

## TECHNICAL ADVANCE

# D-Root: a system for cultivating plants with the roots in darkness or under different light conditions

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## SUMMARY

In nature roots grow in the dark and away from light (negative phototropism). However, most current research in root biology has been carried out with the root system grown in the presence of light. Here, we have engineered a device, called Dark-Root (D-Root), to grow plants *in vitro* with the aerial part exposed to the normal light/dark photoperiod while the roots are in the dark or exposed to specific wavelengths or light intensities. D-Root provides an efficient system for cultivating a large number of seedlings and easily characterizing root architecture in the dark. At the morphological level, root illumination shortens root length and promotes early emergence of lateral roots, therefore inducing expansion of the root system. Surprisingly, root illumination also affects shoot development, including flowering time. Our analyses also show that root illumination alters the proper response to hormones or abiotic stress (e.g. salt or osmotic stress) and nutrient starvation, enhancing inhibition of root growth. In conclusion, D-Root provides a growing system closer to the natural one for assaying Arabidopsis plants, and therefore its use will contribute to a better understanding of the mechanisms involved in root development, hormonal signaling and stress responses.

**Keywords:** root development, light, hormone response, abiotic stress, reactive oxygen species, technical Advance.

## INTRODUCTION

Given their sessile lifestyle, the post-embryonic development of plants is essential for their adaptation to different environments. While adapting to different habitats, the root system plays an essential role. Root system architecture (RSA) is shaped by the three-dimensional distribution of the primary and secondary roots in the soil. The RSA can vary depending on the genotype of the plant (inter- and intra-species), soil composition and the availability of water and mineral nutrients (Lynch, 1995; Malamy, 2005; Gruber *et al.*, 2013). Plants can modulate their RSA by

changing the balance between cell division and differentiation at the root tip, which contributes to differential root elongation, and by altering the number and length of lateral roots (LRs). In Arabidopsis and many crop species, overall root architecture requires the *de novo* organogenesis of LRs, which are specified at intervals along the principal root from a limited number of pericycle cells, called founder cells (Dolan *et al.*, 1993; Casimiro *et al.*, 2003). Founder cells are specified in an auxin-dependent manner and in a regular time-cycle (De Smet *et al.*, 2007; De Rybel

*et al.*, 2010; Moreno-Risueno *et al.*, 2010). Once specified, founder cells, which retain their potential to divide, respond to shootward auxin signaling to initiate a LR primordium that might eventually emerge to develop a new LR (Malamy and Benfey, 1997; Dubrovsky *et al.*, 2008; Lee *et al.*, 2015). Although auxin signaling is essential for root growth and development, several pieces of evidence indicate that other phytohormones, either by modulating auxin activity or signaling independently of auxin, affect root development and RSA (Fukaki and Tasaka, 2009). In addition to hormonal control, formation of a differential gradient of reactive oxygen species (ROS) and redox-mediated signaling in meristems regulate the emergence of LRs and root growth (Tsukagoshi *et al.*, 2010; Manzano *et al.*, 2014; Passaia *et al.*, 2014).

Light is the most important source of energy on the earth. Likewise, light is also a vital signaling cue for plant photomorphogenesis, growth and reproduction during the life of a plant (Kami *et al.*, 2010). In nature, the root system grows in the dark, downward into the soil and away from light (light avoidance). In addition, positive gravitropism reinforces root growth in the dark as they bury underground while following the gravity vector (Mullen *et al.*, 2002). It is particularly intriguing that the majority of light photoreceptors and signaling components, as well as some circadian clock modules, are expressed in roots (Warnasooriya and Montgomery, 2011). In fact, in laboratory conditions, light affects root organogenesis (growth rate and LR production), orientation and pigmentation (Usami *et al.*, 2004; Sassi *et al.*, 2012; Moni *et al.*, 2015). Recently, it was pointed out that root illumination induces a strong burst of response to ROS (Yokawa *et al.*, 2011). As ROS signaling has an important role in controlling RSA (Tsukagoshi *et al.*, 2010; Manzano *et al.*, 2014; Passaia *et al.*, 2014), it is possible that light directly alters root growth and development. Over the last decade, remarkable progress has been made in understanding the molecular and physiological processes that control the development

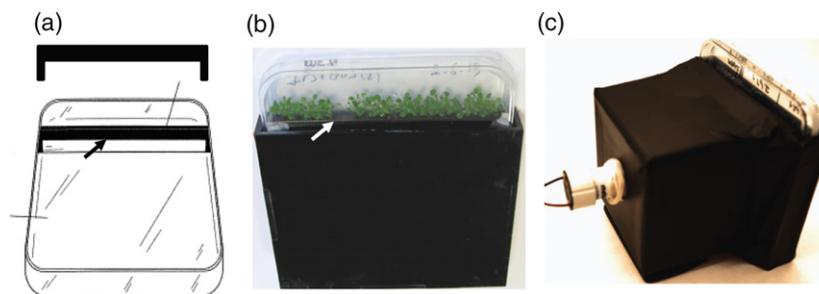
and growth of plant root systems (Kong *et al.*, 2014; Rogers and Benfey, 2015). However, most current research in root biology has been carried out by growing the root system in the presence of light. As roots normally grow underground, light can be considered a non-natural condition. In the case of Arabidopsis, which is typically grown in transparent Petri dishes, root biology studies have exposed roots to light without evaluating the additional effect that light might have on them.

To address to what extent root development and responses are influenced by light, we have engineered a system called Dark-Root (D-Root). In D-Root, the root system can be grown in the dark or under light of a specific wavelength or intensity while the shoot is exposed to standard photoperiods. We have found that light changes root morphology. Light-grown roots generate shorter roots and develop a greater number of emerged LRs than dark-grown roots. Ionomic analyses have shown that light alters ion accumulation, both in the root and the shoot. Furthermore, we demonstrate that light acts as a stress and that its perception by roots modifies their responses to hormonal treatments, abiotic stresses or nutrient deprivation.

