wild-type L*er*-0 (CS20). Explants were exposed to either darkness for 5 d followed by high light (black bar), or continuous high light (white bar) or low light (grey bar). For all experiments, explants (6–7 d post-germination) were placed on callus induction media (CIM) plates for 5 d, then shoot induction media (SIM) for 10 d followed by fresh SIM for the remaining ~3 weeks. See the legend for Figure 3 for explanations of percentages, asterisks and error bars, and Supporting Information Table S2 for statistical analysis. Each histogram is the mean of 5–18 replicates for panels (a,b) and 7–8 replicates for panels (c–g; 26 cotyledons per replicate). The error bar represents the standard error of the mean (SEM). Total experimental n = 10 658.

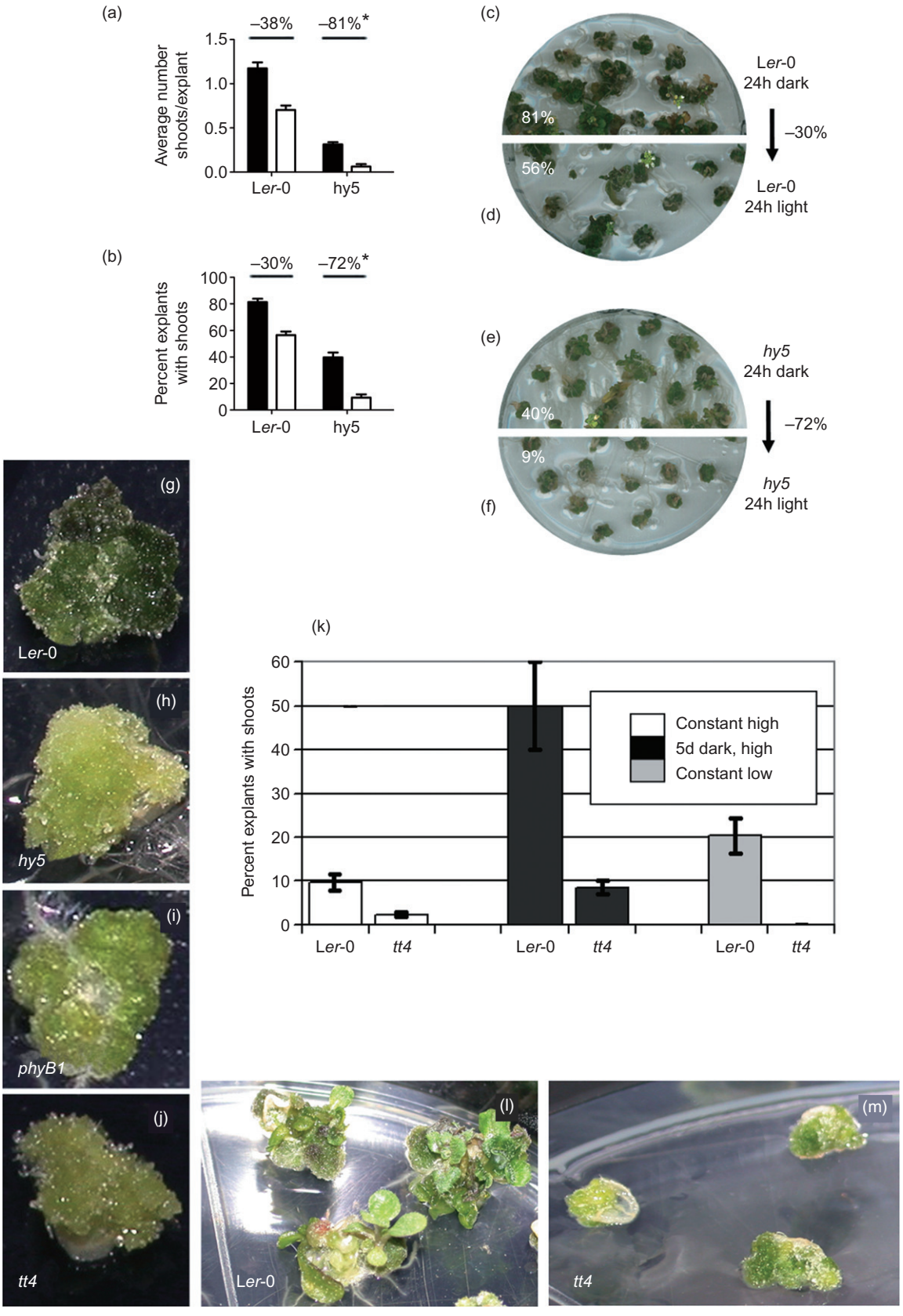
microscope (Leica Microsystems Inc, Wetzlar, Germany) using Northern Eclipse software.

RESULTS

Exposing explants to increasing light intensity decreases adventitious shoot regeneration in several Arabidopsis ecotypes

Following tissue excision, exposure of cotyledon explants to increasing intensities of light has been reported to decrease shoot regeneration in Arabidopsis (Chaudhury & Signer 1989). In an earlier natural variation survey of 60 ecotypes, we identified four ecotypes (L*er*-0, DijG, No-0, Est-1) with diverse responses to regeneration (E. Hewitt and M. Raizada, unpublished results). Cotyledon explants from these ecotypes were exposed to increasing quantities of continuous fluorescent light (Fig. 1). With the exception of Est-1, these ecotypes showed significantly lower shoot regeneration when their explants were exposed to continuous high light rather than low light (Fig. 1b–e; for statistical data, see Supporting Information Table S1). The intrinsic growth rate of an explant appeared to be epistastic to its

Figure 5. Loss-of-function mutants of transcription factor ELONGATED HYPOCOTYL 5 (HY5), and downstream genes that regulate anthocyanin accumulation, cause explants to be hypersensitive to light, resulting in reduced shoot regeneration. (a–f) Effect of the *hy5-1* mutation (CS71) on shoot regeneration compared to wild-type L*er*-0 (CS20), shown graphically (a,b) and by scans of representative 4-week post-excision plates (c–f). Cotyledons were exposed to either darkness (black bars) or high light (~100 μmol m^{-2} s^{-1}, white bars) for 24 h post-excision, then exposed to continuous high light for 4 weeks. See the legend for Figure 3 for explanations of percentages, asterisks and error bars, and Supporting Information Table S2 for statistical analysis. For (a,b) each histogram is the mean of 17 replicates (26 cotyledons per replicate). (g–j) *hy5-1* (CS71) and *phyB-1* (CS6211) mutant calli lack visible anthocyanin compared to wild-type L*er*-0 (CS20), similar to the *tt4-1* (CS85) mutant. (k–m) *tt4-1* (CS85) mutant explants lacking flavonoid anthocyanins have reduced rates of shoot regeneration compared to wild-type L*er*-0 (CS20). The legend indicates the post-excision light treatment. For (k), each histogram is the mean of 3–4 replicates/treatment (26 cotyledons/replicate). (l,m) Shown are explants 4 weeks after excision, exposed to 5 d of darkness after excision followed by 4 weeks of high light. For all experiments, explants (6–7 d post-germination) were placed on callus induction media (CIM) plates for 5 d, then shoot induction media (SIM) for 10 d followed by fresh SIM for the remaining ~3 weeks. In addition, see Supporting Information Fig. S4. Total experimental n = 1712 cotyledons.


(a)
–38%
–81%*
Average number shoots/explant
1.5
1.0
0.5
0.0
Ler-0
hy5
(b)
–30%
–72%*
Percent explants with shoots
100
80
60
40
20
0
Ler-0
hy5
(c)
Ler-0
24h dark
81%
–30%
56%
Ler-0
24h light
(d)
(e)
hy5
24h dark
40%
–72%
9%
hy5
24h light
(f)
(g)
Ler-0
(h)
hy5
(i)
phyB1
(j)
tt4
(k)
Percent explants with shoots
60
50
40
30
20
10
0
Constant high
5d dark, high
Constant low
Ler-0
tt4
Ler-0
tt4
Ler-0
tt4
(l)
Ler-0
(m)
tt4