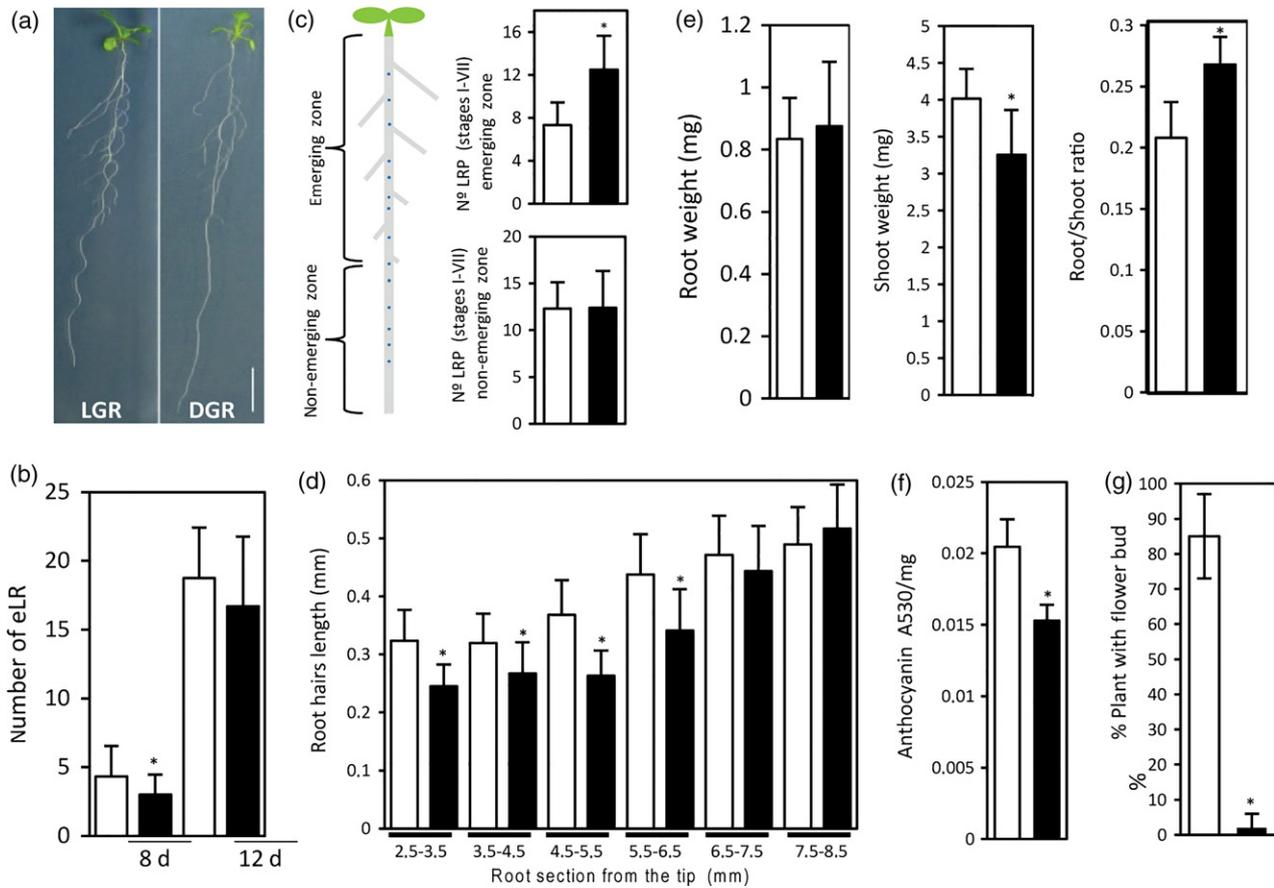
## RESULTS

### D-Root: a device for growing roots in the dark

Roots are an underground tissue with positive gravitropism and negative phototropism. Thus, growing roots in the presence of uniform light, as we normally do in the laboratory, may alter their development, environmental responses and mutant phenotypes, as has been previously suggested (Yokawa *et al.*, 2011). To address this we have engineered a device, D-Root, to grow Arabidopsis seedlings (but not limited to this species) in a Petri dish with the root system in the dark and the shoot in the light (Figure 1a). To simplify root system analysis, Arabidopsis seedlings were grown vertically in a square Petri dish. We designed a black methacrylate box in which the Petri dish



**Figure 1.** The D-Root device allows Arabidopsis seedlings to be grown *in vitro* with the root system in the dark while the shoot is exposed to the light. (a) Diagram of the D-Root device. A methacrylate comb is inserted into the agar. There is a small aperture between the agar and the comb to allow the root to grow. The arrow points to the comb. (b) The Petri dish is inserted into the methacrylate box. The arrow points to the methacrylate comb used to partially block the light coming from the top. (c) Adaptation of the D-Root device to illuminate roots with different wavelengths of light using LED technology or a UV-B lamp (DGR device).



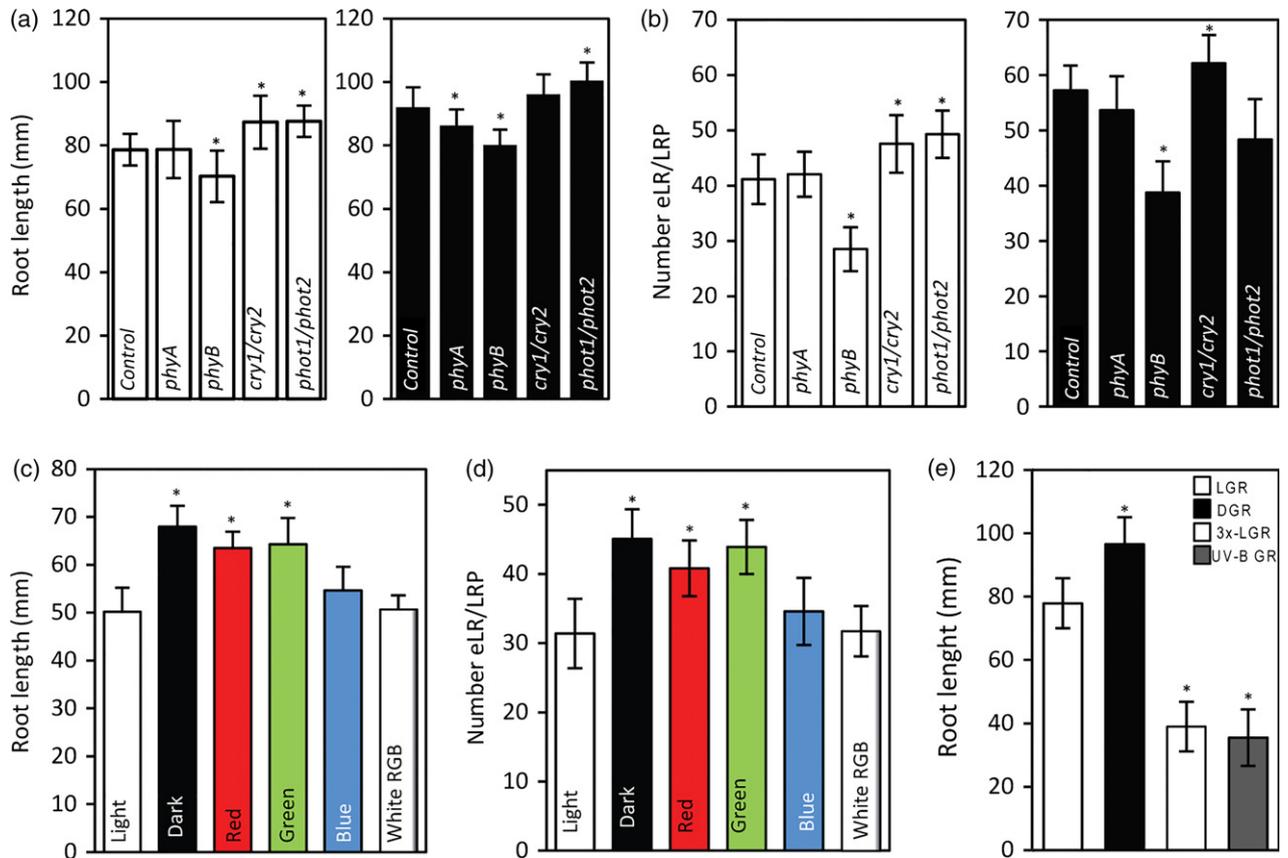
**Figure 2.** The R-Dark device allows *Arabidopsis* seedlings to be grown *in vitro* with the root system in the dark while exposing the shoot to light. (a) Representative pictures of 12-day-old seedlings with roots grown in the light (LGR) or in the dark (DGR). (b) Number of emerged lateral roots (eLR) in 8- or 12-day-old SKP2B::GUS seedlings grown in the LGR or DGR devices. (c) Number of lateral root primordia (LRP; from stages I–VII) in the lateral root emerging and non-emerging zones in 12-day-old LGR or DGR SKP2B::GUS seedlings. (d) Root hair length was measured in different sections along the root, starting from the root tip of LGR and DGR seedlings ( $n \geq 30$ ). (e) Root illumination affects shoot development. Control seedlings were grown for 12 days in the LGR or DGR system and then the dry weight for the root and the shoot (10 plants/sample, 10 samples) were measured ( $n = 60$ ). (f) Anthocyanins were measured as absorbance at 530 nm and referenced per mg of tissue. The mean corresponds to the average of at least six replicates. (g) The percentage of plants that developed flower buds after 4 weeks ( $n \geq 40$ ). Values shown are means  $\pm$  SD. Significance was analyzed by an ANOVA test. \* $P < 0.05$ . The scale bar corresponds to 1 cm.

is introduced to cover the root system. We also designed a black methacrylate comb that was fitted into the agar separating the aerial parts and the root system with the purpose of limiting the amount of light that reaches the roots while they grow downwards (Figure 1a,b). In addition, we have improved D-Root to illuminate roots with different wavelengths, adapting LED lamps with different chromatic lights or a UV-B lamp, or with different light intensities (Figure 1c).

It has been proposed that light is needed for root growth (Laxmi *et al.*, 2008; Xu *et al.*, 2013). To investigate the effect of light on root development, *Arabidopsis* seedlings were grown with the root system in darkness (DGR, dark-grown roots) using the D-Root device, or exposed to light (LGR, light-grown roots) without the methacrylate box but with the comb inserted. The aerial part of the seedlings was grown in a 16-h light/8-h dark photoperiod. Light

reduced root growth by 20–25% (Figure 2a) and promoted the emergence of LRs (Figure 2b), while reducing the total number of non-emerged LR primordia (LRP; Figure 2c). Interestingly, the distribution of LRP along the primary root varied between LGR and DGR. The number of LRP (stages I–VII) in the emergence zone (the root area from the root–shoot junction to the most distal emerged lateral root, eLR) was significantly higher in DGR but was similar in DGR and LGR in the non-emergence zone (Figure 2c). In addition, root hairs closer to the meristem were shorter in DGR (Figure 2d).

Unexpectedly, we also observed changes in the shoots of DGR plants compared with shoots of LGR plants. The former were lighter and accumulated less anthocyanin in the shoot (Figure 2e,f). We also estimated flowering time induction by quantifying the number of plants that showed their first flower bud after 4 weeks of cultivation. The LGR



**Figure 3.** Light affects root growth through photoreceptors.

(a) Root length of 12 day-old light-grown roots (LGR; white bars) and dark-grown roots (DGR; black bars) of controls (SKP2Bp::GUS) and different photoreceptor mutants crossed with SKP2Bp::GUS ( $n \geq 25$ ).

(b) Number of emerged lateral roots (eLR) plus lateral root primordia (LRP) of 12-day-old LGR and DGR SKP2Bp::GUS (control) and different photoreceptor mutants crossed with SKP2Bp::GUS ( $n \geq 25$ ).

(c) Root length of 8-day-old control (SKP2Bp::GUS) plants grown with the roots under different light conditions (LGR, DGR or illuminated with white, red, green blue or a red/green/blue mix using LED lamps) ( $n \geq 20$ ).

(d) Number of eLR plus LRP of 8-day-old control (SKP2Bp::GUS) plants grown with the roots under different light conditions (LGR, DGR or illuminated with white, red, green blue or a red/green/blue mix using LED lamps) ( $n \geq 20$ ).