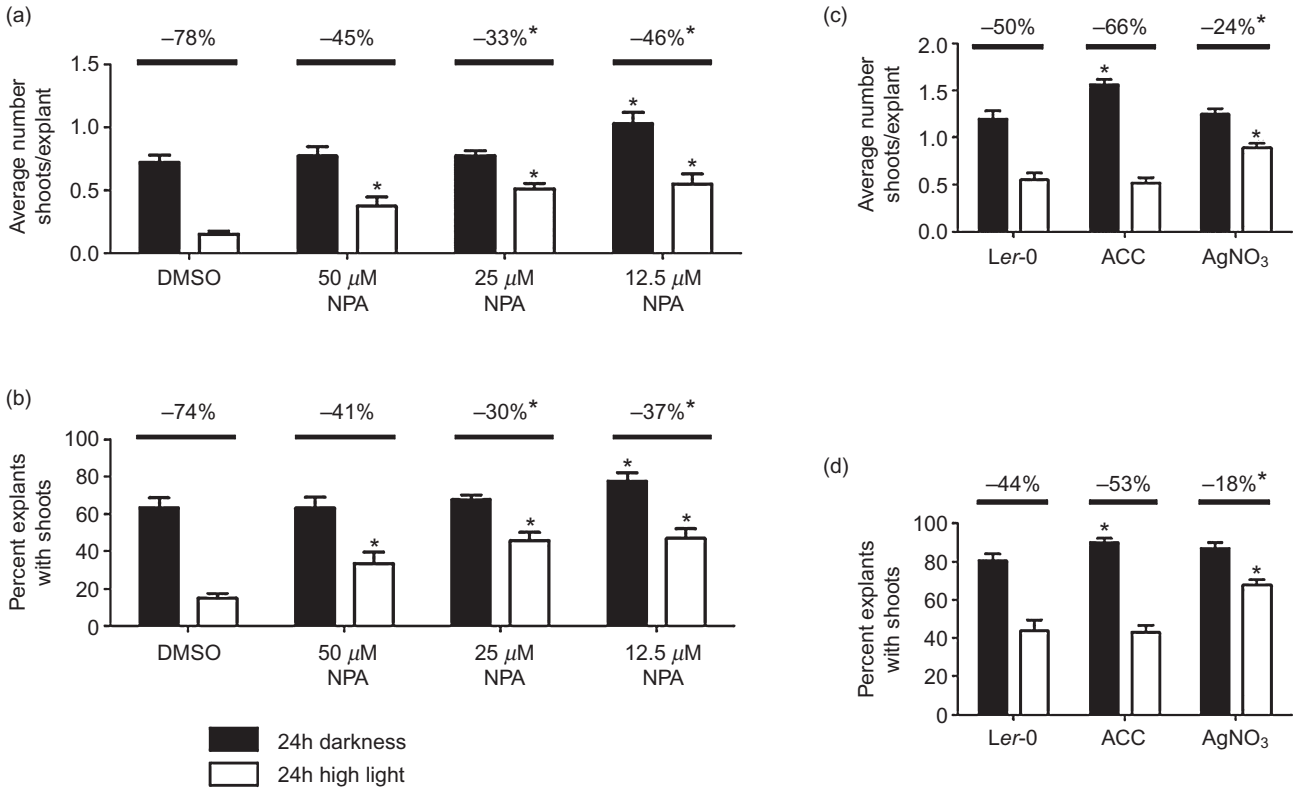


Figure 6. Auxin and ethylene modulate the light sensitivity of new explants with respect to long-term shoot regeneration. (a,b) Shown are the results of applying polar auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) onto 2,4-D-containing callus induction media (CIM) media for the first 24 h after excision using wild-type L*er*-0 (CS20) cotyledons. Cotyledons were exposed to either darkness (black bars) or high light (~100 μmol m^{-2} s^{-1}, white bars) for 24 h after excision, then exposed to continuous high light for another 4 weeks. (c,d) Shown are the results of supplementing CIM media with either the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC, 50 μM) to increase endogenous ethylene levels, or $AgNO_3$ (20 μM), which blocks ethylene signalling. The chemicals were added to CIM media for the first 48 h after excision using wild-type L*er*-0 (CS20) cotyledons. Cotyledons were exposed to either darkness (black bars) or high light (~100 μmol m^{-2} s^{-1}, white bars) for 48 h after excision, and then to continuous high light for 4 weeks. For all experiments, explants (6–7 d post-germination) were placed on CIM plates for 5 d, then shoot induction media (SIM) for 10 d followed by fresh SIM for the remaining ~3 weeks. See the legend for Figure 3 for explanations of percentages, asterisks and error bars, and Supporting Information Table S2 for statistical analysis. Each NPA histogram is the mean of 10 replicates (26 cotyledon cotyledons per replicate) and each ACC/silver nitrate histogram is the mean of 14–20 replicates (26 cotyledons per replicate). Total experimental $n = 4366$ cotyledons.

light response: No-0 explants, which were unusually fast growing, were high light sensitive at 3 weeks after excision, but then recovered (Fig. 1d, Supporting Information Table S1). By contrast, Est-1 explants, which were unusually slow growing, showed similar or improved shoot regeneration under high light, particularly when the total number of shoots per explant was visualized (Fig. 1e, Supporting Information Fig. S1).

The intensity of light exposure during the first 5 d following cotyledon excision determines the frequency of shoot regeneration

To determine if there are critical light intervals for shoot regeneration within the first 4 weeks post-excision, explants were exposed to 16 different light–dark shifting treatments, of which 10 informative treatments are shown (Fig. 1f–n). In L*er*-0 (Fig. 1g), DijG (Fig. 1h), No-0 (Fig. 1i) and surprisingly Est-1 (Fig. 1j), the frequency of shoot regeneration at 4 weeks post-excision was strongly influenced by the intensity of light exposure during the first 5 d after cotyledon excision. In these ecotypes, early high light exposure generally inhibited shoot regeneration, while early darkness promoted shoot regeneration across ecotypes (Fig. 1k–n, Supporting Information Table S1). Although No-0 appeared to be insensitive to early light/dark exposure when scored as the number of explants that regenerated shoots (Fig. 1i), visual inspection revealed that early light exposure caused a severe reduction in the total number of regenerated shoots per explant (Fig. 1m,n). This experiment demonstrates that during the prolonged 3–5 week shoot regeneration process, Arabidopsis cotyledon explants are most sensitive to light during the first 5 d after excision.

Early exposure to high light or later light–dark shifting prevents callus formation

Specific light treatments prevented callus formation from some cotyledon explants (Supporting Information Fig. S2). These cotyledons did not green, but simply lost chlorophyll and died, a dramatic phenotype. In general, exposure of explants to high light early after tissue excision prevented callus formation. Another type of light-shifting treatment especially prevented callus formation in three of the ecotypes: early exposure to high light, followed by darkness and then high light (Supporting Information Fig. S2a–e,i).

Darkness during the initial hours after excision promotes shoot regeneration

For all subsequent experiments, we focused on one ecotype, L*er*-0, due to the availability of relevant mutants in this background. Using L*er*-0 explants, a series of additional dark–light shift experiments were undertaken (Fig. 2a) to uncover any differences in light sensitivity within the critical first 5 d after organ excision. As few as 2–6 h of darkness immediately after excision were sufficient to cause a ~twofold increase in the shoot regeneration frequency (Fig. 2b, Supporting Information Table S1), even though the tissue was then transferred to high light for the remaining 4 weeks. Increasing the duration of darkness immediately after organ excision progressively increased the shoot regeneration frequency and increased the mass of callus and new shoots (Fig. 2c). No abrupt increases in the regeneration frequency were observed suggesting that light was not interacting with a discrete, short-lived signal. The significant benefit gained from darkness primarily occurred during the first 24 h after excision, leading to a ~threefold increase in shoot regeneration over that of continuous high light (Fig. 2b,c). These results show that light exposure during the immediate 2–24 h after cotyledon excision regulates long-term shoot regeneration in Arabidopsis ecotype L*er*-0.

Shoot regeneration is affected by 12 h intervals of light/dark exposure during the first day after organ excision

The reciprocal experiment was then conducted: cotyledon explants were first exposed to varying durations of light followed by a constant, extended dark period (Fig. 2d–f). A final return to light was necessary for shoot organogenesis. Exposing explants to ≤12 h of high light before the dark onset led to significant increases in the shoot regeneration frequency as scored 4 weeks later (Fig. 2e, Supporting Information Table S1). However, if high light continued for an additional 12 h before the dark onset (24 h total), then there was a twofold decrease in shoot regeneration compared to the 12 h light treatment (Fig. 2e, Supporting Information Table S1). The combined callus and shoot mass data was consistent with these results (Fig. 2f). To determine whether the effect of the 24 h early light treatment was due to the 24 h duration being critical or due to dark-to-light shifting at ~day 6 post-excision being important, the dark period was extended, but the differential results became even more exaggerated (Fig. 2g–i, Supporting Information Table S1). L*er*-0 cotyledon explants thus show two discrete phases of sensitivity to light during the first 24 h after excision.

Exposure to moderate intensities of light after excision causes reactive oxygen species (ROS) production

Given that exposure to only a few hours of darkness post-excision was beneficial to shoot regeneration, we hypothesized that newly injured tissues were being damaged by the post-excision light intensity used (~100 μmol m^{-2} s^{-1}). In intact seedlings, however, photo-oxidative damage is associated with much higher levels of light exposure, typically 600–2000 μmol m^{-2} s^{-1}, or following dramatic low-to-high shifts in fluence (Fryer *et al*. 2002). The seedlings in this study were germinated under 60–80 μmol m^{-2} s^{-1} of light and thus the transfer to ~100 μmol m^{-2} s^{-1} light did not represent a dramatic shift in light intensity. The explants did, however, experience an intervening interval of 1–2 h with diminished light (5–10 μmol m^{-2} s^{-1}) in a sterile flow hood during which the tissues were excised. Using indicator dyes, we first asked whether only 100 μmol m^{-2} s^{-1} of light could promote ROS accumulation and/or cause cell death in excised L*er*-0 cotyledons (Fig. 3a–i). Hydrogen peroxide was detected [brown stain, 3,3-diaminobenzidine (DAB)] (Fig. 3b) along with superoxide production [purple stain, nitroblue tetrazolium (NBT)] (Fig. 3e) following 6 h of exposure to 100 μmol m^{-2} s^{-1} light, in contrast to dark-treated controls (Fig. 3c,f; Flohe & Otting 1984; Beyer & Fridovich 1987; Thordal-Christensen *et al*., 1997). In fact, as few as 2 h of light exposure was sufficient to cause an increase in hydrogen peroxide and superoxide accumulation (data not shown). We conclude that only modest levels of light exposure are sufficient to induce ROS production in newly excised Arabidopsis cotyledon explants.

Shoot regeneration is reduced by loss of photoprotective beta-carotene/zeaxanthin pigments and increased photo-oxidative damage

Given that only modest light exposure induces ROS production in new explants, we hypothesized that ROS kills cells critical for shoot regeneration. To test for cell death, Evans Blue was used: it diffuses into dead tissue and stains cells blue, but is excluded by the membranes of living cells (Gaff & Okongoog 1971). In 7-day-old L*er*-0 cotyledons, the cut edge stained blue immediately following excision (Fig. 3g); however, opposite to prediction, the 5 d dark-treated cotyledons stained darker blue (Fig. 3i) than the 5 d high light-treated cotyledons (Fig. 3h) suggesting that darkness causes cell death more than high light, a result that we cannot currently explain.

Because of the inconclusive cell death results, the impact of ROS on shoot regeneration was measured by reducing the levels of photoprotective carotenoids. Explants from the mutant, *non-photochemical quenching 1* (*npq1-2,* L*er*-0 background), were first tested; *npq1* plants have reduced photoprotective zeaxanthin pigmentation in the chloroplast due to a mutation in the enzyme, violaxanthin de-epoxidase (Niyogi, Grossman & Bjorkman 1998). Regardless of early light/dark exposure, this mutation caused dramatic declines in rates of shoot regeneration compared to wild-type L*er*-0 (Fig. 3j,k), suggesting that photo-oxidative damage was responsible for at least part of the light inhibition phenomenon.

Since early darkness prior to weeks of high light exposure was not beneficial to shoot regeneration in *npq1* mutants, explants appeared to have a long-term need for photoprotection. To distinguish between the importance of early versus late photoprotection after organ excision, explants were transiently exposed to chemical inhibitors that blocked photoprotective pigment production or increased ROS immediately after organ excision. The following chemicals were added transiently: dithiothrietol (DTT), a xanthophyll inhibitor that again targets NPQ1 (Yamamoto & Kamite 1972); NF, an inhibitor earlier in the beta-carotene pathway that targets phytoene desaturase (Bramley & Britton, 1993; Jung 2004); or paraquat (PQ), a photosynthesis inhibitor that promotes ROS accumulation (Dodge, Harris & Baldwin 1970; Dodge 1982; Fig. 3l,m). Explants were placed on to media containing the inhibitors or control buffers for 2 d, accompanied by a simultaneous 2 d post-excision dark or high light treatment; the explants were subsequently shifted back to media lacking inhibitors and exposed to high light for >4 weeks. Compared to untreated L*er*-0 cotyledons, the 2 d DTT, NF and PQ treatments dramatically reduced shoot regeneration if applied under early high light but were not inhibitory if applied during early darkness (Fig. 3l,m; Supporting Information Table S2), consistent with photoprotection being critical for shoot regeneration early after organ excision.

Together with the earlier indicator dye and *npq1* data, the pharmacological results demonstrate that exposure to only modest light levels immediately after organ excision promotes ROS accumulation, that extended exposure to ROS inhibits shoot regeneration and that the negative effects of early ROS are at least partially reduced by beta-carotene-derived pigments, in particular zeaxanthin. It should also be noted that attempts were made to reduce ROS using ascorbic acid, a free oxygen radical scavenger, but it caused Phytagel to melt when the plates were simultaneously exposed to light (data not shown).

phyA signalling helps protect explants against high light during the first 24 h after excision

We hypothesized that R/FR photoreceptor-mediated signalling might have also contributed to cotyledons being light sensitive after excision. Of the five R/FR photoreceptors in Arabidopsis, mutants that disrupt the phyA or phyB photoreceptors were initially tested, as they play the dominant roles in most photomorphogenic responses (Casal 2000; Fig. 4a,b). Relative to wild-type L*er*-0 explants (ABRC CS20), only *phyA-201* mutant explants (Nagatani, Reed & Chory 1993) showed statistically differential declines in shoot regeneration in light versus darkness; *phyA-201* explants showed reduced regeneration if immediately exposed to high light, but not when initially exposed to 1 d of darkness (Fig. 4a). As phyA is the primary phytochrome acting under FR light, this result suggests that FR light signalling normally helps to protect explants against early light inhibition of shoot regeneration.

Explants of mutant *phyB-1* (Koornneef, Rolff & Spruit 1980; Nagatani *et al*. 1993) were then tested; the percentage decline in shoot regeneration following exposure of explants to continuous high light compared to 1 d of initial darkness was not significantly different for *phyB-1* explants compared to wild-type (Fig. 4a). However, *phyB-1* cotyledons showed significant reductions in absolute shoot regeneration rates under both light treatments. As phyB is the primary phytochrome acting under red light (Koornneef *et al*. 1980; Nagatani *et al*. 1993), this result suggests that red light signalling is not involved in the early high light inhibition response but may be required to promote efficient shoot regeneration long term. A similar but more severe result was obtained with explants of *hy1-1* (Fig. 4a,b), a tetrapyrrole biosynthetic mutant that disrupts the chromophores of all five phytochromes (Koornneef *et al*. 1980; Muramoto *et al*. 1999), and that is allelic to a suppressor of *flu* (*fluorescent*), a mutant that makes plants hypersensitive to light by production of singlet oxygen (Goslings *et al*. 2004).

Inhibition of shoot regeneration by early post-excision light requires blue/UV-A wavelengths and cryptochrome 1 (CRY1)

Next, we tested whether blue/UV-A wavelengths mediate early high light inhibition of shoot regeneration (Fig. 4c–g). Explants of a quadruple mutant defective in all four blue/UV-A photoreceptors (*cry1cry2phot1phot2*; Ohgishi *et al*. 2004) were initially tested but the results were difficult to interpret (Supporting Information Fig. S3); as the mutant genotype was constructed in a mixed genetic background (Ler-0/Ws2), segregating genetic modifiers may have confounded the phenotype. As an alternative, in order to block nearly all blue light responses and to do so transiently, a filter that blocked most of the blue/UV-A spectrum was placed above wild-type L*er*-0 cotyledons for the first 5 d post-excision, and then removed (Fig. 4c–f). The experiment was replicated using ecotype Ws2. There were five initial light treatments, all subsequently followed by continuous (white) high light (100 μmol m^{-2} s^{-1}). The initial treatments were 5 d of darkness, 5 d of high light, 5 d of medium light (60 μmol m^{-2} s^{-1}), 5 d of blue/UV-A-deficient high light (BDF high) and 5 d of blue/UV-A-deficient medium light (BDF medium). Lights were adjusted to maintain a constant light quantity (100 or 60 μmol m^{-2} s^{-1})

at the tissue level. Both L*er*-0 and Ws2 explants showed dramatic increases in shoot regeneration when blue/UV-A light was reduced for the first 5 d after excision (BDF treatments, Fig. 4c–f). The regeneration response differed however between the two ecotypes and was dependent on the light quantity: Ws explants showed improved regeneration following exposure to both BDF medium light and BDF high light (blue/UV-A wavelengths contributed 41 and 54% of the decline, respectively), whereas L*er*-0 explants only showed improved regeneration following exposure to BDF medium light (blue/UV-A wavelengths contributed 57% of the decline). Consistent with this observation, in an independent experiment, when explants from a *cry1* single mutant (*hy4-1*; Koornneef *et al*. 1980; Ahmad & Cashmore 1993) in a L*er*-0 background, were exposed to constant low (white) light (20–30 μmol m^{-2} s^{-1}), a 76% increase in shoot regeneration was observed compared to wild type (Fig. 4g). In contrast, the *cry1* (L*er*-0) allele had no significant effect under high light (Fig. 4g). Together, these results show that exposure of freshly excised explants to blue/UV-A light inhibits subsequent shoot regeneration, and that CRY1 at least partially mediates this response. Genotypes may differ in their sensitivity to blue/UV-A wavelengths depending on the total light exposure received.

A mutation in transcription factor HY5 causes explants to be hypersensitive to light both early and late after excision

Next, we tested a mutant allele of the transcription factor ELONGATED HYPOCOTYL 5 (HY5) (Osterlund *et al*. 2000) because it is a major downstream effector of R/FR and blue/UV-A photoreceptors (Chattopadhyay *et al*. 1998). HY5 was also of interest as it coordinates light and auxin/cytokinin signalling pathways (Oyama, Shimura & Okada 1997) as well as photoprotective pigment biosynthesis pathways (Liu *et al*. 2004). Here, mutant *hy5-1* explants were observed to have dramatically reduced rates of shoot regeneration compared to wild-type, regardless of the post-excision light treatment (Fig. 5a–f). This data was consistent with previous results showing that *hy5-1* explants have reduced callus and shoot regeneration rates (Cluis, Mouchel & Hardtke 2004). However, *hy5* shoot regeneration was significantly more inhibited when newly excised explants were initially exposed to 1 d of high light rather than darkness prior to the extended (>4 weeks) high light treatment (Fig. 5a–f). These data suggest that HY5 mediates cotyledon explant photoprotection, particularly during the first 24 h following excision, but also during subsequent weeks.