(e) Root length of 12-day-old LGR, DGR, 3 × LGR and UV-BGR (blue/green/red) SKP2Bp::GUS seedlings. The 3 × LGR: roots were illuminated with a higher white light intensity (three times higher). For UV-BGR: roots were illuminated with a UV-B lamp ( $n \geq 20$ ).

B-GR, blue light grown roots; UV-B GR, ultraviolet-B grown roots. Values shown are means  $\pm$  SD. Significance was analyzed by a one-way ANOVA test. \* $P < 0.01$ . Bars show means  $\pm$  SE.

plants bolted significantly earlier than the DGR plants (Figure 2g). These results suggest the existence of root to shoot signaling mechanisms that appeared to affect general plant development.

### Role of photoreceptors in root development

The majority of photoreceptors and light signaling modules are expressed in roots (Warnasooriya and Montgomery, 2011). As light modifies root growth, we decided to study the role of photoreceptors in root development using D-Root. We found that in the presence of light, only the double *cry1/cry2* or *phot1/phot2* mutants showed longer main roots than control LGR (Figure 3a). In addition, *cry1/cry2* and *phot1/phot2* LGR had a similar length to the DGR control, suggesting that these photoreceptors

largely contribute to reduce root growth in response to light. We did not detect significant changes between LGR or DGR in *phyA* mutants and the respective controls. Conversely, *phyB* roots grew significantly less than control LGR or DGR (Figure 3a), indicating that PHYB is required for root growth. We also quantified the LR system using the SKP2Bp::GUS reporter, which marks eLRs and LRP at all stages of development (Manzano *et al.*, 2012). We found that *cry1/cry2* mutants developed a greater number of eLRs plus LRP (eLR/LRP) in both LGR and DGR conditions than control plants. Conversely, the *phyB* mutant formed fewer eLR/LRP than control plants (Figure 3b). As photoreceptors perceive differences in the quality of light, we decided to analyze the effect of different wavelengths of light on root growth. We adapted D-Root to illuminate roots with blue,

green, red or UV-B light (Figure 3c–e). The length of main roots illuminated with red or green light was similar to DGR, indicating that these types of light are not normally detected by roots. However, blue light, or a mix of the blue, green and red light (White RGB), reduced root growth to a similar extent to white light (Figure 3c). Using the SKP2Bp::GUS reporter line, we found that the total number of eLR/LRP increased when the roots were grown in the dark or under red or green light (Figure 3d). Type B UV light controls many aspects of plant development, including root growth (Tanaka *et al.*, 2002). Growth was significantly reduced when roots were exposed to UV-B light, indicating that UV-B is directly perceived by roots (Figure 3e). The LGR cultivated with a higher light intensity showed a three-fold reduction in root growth, similar to that observed for UV-B-grown roots, supporting the idea that illumination is a stress for roots. Taken together, our results show that light inhibits root growth, likely through perception of blue light and/or UV-B.

#### Accumulation of ions is affected by root illumination

As root illumination modifies RSA, we wondered whether or not light could also alter nutrient uptake. The term ionome refers as all the mineral nutrient and trace elements found in a living organism (Salt *et al.*, 2008). We analyzed the accumulation of different ions in both roots and shoots of LGR or DGR plants. Root illumination statistically reduced the accumulation of potassium, sodium and

molybdate, while iron was the only element that significantly accumulated in LGR roots (Table 1). It therefore appears that light has a negative effect on nutrient accumulation. A similar ionic analysis of shoots showed that iron also accumulated at higher levels in the aerial parts of LGR compared with DGR while other ions showed no significant change (Table 1).

#### Root responses to hormones are affected by light

Phytohormones build a signaling network that controls plant development, including root growth. We decided to evaluate the combined effect of root illumination and hormone treatment on root growth. As shown in Figure 4(a), DGR and LGR displayed differential growth in a medium containing the synthetic hormone 2,4-dichlorophenoxyacetic acid (2,4-D), brassinosteroids, abscisic acid (ABA), cytokinin or 1-*N*-naphthylphthalamic acid (NPA) whereas other hormones treatments did not change root growth.

Auxin plays a pivotal role in root development (De Smet and Jurgens, 2007; Laskowski *et al.*, 2008; Chapman and Estelle, 2009). To evaluate the effect of light in auxin signaling, we grew *Arabidopsis* seedlings in D-Root with the addition of the synthetic auxin 2,4-D or natural indole-3-acetic acid (IAA). Interestingly, we found significant, but opposite, differences in the inhibition of root growth when seedlings were treated with 2,4-D or with IAA. In the presence of 2,4-D, LGR were shorter than DGR, indicating that light eases inhibition of root growth by 2,4-D. However, in

**Table 1** Ion accumulation in roots and shoots of plants grown with the roots illuminated (LGR) or in the dark (DGR)

Ions	Root					Shoot				
	Light		Dark		% diff. L/D	Light		Dark		% diff. L/D
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
B	38.65	11.5	48.78	6.48		43.95	5.7	45.75	6.35	
Ca	3879.25	626.75	3897.75	220.38		5161	388	5647	344	
Cd	0.08	0.07	0.19	0.1		0.05	0.03	0.07	0.02	
Cr	0.48	0.14	0.54	0.12		0.21	0.14	0.2	0.02	
Cu	11.97	4.49	7.35	1.73		3.96	1	3.24	0.6	
Fe	706.5	136	278.5**	88.25	+2.54x**	148.5	13.5	119.5*	3	+1.3x*
K	39466	3148.5	46028.25*	1354.88	-1.16x*	53054	6728	49045.75	452.75	
Mg	1388.25	75.75	1528.5	101.5		2480.25	123.63	2459.75	123.25	
Mn	79.03	16.28	77.48	8.11		188.75	14.25	218	16.5	
Mo	0.76	0.13	1.12**	0.05	-1.47x**	1.58	0.29	1.76	0.26	
Na	662.75	103.25	1034**	59	-1.56x**	1206.25	62.25	1367.25	81.75	
Ni	1.92	1.1	0.84	0.63		0.66	0.35	0.24	0.14	
P	5667	392.5	6339.75	367.13		7171.5	934	7576.5	274.5	
S	6434.75	288.75	6412.25	188.75		6556.5	282.5	6851.25	206.75	
Sr	20.23	3.03	20.55	1.78		12.23	1.13	13.15	0.57	
Zn	305.25	72.88	314	31		110.2	9.8	121	5	

Cr, Chromium; Zn, zinc; Ni, nickel; Ca, Calcium; Sr, Strontium; B, Boron; Mn, magnesium; P, phosphate; Na, sodium; Fe, iron; Mg, magnesium; Cu, copper; K, potassium; Cd, cadmium; S, sulfur; Mo, molybdenum.