HY5 and phyB promote chalcone synthase (*TT4*)-dependent accumulation of photoprotective anthocyanin in explants

We investigated how transcription factor HY5 suppresses post-excision high light inhibition of shoot regeneration. In Arabidopsis, HY5 directly activates transcription of *TRANSPARENT TESTA 4* (*TT4*), which encodes chalcone synthase (ChS), a rate-limiting step in the biosynthesis of anthocyanins (Shirley *et al*. 1995; Gould 2004; Lee *et al*. 2007). This observation was of interest as anthocyanins have been shown to shield tissues from high-energy, blue-green wavelengths, thus preventing production of ROS (Neill & Gould 1999; Barnes *et al*. 2000; Havaux & Kloppstech 2001; Gould, Neill & Vogelmann 2002a; Gould *et al*. 2002b). We first asked whether anthocyanin accumulation in regenerating calli was dependent on HY5. Visually, wild-type L*er*-0 callus accumulated significant anthocyanin (Fig. 5g), whereas *hy5* mutant explants showed no anthocyanin accumulation (Fig. 5h) similar to the *tt4* mutant control (Fig. 5j). Whereas wild-type L*er*-0 explants accumulated visible anthocyanin under all light conditions tested, *hy5* mutant explants failed to accumulate any visible anthocyanin regardless of whether the tissues were first exposed to high light (100 μmol m^{-2} s^{-1}), 1 or 5 d of darkness followed by 4 weeks of high light, or constant low light (20–30 μmol m^{-2} s^{-1}; Supporting Information Fig. S4).

We also asked whether *phyA*, *phyB*, *hy1* or *cry1* single mutants affected visible anthocyanin accumulation in callus: only *phyB* explants were unable to accumulate significant anthocyanin under a subset of light/dark treatments (Fig. 5i; Supporting Information Fig. S4). phyB has previously been implicated as the major phytochrome regulating anthocyanin production (Nagy & Schafer 2002). If *phyB* mutant explants were first exposed to 5 d of darkness, but not 1 d of darkness, prior to 4 weeks of high light, then anthocyanin production was restored, suggesting that prolonged post-excision darkness can bypass the requirement for phyB (Supporting Information Fig. S4).

Finally, the *tt4* mutant was tested to determine whether loss of anthocyanin accumulation affected shoot regeneration (Fig. 5k–m). Compared to wild-type L*er*-0 explants, mutant *tt4* explants showed a >80% reduction in shoot regeneration under all light treatments including early exposure to darkness (Fig. 5k). These results suggest that flavonoids downstream of chalcone synthase (*TT4*), likely anthocyanins, are required to permit efficient shoot regeneration, and that these pigments are required both early and late after organ excision.

As *TT4* has previously been shown to be the downstream target of HY5 (Ahmad, Jarillo & Cashmore 1998; Lee *et al*. 2007), and since explants of both *phyB* and *hy5* mutants lack visible anthocyanin in early high light (Fig. 5h,i), then together we conclude that phyB and its downstream target, HY5, suppress light inhibition of shoot regeneration, perhaps in part by activating enzymes (e.g. chalcone synthase/*TT4*) required for the biosynthesis of photoprotective flavonoids/anthocyanins.

Cytokinin may have a secondary beneficial effect on shoot regeneration by mediating photoprotection

Cytokinin is well known to increase rates of shoot regeneration (Skoog & Miller 1957). However, in Arabidopsis,

cytokinin has also been shown to stimulate the biosynthesis of photoprotective anthocyanins by activating *TT4* transcription via a HY5-dependent pathway (Koornneef *et al*. 1980; Nagatani *et al*. 1993; Nagy & Schafer 2002; Lee *et al*. 2007; Vandenbussche *et al*. 2007). Therefore, we asked whether cytokinin has a secondary role *in vitro,* namely to suppress the negative effects of light in explants. We exposed explants of a cytokinin-overexpression line (CaMV35S-isopentyl transferase, IPT161, ecotype C24; Karpinski *et al*. 1999) to different light/dark treatments (Supporting Information Fig. S5). Compared to non-transgenic C24 explants, explants from the cytokinin-overexpression line were less sensitive to continuous high light exposure than the same explants initially placed in darkness for 5 d prior to >4 weeks at high light (88% versus 55% decline in shoot regeneration, respectively; Supporting Information Fig. S5a). Cytokinin overexpression was associated with increased anthocyanin production as predicted (Supporting Information Fig. S5a–e). Transgenic explants from the 5 d dark subgroup had both the highest regeneration rates and the highest visible anthocyanin accumulation (Supporting Information Fig. S5d,e). These results suggest that cytokinin may have a modest, secondary role in promoting shoot regeneration by boosting levels of photoprotective anthocyanin.

Light inhibition of shoot regeneration requires polar auxin transport (PAT)

In addition to coordinating light signalling with pigment biosynthetic pathways, HY5 also coordinates light with auxin signalling; specifically, HY5 has been shown to regulate the auxin efflux PINOID (PIN) carrier proteins (Lee *et al*. 2007; Laxmi *et al*. 2008). Upstream of HY5, both phyA and CRY1, which affect light inhibition of shoot regeneration (Fig. 4), have also been shown to alter polar auxin transport (PAT; Jensen *et al*. 1998; Canamero *et al*. 2006). For these reasons, we asked whether PAT mediates or mitigates the effects of light exposure on shoot regeneration. Auxin efflux can be inhibited transiently and non-competitively by the PAT inhibitor, 1-N-naphthylphthalamic acid (NPA; Petrasek *et al*. 2006). NPA was added to CIM media during the first 24 h after organ excision, and then the cotyledons were transferred on to CIM media lacking NPA; explants were simultaneously exposed to 24 h of high light or darkness, followed by 4 weeks of high light. NPA was predicted primarily to affect endogenous tissue auxin (e.g. IAA) not CIM-derived 2,4-D (Estelle 1998; Petrasek *et al*. 2006). The NPA treatment was observed to significantly alleviate early high light inhibition of shoot regeneration compared to the DMSO buffer control (Fig. 6a,b). In fact, transient blockage of PAT using 25 μM NPA prevented 63% of the decline in shoot regeneration associated with early light exposure compared to early dark exposure (Fig. 6a). There was no significant difference in regeneration when NPA was added to explants transiently exposed to darkness, except at the most diluted NPA dosage (Fig. 6a,b). These results suggest that PAT mediates light-dependent inhibition of shoot regeneration.

Ethylene affects shoot regeneration in a light-dependent manner

Finally, we tested the impact of ethylene on light inhibition of shoot regeneration for three reasons. Firstly, ethylene regulates auxin signalling (Fukaki & Tasaka 2009). Secondly, ethylene strongly promotes shoot regeneration from Arabidopsis cotyledon explants (Chatfield & Raizada 2008). Finally, ethylene has been shown to be regulated by light: ethylene emission from Arabidopsis seedlings increases when plants are shifted from light into darkness, but decreases when seedlings are shifted to light (Vandenbussche *et al*. 2003). To increase ethylene levels transiently, explants were exposed for 48 h to ACC, the biosynthetic precursor for ethylene (Abeles, Morgan & Saltveit 1992). To reduce ethylene signalling transiently, we similarly applied silver nitrate ($AgNO_3$), an inhibitor of ethylene perception (Beyer 1979). ACC and silver nitrate were applied at concentrations previously defined (Chatfield & Raizada 2008). Explants were simultaneously exposed to 48 h of high light or darkness, prior to 4 weeks of high light. ACC caused an increase in the percentage of explants that regenerated shoots but only if applied alongside an early dark treatment (Fig. 6c,d). Conversely, when ethylene perception was reduced using $AgNO_3$, there was a significant increase in shoot regeneration if explants were simultaneously exposed to early high light, but not darkness (Fig. 6c,d). We conclude that ethylene promotes cotyledon explants to regenerate under darkness, but inhibits shoot regeneration under light.

DISCUSSION

Since its beginnings in the 1950s (Skoog & Miller 1957), *in vitro* plant regeneration has suffered from considerable variability, a phenomenon that has limited plant stem cell research and applied efforts towards gene transformation, propagation of viral-free crops in developing nations and propagation of horticultural, medicinal and forest plant species (Steeves & Sussex 1989). By undertaking systematic testing of excised Arabidopsis cotyledon explants, we have shown here that variability in plant tissue cultures may arise from a previously unappreciated source, the dosage and quality of light experienced by a tissue within the initial hours after it has been excised, combined with interactions with hormones.

Inadvertent early light or dark exposure may contribute to variable responses by plant tissue culture explants

Newly excised tissues appear to be hypersensitive to light quantity, whereas 500–2000 μmol m^{-2} s^{-1} of light is needed to cause ROS accumulation in non-acclimated intact

seedlings (Karpinski *et al*. 1999), here only 2–6 h of 100 μmol m^{-2} s^{-1} of fluorescent light were observed to be sufficient to elicit ROS in newly detached cotyledons of Arabidopsis ecotype L*er*-0 (Fig. 3a–f). Treatments that reduced accumulation of photoprotective xanthophyll pigments or resulted in overproduction of ROS for the initial 48 h after excision (Fig. 3j–m) made explants hypersensitive to light, suggesting these pathways may be mis-regulated following tissue excision. Plant tissue researchers may need to be vigilant of both the ambient light quantity and quality at the time of explanting, such as during the time needed to excise tissues in a sterile flow hood prior to moving explants to a controlled chamber. For example, extrapolating from this study, a dark cover (e.g. aluminium foil) placed on explants for only 2 h after excision may stimulate long-term shoot regeneration (Fig. 2). Furthermore, exposure to sunlight from a nearby window may elicit damaging ROS (Fig. 3; Tyystjarvi 2008). Early exposure to indoor or flow hood fluorescent light (2–100 μmol m^{-2} s^{-1}), the lamp type most commonly used in plant tissue culture research for cost-effectiveness and low heat emission, may inhibit regeneration as it is rich in blue/UV-A and has low FR (Supporting Information Fig. S6). We predict that indiscriminate usage of different brands of fluorescent bulbs may also be causing tissue culture variation, as they can vary both in blue and FR wavelengths. Variable early exposure of explants to incandescent light, which is rich in FR (Supporting Information Fig. S6), may also be causing variation in tissue regeneration. We hypothesize that genotypes varying in elicitation of ROS or activation of photoprotective anthocyanin or xanthophylls after excision (Fig. 5; Supporting Information Fig. S4–S6) may also contribute to the variation observed in shoot regeneration responses within a species (e.g. Fig. 1, 2).

As for the underlying mechanisms of light inhibition of shoot regeneration, firstly, blue/UV-A wavelengths may inhibit shoot regeneration because they are high-energy wavelengths absorbed by chlorophyll, leading to photosystem II damage (Peterman *et al*. 1997). We have also demonstrated that blue/UV-A wavelengths, even in low fluence light (20–30 μmol m^{-2} s^{-1}), can inhibit long-term shoot regeneration via a photoreceptor CRY1-mediated signalling pathway (Fig. 4g). In terms of the mechanism by which low-FR light exposure is detrimental to explants in the initial 24 h after excision, we infer it is because the primary receptor mediating FR responses, phyA, cannot properly activate photoprotective pathway(s) (Fig. 4a,b). phyA has previously been shown to up-regulate carotenoid/xanthophyll production in intact seedlings (von Lintig *et al*. 1997). Exposure of explants to low FR might thus reduce chloroplast xanthophyll pigments that could otherwise dissipate excess photosynthetic energy as heat instead of producing ROS (Demmig-Adams & Adams 1992). Consistent with this result, we observed that transient disruption of xanthophylls in the first 48 h after excision, during light exposure, inhibited long-term shoot regeneration (Fig. 3j–m). It is also noteworthy that both phyA and phyB have been shown to regulate pathways involving auxin and ethylene (Foo *et al*. 2006), both of which were shown to interact with light to regulate shoot regeneration (Fig. 6).

The duration of light or dark exposure experienced by explants in the 24 h after their excision may also affect shoot regeneration rates. Researchers often place explants in a continuous light or dark treatment immediately after excision (Zhao *et al*. 2002), but our data suggests that explants may be highly susceptible to 12 h diurnal effects in the first day following excision (Fig. 2d–i). We observed that a delay ($\leq$12 h) in the initial onset to darkness following excision improved regeneration, yet remarkably, delaying dark onset by another 12 h caused the beneficial effects of darkness to be completely negated (Fig. 2d–i). Given that exposure to 6 h of ~100 μmol m^{-2} s^{-1} light was sufficient to induce ROS in fresh explants (Fig. 3a–f), and that 6 h of early darkness promoted shoot regeneration (Fig. 2a), it is surprising that up to 12 h of 90–120 μmol m^{-2} s^{-1} light, prior to an extended dark period, promoted shoot regeneration (Fig. 2d–i). It may be that extended periods of darkness can mitigate earlier ROS exposure as long as explants are not exposed to ROS beyond a critical threshold. This explanation begs the question as to why $\leq$12 h of high light, prior to an extended dark period, would be beneficial, rather than neutral, to shoot regeneration. One clue may come from recent studies showing that mild levels of ROS can act as developmental signalling molecules, perhaps by forming concentration gradients that interact with auxin gradients (Tognetti, Muhlenbock & Van Breusegem 2012).

Finally, our data suggests that in the days following explanting, a power failure or deliberate exposure to darkness interrupting a continuous light treatment, could lead to dramatic declines in both shoot regeneration and callus production (Supporting Information Fig. S2). It may be that two light/dark shifts are particularly stressful to explants.

Hormone supplements interact with light in the initial days after tissue excision

The results from our hormone experiments also have implications for plant tissue culture researchers. This study predicts that polar auxin transport inhibitors commonly used in plant tissue culture (e.g. 2,3,5-triiodobenzoic acid, TIBA; Steeves & Sussex 1989) may actually reduce the negative effects of inadvertent or deliberate early light exposure with respect to shoot regeneration (Fig. 6a,b). Surprisingly, polar transportable forms of auxin that are commonly added as hormone supplements (e.g. NAA) may switch from stimulating shoot regeneration to inhibiting shoot regeneration, depending on whether a fresh explant is exposed to darkness or light, respectively, for the first 24–48 h after excision (Fig. 6a,b).

In addition to auxin, this study suggests that researchers should be aware that inadvertent or deliberate manipulation of ethylene in the initial days after organ excision may have long-term impacts on shoot regeneration. Previously, we showed that ethylene stimulated shoot regeneration

from Arabidopsis cotyledon explants (Chatfield & Raizada 2008). In this study, ethylene was shown to have opposite effects on shoot regeneration rates depending on whether new explants were exposed to light or darkness in the initial days after excision (Fig. 6c,d). Ethylene gas build-up within Petri dishes can occur due to the use of sealing tape, placing dishes within sealed container boxes or use of ethylene antagonists such as $AgNO_3$, all of which are routine practices in plant tissue culture research (Steeves & Sussex 1989).

Finally, we observed that cytokinin interacts with light after organ excision. Cytokinin appears to partially suppress photoinhibition, perhaps by stimulating accumulation of vacuolar anthocyanin, known to shield against high-energy blue-green light (Gould *et al.* 2002b; Supporting Information Fig. S5–S6). Cytokinin may thus play a previously unreported secondary role in plant tissue culture. Perhaps of greater practical importance is the observation that our most damaging light treatment with respect to shoot regeneration (5 d high – 10 d dark – constant high) also blocked cytokinin-induced anthocyanin accumulation in callus (Supporting Information Fig. S5c). This result suggests that light may interfere with signalling by cytokinin, a hormone critical for efficient shoot regeneration (Skoog & Miller 1957).

HY5 may help coordinate a complex light signalling network in the initial hours after tissue excision

At the molecular level, here we showed that the light-activated transcription factor HY5 (Chattopadhyay *et al.* 1998) is critical to shoot regeneration (Fig. 5). HY5 may be an important integrator of the different signalling pathways suggested by this study to act in the initial hours and days after a tissue is excised (Fig. 7). Specifically, HY5 has been shown to bind promoters of genes encoding anthocyanin biosynthetic enzymes (chalcone synthase/TT4, F3H, FLS; Cluis *et al.* 2004), auxin transport proteins (PIN1 and PIN3), auxin signalling proteins (9 AUX/IAAs including AXR2/IAA and SLR/IAA14; 6 ARFs), ethylene biosynthetic enzymes and signalling proteins (ACS7 and ACS8; 6 ERFs), cytokinin signalling proteins (ARR4, ARR9; Ahmad *et al.* 1998; Lee *et al.* 2007) and diurnal clock regulation proteins (morning CCA1/LHY, evening TOC1/ELF4). Our result that HY5 is important for anthocyanin accumulation in callus (Fig. 5) is consistent with previous studies (Nagy & Schafer 2002; Vandenbussche *et al.* 2007).