All elements are presented as  $\mu\text{g g}^{-1}$  of dry plant. Data represent the average of four independent replicates of pooled *Arabidopsis* roots or shoots grown for 12 days in LGR or DGR devices. SD represents the standard deviation. The asterisk denotes statistical differences by a *t*-test (*P*-value < 0.05).

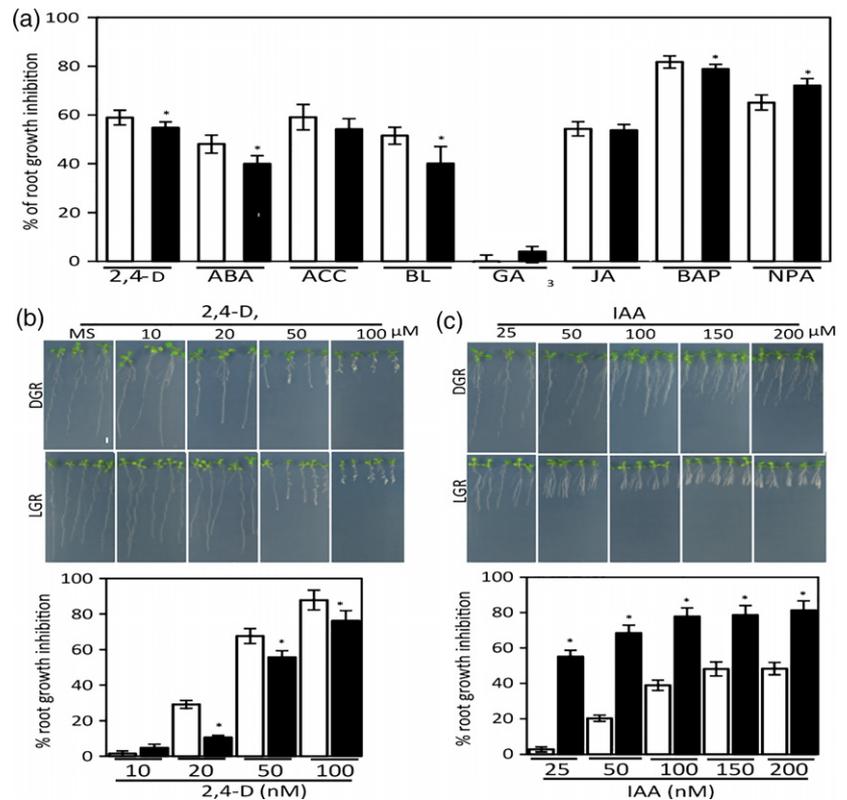
**Figure 4.** Effect of light and hormones.

(a) Arabidopsis seedlings were grown in MS1/2 for 4 days in devices for growing roots in the light (LGR) or dark (DGR). Afterwards, seedlings were transferred to fresh medium containing different hormones [synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D); abscisic acid (ABA); ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC); brassinolide (BL); gibberellic acid ( $GA_3$ ); jasmonic acid (JA); cytokinin, 6-benzylaminopurine (BAP); or auxin transport inhibitor, 1-N-naphthylphthalamic acid (NPA)] for an extra 8 days. The graph represents the percentage of root growth inhibition with respect to the growth in MS1/2 medium.

(b) Inhibition of root growth by treatment of LGR or DGR control seedlings with 2,4-D. The values correspond to the percentage of inhibition with respect to root growth without the hormone ( $n = 30$ ).

(c) Inhibition of root growth by IAA treatment of LGR or DGR seedlings. The values correspond to the percentage of inhibition with respect to root growth without the hormone ( $n = 30$ ).

Significance was analyzed by a one-way ANOVA test. \* $P < 0.05$ . The scale bar corresponds to 1 cm.



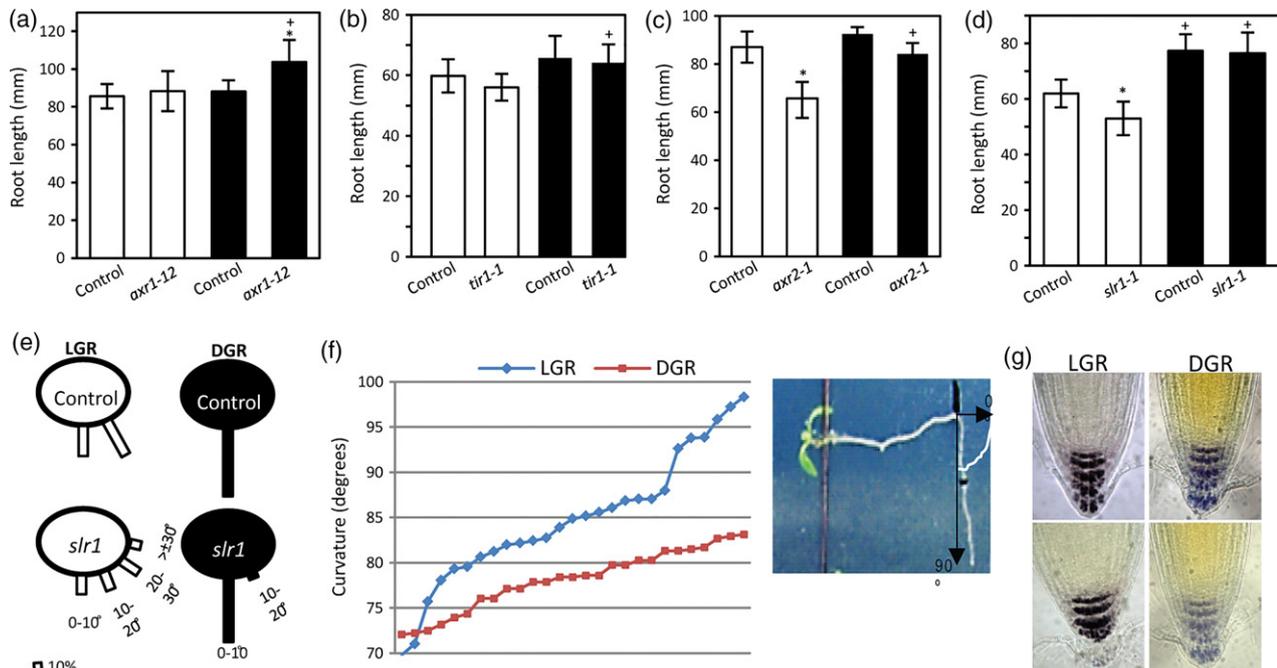
the presence of IAA, LGR were longer than DGR (Figure 2b,c). These data suggest that catabolism of or signaling by 2,4-D and IAA are different in the presence or absence of light in the root, which is in agreement with the idea that IAA is a photolabile molecule (Leasure *et al.*, 2013). As auxin response was significantly different between DGR and LGR, we decided to analyze the effect of light on root growth in various auxin signaling mutants. We found that reduction in root growth mediated by light in *axr1-12* and *tir1-1* mutants was similar to that found in control plants (Figure 5a,b). We also analyzed the number of emerged LR in these auxin signaling mutants. Both DGR *axr1-12* and DGR *tir1-1*, but not DGR control seedlings, developed a significantly higher number of eLR (Figure S1a). Nevertheless, light had a stronger impact on root growth in *axr2* and *slr1* plants. The LGR *axr2-1* and *slr1-1* mutants showed a reduction in root growth of 35 and 28%, respectively, compared with LGR controls (Figure 5c,d). The roots of DGR *axr2-1* mutants were also significantly shorter than the DGR control (Figure 5c). However, DGR *slr1-1* and DGR control showed similar root lengths (Figure 5d). This indicates that *axr2-1* and *slr1-1* mutants are more sensitive to inhibition of root growth by light.

The *slr1-1* mutant displays an agravitropic phenotype (Fukaki *et al.*, 2002). Interestingly, DGR of *slr1-1* showed reduced agravitropic defects compared with *slr1-1* LGR (Figure 5e). We did not find such reversion in the *aux1-7*

mutant, a well-characterized agravitropic mutant (Figure S1b,c). These results suggest that light has a specific impact on *slr1-1* signaling that affects root development. Based on these results, we decide to analyze the gravitropic response in DGR and LGR. We found that illuminated roots responded faster than DGR to gravistimulation (Figure 5f). However, we did not find differences in starch staining in root tips – accumulation was similar in both conditions (Figure 5g). Our results show that root illumination affects auxin signaling and auxin-mediated gravitropic responses in roots.

#### Root illumination alters the responses to salt and osmotic stresses

As light has a profound impact on the production of ROS in roots (Yokawa *et al.*, 2011, 2014; Hasanuzzaman *et al.*, 2013) and abiotic stresses induce the accumulation of ROS (Jaspers and Kangasjärvi, 2010), we wondered whether light might modify the responses of roots to stress. To test this idea we selected salt and osmotic stresses, as they involve ROS production (Tamás *et al.*, 2010; Xie *et al.*, 2011; Kurusu *et al.*, 2015; Wang *et al.*, 2015). In the presence of 100 mM of NaCl, LGR plants showed a 40% inhibition in root growth while DGR plants only showed 27% inhibition (Figure 6a,b). In the case of mannitol treatment (250 mM), root growth of LGR plants was inhibited by 55% while that of DGR plants was inhibited by 44% (Figure 6a,



**Figure 5.** Auxin and gravitropic responses are altered by root illumination.

(a) Root length of 12-day-old light-grown (LGR) or dark-grown (DGR) controls or the *axr1-12* mutant ( $n \geq 20$ ). (b) Root length of 12-day-old LGR or DGR controls or the *tir1-1* mutant ( $n \geq 20$ ). (c) Root length of 12-day-old LGR or DGR controls or the *axr2-1* mutant ( $n \geq 15$ ). (d) Root length of 12-day-old LGR or DGR controls or the *slr1-1* mutant ( $n \geq 20$ ). (e) Gravitropic response of control roots and *slr1-1* LGR or DGR ( $n \geq 30$ ). (f) Gravitropic response of control LGR or DGR after gravistimulation of  $90^\circ$  ( $n = 27$ ). (g) Lugol staining of statoliths in LGR and DGR. Values shown are means  $\pm$  SD. Significance was analyzed by a one-way ANOVA test. \* $P < 0.05$  (mutant compared with control plants); \* $P < 0.01$  (effect of light on similar genotypes).

b). These data suggest that light illumination has an additive negative effect on root growth when combined with salt or mannitol stresses. Interestingly, ROS levels were higher in DGR than in LGR roots (Figure 6c,d). Nevertheless, when plants were treated with salt or mannitol, ROS levels increased in root tips, but, remarkably, these levels were still significantly higher in DGR than in LGR roots (Figure 6c,d).

#### Root illumination alters the response to nitrogen deprivation

It has also been shown that ROS play a role in the response of roots to nitrogen (N) starvation (Shin and Schachtman, 2004). To evaluate the effect of root illumination on root-specific responses to N deprivation, we used D-Root to grow seedlings in media with and without N. As shown in Figure 7(a,b), in the presence of light N-deprived roots were shorter than in the dark, indicating that light has a negative affect on root growth during response to N starvation. We also analyzed whether root illumination affected the plant ionome during N deprivation. We analyzed the differential accumulation of elements in roots and shoots of LGR or DGR plants grown in MS medium with (+N) or without (-N) nitrogen. As shown in Figure S2, light significantly altered the accumulation of several ions,

such as iron, sodium, cadmium or copper, in both shoots and roots during response to N starvation.