Particularly intriguing, HY5 may be mediating the critical interaction between light and auxin transport in new tissue explants (Fig. 6a,b). This result is consistent with chromatin immunoprecipitation (ChiP) experiments, which revealed putative binding sites for HY5 within the promoters of the auxin efflux carriers, PIN1, a candidate PIN3, and the protein kinase PINOID (PID; Lee *et al.* 2007), the latter shown to control the polar localization of PIN1 (Friml *et al.* 2004). Furthermore, correct localization of PIN2 to the plasma membrane was shown to require

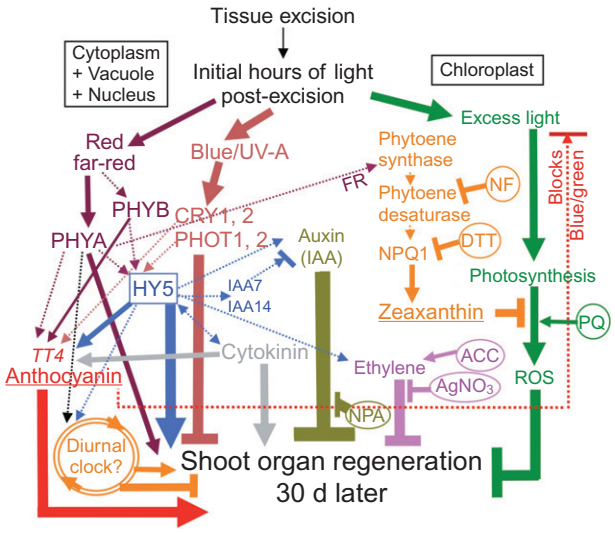


Figure 7. Speculative model to summarize proposed genetic pathways that act in newly excised Arabidopsis cotyledons exposed to high light, their impact on shoot regeneration and possible genetic interactions. A solid line represents a result from this study. The thickness of the line indicates our proposed level of importance of that pathway for regulating shoot regeneration: perpendicular lines indicate a negative effect on shoot regeneration, while arrows indicate a positive effect. A dotted line indicates a possible genetic interaction based on the literature but not shown in regeneration studies. Chemical inhibitors or elicitors used in this study are circled. This model primarily reflects results from 6–7-day-old L*er*-0 cotyledon explants, placed on to callus induction media (CIM) media for 5 d then transferred to shoot induction media (SIM) media for 4 weeks.

HY5, whereas darkness caused PIN2 to be degraded in the vacuole (Laxmi *et al.* 2008). Therefore, if a dark treatment impacts auxin efflux proteins by interrupting directional auxin transport, the beneficial application of the PAT inhibitor NPA in the light (Fig. 6a,b) may be phenocopying dark-mediated degradation of HY5. It is possible that interrupting auxin transport by exposure to darkness leads to auxin maxima, which in turn promotes initiation of lateral root meristems shown to be the source of stem cells for shoot regeneration (Atta *et al.* 2009; Duclercq *et al.* 2011). HY5 has been shown to regulate adventitious root regeneration (Oyama *et al.* 1997; Cluis *et al.* 2004). In contrast, early exposure to high light may be increasing auxin transport in explants, leading to reduced points of auxin accumulation and ultimately reduced shoot regeneration. Testing of these hypotheses will require introgression of auxin sensitivity/transport mutant alleles from Col-0 (where most were isolated), into ecotype backgrounds more amenable to the regeneration system described here.

In conclusion, our data has revealed that the developmental signalling pathways underlying shoot meristem regeneration are regulated by a complex signalling network that includes light signalling (Fig. 7). This network acts in the initial hours-to-days after tissue excision in Arabidopsis.

The importance of this network and its timing must now be tested in other species and/or explant types. We nevertheless suggest that caution, strict reporting and uniform standards (e.g. lamp types) should be adopted by plant tissue culture researchers, related to light exposure during the first hours after excision. A minor change in tissue handling immediately following excision appears to have a dramatic effect on the capacity of a tissue to regenerate a new shoot apical meristem.

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REFERENCES

Abeles F.B., Morgan P.W. & Saltveit M.E., Jr (1992) *Ethylene in Plant Biology* 2nd edn, Academic Press, Inc., San Diego, CA, USA.

Ahmad M. & Cashmore A.R. (1993) *HY4* gene of *A. thaliana* encodes a protein with characteristics of a blue-light photoreceptor. *Nature* **366,** 162–166.

Ahmad M., Jarillo J.A. & Cashmore A.R. (1998) Chimeric proteins between CRY1 and CRY2 Arabidopsis blue light photoreceptors indicate overlapping functions and varying protein stability. *The Plant Cell* **10,** 197–207.

Atta R., Laurens L., Boucheron-Dubuisson E., Guivarc'h A., Carnero E., Giraudat-Pautot V., Rech P. & Chriqui D. (2009) Pluripotency of Arabidopsis xylem pericycle underlies shoot regeneration from root and hypocotyl explants grown *in vitro*. *The Plant Journal* **57,** 626–644.

Baldwin I.T., Kessler A. & Halitschke R. (2002) Volatile signaling in plant-plant-herbivore interactions: what is real? *Current Opinion in Plant Biology* **5,** 351–354.

Banno H., Ikeda Y., Niu Q.W. & Chua N.H. (2001) Overexpression of Arabidopsis ESR1 induces initiation of shoot regeneration. *The Plant Cell* **13,** 2609–2618.

Barnes P.W., Searles P.S., Ballare C.L., Ryel R.J. & Caldwell M.M. (2000) Non-invasive measurements of leaf epidermal transmittance of UV radiation using chlorophyll fluorescence: field and laboratory studies. *Physiologia Plantarum* **109,** 274–283.

Bertram L. & Lercari B. (2000) Evidence against the involvement of phytochrome in UVB-induced inhibition of stem growth in green tomato plants. *Photosynthesis Research* **64,** 107–117.

Beyer E.M. (1979) Effect of silver ion, carbon-dioxide, and oxygen on ethylene action and metabolism. *Plant Physiology* **63,** 169–173.

Beyer W.F. & Fridovich I. (1987) Assaying for superoxide-dismutase activity – some large consequences of minor changes in conditions. *Analytical Biochemistry* **161,** 559–566.

Birnbaum K.D. & Alvarado A.S. (2008) Slicing across kingdoms: regeneration in plants and animals. *Cell* **132,** 697–710.

Bramley P.M. & Britton G. (1993) Inhibition of carotenoid biosynthesis. In *Carotenoids in Photosynthesis* (eds A.J. Young & G. Britton) pp. 127–159. Chapman & Hall, London, UK.

Buchanan B.B., Gruissem W. & Jones R.L. (2000) *Biochemistry and Molecular Biology of Plants*. American Society of Plant Physiologists, Rockville, MD, USA .

Canamero R.C., Bakrim N., Bouly J.P., Garay A., Dudkin E.E., Habricot Y. & Ahmad M. (2006) Cryptochrome photoreceptors CRY1 and CRY2 antagonistically regulate primary root elongation in *Arabidopsis thaliana*. *Planta* **224,** 995–1003.

Casal J.J. (2000) Phytochromes, cryptochromes, phototropin: photoreceptor interactions in plants. *Photochemistry and Photobiology* **71,** 1–11.

Chatfield S.P. & Raizada M.N. (2008) Ethylene and shoot regeneration: *Hookless1* modulates *de novo* shoot organogenesis in *Arabidopsis thaliana*. *Plant Cell Reports* **27,** 655–666.

Chattopadhyay S., Ang L.H., Puente P., Deng X.W. & Wei N. (1998) Arabidopsis bZIP protein HY5 directly interacts with light-responsive promoters in mediating light control of gene expression. *The Plant Cell* **10,** 673–683.

Chaudhury A.M. & Signer E.R. (1989) Relative regeneration proficiency of *Arabidopsis thaliana* ecotypes. *Plant Cell Reports* **8,** 368–369.

Che P., Lall S., Nettleton D. & Howell S.H. (2006) Gene expression programs during shoot, root, and callus development in Arabidopsis tissue culture. *Plant Physiology* **141,** 620–637.

Christianson M.L. & Warnick D.A. (1983) Competence and determination in the process of *in vitro* shoot organogenesis. *Developmental Biology* **95,** 288–293.

Cluis C.P., Mouchel C.F. & Hardtke C.S. (2004) The Arabidopsis transcription factor HY5 integrates light and hormone signaling pathways. *The Plant Journal* **38,** 332–347.

Decook R., Lall S., Nettleton D. & Howell S.H. (2006) Genetic regulation of gene expression during shoot development in Arabidopsis. *Genetics* **172,** 1155–1164.

Demmig-Adams B. & Adams W.W., III (1992) Photoprotection and other responses of plants to high light stress. *Annual Review of Plant Physiology and Plant Molecular Biology.* **43,** 599–626.

Dodge A.D. (1982) The role of light and oxygen in the action of photosynthetic inhibitor herbicides. *ACS Symposium Series* **181,** 57–77.

Dodge A.D., Harris N. & Baldwin B.C. (1970) The mode of action of paraquat and diquat. *Biochemical Journal* **118,** 43–44.

Dong N., Montanez B., Creelman R.A. & Cornish K. (2006) Low light and low ammonium are key factors for Guayule leaf tissue shoot organogenesis and transformation. *Plant Cell Reports* **25,** 26–34.

Duclercq J., Sangwan-Norreel B., Catterou M. & Sangwan R.S. (2011) *De novo* shoot organogenesis: from art to science. *Trends in Plant Science* **16,** 597–606.

Estelle M. (1998) Polar auxin transport: new support for an old model. *The Plant Cell* **10,** 1775–1778.

Flohe L. & Otting F. (1984) Superoxide-dismutase assays. *Methods in Enzymology* **105,** 93–104.

Foo E., Ross J.J., Davies N.W., Reid J.B. & Weller J.L. (2006) A role for ethylene in the phytochrome-mediated control of vegetative development. *The Plant Journal* **46,** 911–921.