#### DISCUSSION

Arabidopsis was established as a model plant almost 70 years ago because its characteristics made it particularly suitable for laboratory work. Since that time, Arabidopsis has been grown in Petri dishes and the vast majority of experimental work in root biology has been done with the root system exposed to light. Recently, some works have highlighted that light appears to have a direct effect on root growth and responses (Yokawa *et al.*, 2014; Meng, 2015; Moni *et al.*, 2015). It has been proposed that light is a positive stimulus for root growth, since etiolated- or soil-grown seedlings developed shorter roots than illuminated plants (Laxmi *et al.*, 2008; Xu *et al.*, 2013). It is possible that soil-grown plants develop shorter roots because they have to overcome soil resistance, but also because photosynthetic activity needs to be established. Closer analysis of etiolated seedlings indicates that the apparent direct effect of light on root growth can be related to deficient auxin transport from the shoot to the root in these seedlings (Sassi *et al.*, 2012). Therefore, the positive effect of light on root development can be related to the necessary development of the aerial part or its photo-mor-

**Figure 6.** Responses of roots to high salt and osmotic stresses are altered by light.

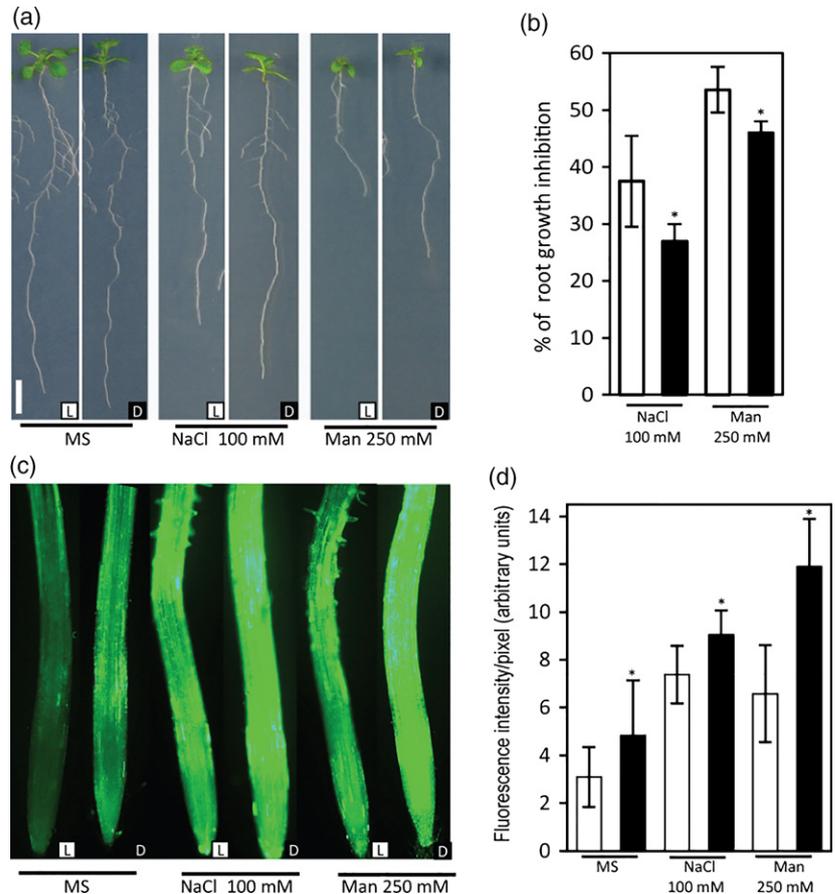
(a) Representative picture of LGR (L) or DGR (D) Arabidopsis seedlings grown in MS1/2 for 4 days and then transferred to a MS1/2 medium containing 0 or 100 mM of NaCl and 0 or 250 mM of mannitol for an extra 8 days.

(b) Percentage inhibition of root growth in LGR and DGR seedlings by the effect of salt or mannitol grown as in (a) ( $n \geq 30$ ).

(c) Representative pictures of roots from 12-day-old LGR and DGR Arabidopsis and those treated with NaCl or mannitol stained with fluorescein diacetate to visualize reactive oxygen species.

(d) Graph representing the average fluorescence intensity in the root tips in (c) per pixel per 100 000 (arbitrary units) ( $n = 12$ ).

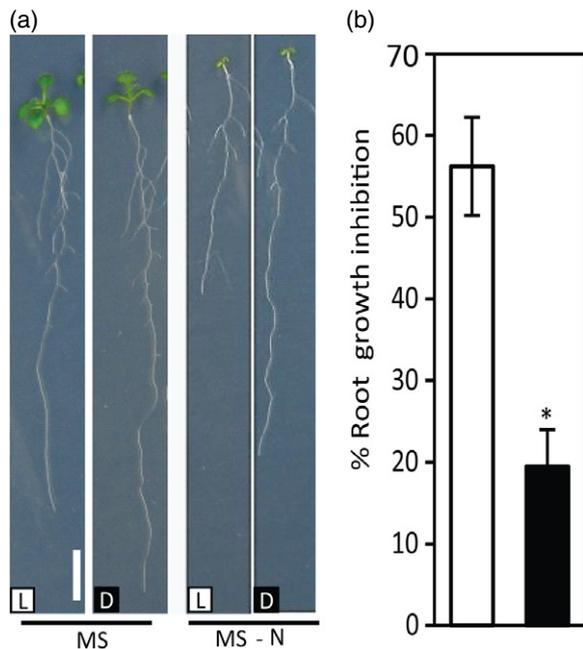
Significance was analyzed by a one-way ANOVA test. \* $P < 0.05$ . Scale bars = 1 cm.



phogenesis that, in turn, would activate auxin transport or other shoot-derived signals. Here, we have engineered a device for cultivating plants with the shoot growing under a normal photoperiod (light–dark cycles) while the roots are maintained in the dark or specific light conditions. Using this system, we present convincing evidences that light reduces root growth, promotes the emergence of LR and reduces LRP specification, overall altering the RSA of the plant. Our data also support the view that root illumination generates a stress that might be additive to other stresses, such as high salt or mannitol or nutrient deprivation.