Friml J., Yang X., Michniewicz M., *et al*. (2004) A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. *Science* **306,** 862–865.

Fryer M.J., Oxborough K., Mullineaux P.M. & Baker N.R. (2002) Imaging of photo-oxidative stress responses in leaves. *Journal of Experimental Botany* **53,** 1249–1254.

Fukaki H. & Tasaka M. (2009) Hormone interactions during lateral root formation. *Plant Molecular Biology* **69,** 437–449.

Gaff D.F. & Okongoog O. (1971) Use of non-permeating pigments for testing survival of cells. *Journal of Experimental Botany* **22,** 756–758.

Gallois J.L., Nora F.R., Mizukami Y. & Sablowski R. (2004) WUSCHEL induces shoot stem cell activity and developmental plasticity in the root meristem. *Genes & Development* **18,** 375–380.

Goslings D., Meskauskiene R., Kim C.H., Lee K.P., Nater M. & Apel K. (2004) Concurrent interactions of heme and FLU with Glu tRNA reductase (HEMA1), the target of metabolic feedback inhibition of tetrapyrrole biosynthesis, in dark- and light-grown Arabidopsis plants. *The Plant Journal* **40,** 957–967.

Gould K.S. (2004) Nature's Swiss Army knife: the diverse protective roles of anthocyanins in leaves. *Journal of Biomedicine and Biotechnology* **4,** 314–320.

Gould K.S., Neill S.O. & Vogelmann T.C. (2002a) A unified explanation for anthocyanins in leaves? *Advances in Botanical Research* **37,** 167–192.

Gould K.S., Vogelmann T.C., Han T. & Clearwater M.J. (2002b) Profiles of photosynthesis within red and green leaves of *Quintinia serrata*. *Physiologia Plantarum* **116,** 127–133.

Havaux M. & Kloppstech K. (2001) The protective functions of carotenoid and flavonoid pigments against excess visible radiation at chilling temperature investigated in Arabidopsis *npq* and *tt* mutants. *Planta* **213,** 953–966.

Hibara K., Takada S. & Tasaka M. (2003) *Cuc1* gene activates the expression of sam-related genes to induce adventitious shoot formation. *The Plant Journal* **36,** 687–696.

Hunter D.C. & Burritt D.J. (2004) Light quality influences adventitious shoot production from cotyledon explants of lettuce (*Lactuca sativa* L.) *In Vitro Cellular & Developmental Biology-Plant* **40,** 215–220.

Jensen P.J., Hangarter R.P. & Estelle M. (1998) Auxin transport is required for hypocotyl elongation in light-grown but not dark-grown Arabidopsis. *Plant Physiology* **116,** 455–462.

Jung S. (2004) Effect of chlorophyll reduction in *Arabidopsis thaliana* by methyl jasmonate or norflurazon on antioxidant systems. *Plant Physiology and Biochemistry* **42,** 225–231.

Karpinski S., Reynolds H., Karpinska B., Wingsle G., Creissen G. & Mullineaux P. (1999) Systemic signaling and acclimation in response to excess excitation energy in Arabidopsis. *Science* **284,** 654–657.

Kimura M. & Kagawa T. (2006) Phototropin and light-signaling in phototropism. *Current Opinion in Plant Biology* **9,** 503–508.

Koornneef M., Rolff E. & Spruit C.J.P. (1980) Genetic-control of light-inhibited hypocotyl elongation in *Arabidopsis thaliana* (L) Heynh. *Zeitschrift Fur Pflanzenphysiologie* **100,** 147–160.

Laxmi A., Pan J., Morsy M. & Chen R. (2008) Light plays an essential role in intracellular distribution of auxin efflux carrier PIN2 in *Arabidopsis thaliana*. *PLoS ONE* **3,** e1510.

Lee J., He K., Stolc V., Lee H., Figueroa P., Gao Y., Tongprasit W., Zhao H.Y., Lee I. & Deng X. (2007) Analysis of transcription factor HY5 genomic binding sites revealed its hierarchical role in light regulation of development. *The Plant Cell* **19,** 731–749.

von Lintig J., Welsch R., Bonk M., Giuliano G., Batschauer A. & Kleinig H. (1997) Light-dependent regulation of carotenoid biosynthesis occurs at the level of phytoene synthase expression and is mediated by phytochrome in *Sinapis alba* and *Arabidopsis thaliana* seedlings. *The Plant Journal* **12,** 625–634.

Liu H.X., Wang Y., Xu J., Su T.B., Liu G.Q. & Ren D.T. (2008) Ethylene signaling is required for the acceleration of cell death induced by the activation of AtMEK5 in Arabidopsis. *Cell Research* **18,** 422–432.

Liu Y.S., Roof S., Ye Z.B., Barry C., Van Tuinen A., Vrebalov J., Bowler C. & Giovannoni J. (2004) Manipulation of light signal transduction as a means of modifying fruit nutritional quality in tomato. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 9897–9902.

Muramoto T., Kohchi T., Yokota A., Hwang I. & Goodman H.M. (1999) The Arabidopsis photomorphogenic mutant *hy1* is deficient in phytochrome chromophore biosynthesis as a result of a mutation in a plastid heme oxygenase. *The Plant Cell* **11,** 335–348.

Nagatani A., Reed J.W. & Chory J. (1993) Isolation and initial characterization of Arabidopsis mutants that are deficient in phytochrome A. *Plant Physiology* **102,** 269–277.

Nagy F. & Schafer E. (2002) Phytochromes control photomorphogenesis by differentially regulated, interacting signaling pathways in higher plants. *Annual Review of Plant Biology* **53,** 329–355.

Neill S. & Gould K.S. (1999) Optical properties of leaves in relation to anthocyanin concentration and distribution. *Canadian Journal of Botany* **77,** 1777–1782.

Nishimura A., Ashikari M., Lin S., Takashi T., Angeles E.R., Yamamoto T. & Matsuoka M. (2005) Isolation of a rice regeneration quantitative trait loci gene and its application to transformation systems. *Proceedings of the National Academy of Sciences of the United States of America* **102,** 11940–11944.

Nishimura T., Mori Y., Furukawa T., Kadota A. & Koshiba T. (2006) Red light causes a reduction in IAA levels at the apical tip by inhibiting *de novo* biosynthesis from tryptophan in maize coleoptiles. *Planta* **224,** 1427–1435.

Niyogi K.K., Grossman A.R. & Bjorkman O. (1998) Arabidopsis mutants define a central role for the xanthophyll cycle in the regulation of photosynthetic energy conversion. *The Plant Cell* **10,** 1121–1134.

Ohgishi M., Saji K., Okada K. & Sakai T. (2004) Functional analysis of each blue light receptor, CRY1, CRY2, PHOT1, and PHOT2, by using combinatorial multiple mutants in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 2223–2228.

Osterlund M.T., Hardtke C.S., Wei N. & Deng X.W. (2000) Targeted destabilization of HY5 during light-regulated development of Arabidopsis. *Nature* **405,** 462–466.

Oyama T., Shimura Y. & Okada K. (1997) The Arabidopsis *HY5* gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. *Genes & Development* **11,** 2983–2995.

Ozawa S., Yasutani I., Fukuda H., Komamine A. & Sugiyama M. (1998) Organogenic responses in tissue culture of *srd* mutants of *Arabidopsis thaliana*. *Development* **125,** 135–142.

Peterman E.J.G., Gradinaru C.C., Calkoen F., Borst J.C., Vangrondelle R. & Vanamerongen H. (1997) Xanthophylls in Light-Harvesting Complex II of higher plants: light harvesting and triplet quenching. *Biochemistry* **36,** 12208–12215.

Petrasek J., Mravec J., Bouchard R., *et al*. (2006) PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science* **312,** 914–918.

Qin Y.H., Zhang S.L., Asghar S., Zhang L.X., Qin Q.P., Chen K.S. & Xu C.J. (2005) Regeneration mechanism of Toyonoka strawberry under different color plastic films. *Plant Science* **168,** 1425–1431.

Rahman A., Bannigan A., Sulaman W., Pechter P., Blancaflor E.B. & Baskin T.I. (2007) Auxin, actin and growth of the *Arabidopsis thaliana* primary root. *The Plant Journal* **50,** 514–528.

Saitou T., Tachikawa Y., Kamada H., Watanabe M. & Harada H. (1993) Action spectrum for light-induced formation of adventitious shoots in hairy roots of horseradish. *Planta* **189,** 590–592.

Saitou T., Tokutomi S., Harada H. & Kamada H. (1999) Overexpression of phytochrome A enhances the light-induced formation of adventitious shoots on horseradish hairy roots. *Plant Cell Reports* **18,** 754–758.

Saitou T., Hashizume A. & Kamada H. (2000) Genes for phytochrome a in horseradish: isolation of cDNAs and analysis of expression during light-induced formation of adventitious shoots from hairy roots. *Plant Cell Reports* **19,** 1212–1218.

Saitou T., Hashidume A., Tokutomi S. & Kamada H. (2004) Reduction of phytochrome level and light-induced formation of adventitious shoots by introduction of antisense genes for phytochrome a in horseradish hairy roots. *Plant Cell Tissue and Organ Culture* **76,** 45–51.

Shirley B.W., Kubasek W.L., Storz G., Bruggemann E., Koornneef M., Ausubel F.M. & Goodman H.M. (1995) Analysis of Arabidopsis mutants deficient in flavonoid biosynthesis. *The Plant Journal* **8,** 659–671.

Skoog F. & Miller C.O. (1957) Chemical regulation and growth of organ formation in plant tissue cultured *in vitro*. *Symposia of the Society for Experimental Biology* **11,** 118–131.

Smets R., Le J., Prinsen E., Verbelen J.P. & Van Onckelen H.A. (2005) Cytokinin-induced hypocotyl elongation in light-grown Arabidopsis plants with inhibited ethylene action or indole-3-acetic acid transport. *Planta* **221,** 39–47.

Steeves T.A. & Sussex I.M. (1989) *Patterns in Plant Development*. 2nd edn, Cambridge University Press, New York, NY, USA.

Strack D. (1997) Phenolic metabolism. In *Plant Biochemistry* (eds P.M. Dey & J.B. Harborne) pp. 387–416. Academic Press, London, UK.

Sugimoto K., Jiao Y.L. & Meyerowitz E.M. (2010) Arabidopsis regeneration from multiple tissues occurs via a root development pathway. *Developmental Cell* **18,** 463–471.

Sugimoto K., Gordon S.P. & Meyerowitz E.M. (2011) Regeneration in plants and animals: dedifferentiation, transdifferentiation, or just differentiation? *Trends in Cell Biology* **21,** 212–218.

Thordal-Christensen H., Zhang Z.G., Wei Y.D. & Collinge D.B. (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *The Plant Journal* **11,** 1187–1194.

Tognetti V.B., Muhlenbock P. & Van Breusegem F. (2012) Stress homeostasis – the redox and auxin perspective. *Plant, Cell & Environment* **35,** 321–333.

Tyystjarvi E. (2008) Photoinhibition of photosystem II and photodamage of the oxygen evolving manganese cluster. *Coordination Chemistry Reviews* **252,** 361–376.

Vandenbussche F., Vriezen W.H., Smalle J., Laarhoven L.J.J., Harren F.J.M. & Van Der Straeten D. (2003) Ethylene and auxin control decreased light intensity. *Plant Physiology* **133,** 517–527.

Vandenbussche F., Habricot Y., Condiff A.S., Maldiney R., Van Der Straeten D. & Ahmad M. (2007) HY5 is a point of convergence between cryptochrome and cytokinin signalling pathways in *Arabidopsis thaliana*. *The Plant Journal* **49,** 428–441.

Yamamoto H.Y. & Kamite L. (1972) Effects of dithiothreitol on violaxanthin deepoxidation and absorbance changes in 500-nm region. *Biochimica et Biophysica Acta* **267,** 538–543.

Zhang S.B. & Lemaux P.G. (2004) Molecular analysis of *in vitro* shoot organogenesis. *Critical Reviews in Plant Sciences* **23,** 325–335.

Zhao Q.H., Fisher R. & Auer C. (2002) Developmental phases and STM expression during Arabidopsis shoot organogenesis. *Plant Growth Regulation* **37,** 223–231.

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SUPPORTING INFORMATION

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

Figure S1. Representative images of regenerating explants of the unusual ecotype Est-1. Shown are explants regenerating shoots when exposed to: (a) continuous low light (20–25 μmol m^{-2} s^{-1}), (b) continuous medium light (44–55 μmol m^{-2} s^{-1}), (c) continuous high light (80–94 μmol m^{-2} s^{-1}), (d) 5 days darkness followed by continuous low light, (e) 5 days darkness followed by continuous medium light, (f) 5 days darkness followed by continuous high light. Though significant differences in shoot regeneration were not observed at these light levels when regeneration was quantified as percent explants with shoots (Fig. 1), these scans, which show the total number of regenerated shoots per explant, demonstrate that shoot regeneration from Est-1 explants was promoted by increasing light intensity following excision.

Figure S2. Exposure of Arabidopsis cotyledon explants to early high light, or high light followed by darkness, promotes callus failure. (a) Shown is the experimental design, including media (CIM, SIM) and light–dark shifting treatments. (b-e) Shown are the percentages of cotyledon explants that failed to regenerate any visible callus 5 weeks after excision for ecotypes: (b) L*er*-0, (c) DijG, (d) No-0 and (e) Est-1. One particular treatment (high light, followed by darkness, followed by high light) promoted the highest rates of callus failure. Each histogram represents 78 pooled cotyledons. (f-j) Pictures of ecotype DijG showing the callus failure response, where reduced green callus can be observed on selected treatments. The treatments shown are: (f) constant low light, (g) constant high light, (h) 10 days of darkness followed by high light, (i) 5 days of high light, followed by 10 days of darkness, followed by high light, (j) 5 days of low light, followed by 10 days of darkness, followed by low light. Early high light as in (g) and (i) caused considerable callus failure, two examples of which are boxed in yellow. All ecotypes shown were from Lehle Seeds: DijG (WT-10), Est-1 (WT-6A), L*er*-0 (WT-4) and No-0 (WT-9). For all experiments, explants (6–7 days post-germination) were placed on CIM plates for 5 days, then SIM for 10 days followed by fresh SIM for the remaining ~3 weeks.

Figure S3. Effect of the quadruple blue/UVA light photoreceptor mutant *cry1cry2phot1phot2* (quadblue) on shoot regeneration. Shown are the two wild-type parents of quadblue (Ohgishi *et al*. 2004), ecotypes L*er*-0 (CS20) and Ws2 (CS2360/CS22659). Graphed are the (a) average number of regenerated shoots/explant and (b) percent explants with shoots, scored 4 weeks following excision. For (a,b), cotyledons were exposed to either darkness (black bars) or high light (~100 μmol m^{-2} s^{-1}, white bars) for 24 h after excision, then treated for another 4 weeks with continuous high light. For all experiments, explants (6–7 days post-germination) were placed on CIM plates for 5 days, then SIM for 10 days followed by fresh SIM for the remaining ~3 weeks. See the legend for Figure 3 for explanations of percentages, asterisks and error bars, and Supporting Information Table S2

for statistical analysis. Each histogram is the mean of 5–18 replicates (26 cotyledons per replicate).

Figure S4. Effect of mutations on visible anthocyanin accumulation in cotyledon explants four weeks after excision. Explants (6–7 days post-germination) were placed on CIM plates for 5 days, then SIM for 10 days followed by fresh SIM for the remaining ~3 weeks. Explants were exposed to constant high light (~100 μmol m^{-2} s^{-1}) for ~4 weeks, constant low light (~20–30 μmol m^{-2} s^{-1}) for ~4 weeks, 1 or 5 days of darkness followed by constant high light for ~4 weeks. Pictures of representative explants were taken at the end of the experiments. Shown are explants of the following ecotypes: (a-d) wild-type L*er*-0 (CS20), (e-h) *phyA-203* (CS6221), (i-l) *phyB-1* (CS6211), (m-p) *hy1-1* (CS67), and (q-t) *cry1* (*hy4-1*, CS70). The asterisks denote treatment/genotype combinations that resulted in extremely low visible anthocyanin accumulation ($n = 72$).

Figure S5. The effect of a cytokinin-overexpressing transgene (CaMV35S-IPT161) on shoot regeneration and anthocyanin accumulation. (a) Shoot regeneration of explants containing the CaMV35S-IPT161 transgene (pCYT::IPT, CS117) compared to wild-type ecotype C24 (CS906). Four light treatments were used: continuous high light (white, ~100 μmol m^{-2} s^{-1}) for ~4 weeks, continuous low light (light grey, ~20–30 μmol m^{-2} s^{-1}) for ~4 weeks, five days of high light followed by 10 days of darkness followed by continuous high light (white with diagonal lines), and five days of darkness post-excision then continuous high light (black). (b-e) Transgene effects on anthocyanin accumulation. The two treatments were: (b,d) extended high light (100 μmol m^{-2} s^{-1}), followed by extended darkness, followed by high light (double shift), or (c,e) 5 days of darkness after excision and 4 more weeks of high light. For all experiments, explants (6–7 days post-germination) were placed on CIM plates for 5 days, then SIM for 10 days followed by fresh SIM for the remaining ~3 weeks. The error bar is the standard error of the mean (SEM). Total experimental $n = 732$ cotyledons.

Figure S6. Pigment absorbance spectra and light emission spectra. Absorbance spectra of (a) chlorophylls and carotenoids (Buchanan, Gruissem & Jones 2000) and (b) anthocyanins (cyanidin-3-glucoside) (Strack, 1997; Gould *et al*. 2002b) as well as emission spectra of (c) cool white fluorescent light emitted from Sylvania F72T12CW/VHO bulbs and (d) incandescent Sylvania light bulbs (http://www.sylvania.com).

Table S1. Statistical testing for significant differences between mean shoot regeneration rates and regenerative tissue biomass, for initial light/dark treatments.

Table S2. Statistical testing for significant differences between mean shoot regeneration rates for all mutant, filter or pharmacological treatments presented.