Changes in RSA might lead to different nutrient/ion uptake. It is likely that roots and shoots continuously communicate to coordinate nutrient demand and uptake, and to respond to environmental changes. Recently, it has been shown that RSA is highly dependent on nutrient availability (Gruber *et al.*, 2013; Kellermeier *et al.*, 2014). The combination of different nutrient deficiencies did not significantly affect the nutritional status in the shoot compared with an individual deficiency, except for iron which seems to be additive to phosphate or nitrate starvation (Kellermeier *et al.*, 2014). These authors also found differences in the RSA in plants grown under one or more nutri-

ent deficiencies and different photoperiod lengths. However, the effect of light on roots was not considered. Using D-Root, we show that root illumination alters ion accumulation. Light reduces the accumulation of potassium, sodium and molybdenum in the root while significantly increasing the uptake of iron in both the root and the shoot. Iron is the only ion differentially accumulated in the shoot. Iron solubilization occurs through redox reactions (Geisler *et al.*, 2011). As light can photo-catalyze the production of ROS in roots, it is possible that iron accumulation occurred in LGR as a result of activation of certain ROS in the root system but also through modification of iron uptake, transport and storage mechanisms. Iron is an essential element for all organisms and may be a growth-limiting resource for plants as it is a basic element in redox processes during photosynthesis and respiration. Remarkably, light increases the rate of uptake of iron in *Microcystis aeruginosa* (Fujii *et al.*, 2011), a freshwater cyanobacterium, and in some strains of *Prochlorococcus*, small marine cyanobacteria (Thompson *et al.*, 2011). In addition, a mitochondrial iron transporter is essential for the growth of rice (Bashir *et al.*, 2011). According to the endosymbiotic theory, chloroplasts of plants and eukaryotic algae evolved from cyanobacterial ancestors. Based



**Figure 7.** Illumination of roots alters the response to nitrogen deprivation. (a) Representative pictures of control seedlings grown in MS1/2 medium with (MS+N) or without nitrogen (MS-N). (b) Percentage inhibition of root growth of light-grown (LGR, white bar) and dark-grown (DGR, dark bar) control plants grown in a medium without nitrogen for 12 days with respect to a medium with nitrogen ( $n \geq 30$ ). Values shown are means  $\pm$  SD. Significance was analyzed by a one-way ANOVA test. \* $P < 0.01$ . Scale bars = 1 cm.

on this, it is tempting to speculate that regulation of iron uptake by light might have been maintained during this endosymbiotic process and through evolution.

Plant development is largely controlled by the function of different hormones and their interactions (Santner and Estelle, 2009). Among plant hormones, auxin plays a key role in controlling many biological processes, including root development (Del Pozo and Manzano, 2014). In many experiments, hormones are added to the medium to analyze their effect on root growth and responses. Using D-Root we have found that DGR respond differently to hormone treatments than LGR. Thus, root illumination might alter the results of experiments that imply the use of hormones, masking phenotypes and/or responses. This is well exemplified for auxin: DGR have a higher hypersensitivity to natural auxin than LGR, probably due to the fact that IAA is not photodegraded. Remarkably, we found that DGR of *axr1-12* and *tir1-1* seedlings developed a higher number of eLR than controls. These mutants also increased the number of eLR in response to a higher concentration of auxin in the medium (Knee and Hangarter, 1996; Ruegger *et al.*, 1998). Thus, taking in account that IAA is photolabile, DGR might accumulate a higher amount of IAA, increasing the auxin response. In the root meristem, an auxin maximum, which is located in the quiescent

center and adjacent cells (Pettersson *et al.*, 2009), is formed by polar auxin transport through plasma membrane-localized carriers, which can function as auxin efflux carriers (PIN-FORMED proteins) or as influx auxin carriers (AUX/LAX proteins). In etiolated plants, PIN2 is primarily detected in vacuolar compartments of root cells, while in LGR PIN2 is predominantly localized in the plasma membrane (Laxmi *et al.*, 2008). These defects were also observed in aerial parts of seedlings which had not undergone photo-morphogenesis (Sassi *et al.*, 2012), indicating that auxin transport from the shoot to the root is required for root development and can act as a shoot signal upon light perception.

An improved plant growth (IPG) system, in which roots can be grown in the dark, was previously presented by Xu *et al.* (2013). These authors showed that expression of PIN2 protein was reduced and delocalized from the plasma membrane into endosomal-like compartments when roots were grown in the dark as compared with roots grown in the presence of light (Xu *et al.*, 2013). Sassi *et al.* (2012) showed that PIN2 de-localization also relates to changes in the shoot rather than root illumination. It should be highlighted that in the IPG system the shoot is grown outside of plates and the roots inside the *in vitro* plate, generating different environmental conditions than in D-Root, which might contribute, at least in part, to the alteration of PIN2 accumulation and distribution. Using the D-Root device, we found that PIN2-GFP levels or localization did not change between LGR and DGR plants (Figure S3), reinforcing the idea that shoot illumination, rather than root illumination, controls PIN2 levels and polarity.

Root illumination induces a burst of ROS (Yokawa *et al.*, 2011). Plants are sessile organisms that have to cope with the surrounding environment and its stresses. One of the most general and well-characterized responses to various stresses is the generation of ROS (Jaspers and Kangasjärvi, 2010). Among other processes, high ROS levels might influence the balance between cell division and differentiation, root growth and LR emergence (Tsukagoshi *et al.*, 2010; Manzano *et al.*, 2014; Passaia *et al.*, 2014). In addition to being a signal, ROS are strong oxidizers that react with a large variety of biological molecules that may result in severe damage to plant tissues (Petrov and Van Breusegem, 2012). For this reason plants also generate ROS scavengers to counteract the excess of ROS and balance developmental and physiological responses (Cruz de Carvalho, 2008). It is noteworthy that ROS levels were slightly higher in DGR than in LGR plants. This difference could be due to the induction of scavengers in the presence of light. These ROS scavengers might also limit accumulation of ROS during stress responses, as we observed after subjecting LGR plants to salt or osmotic stresses.

In conclusion, we think that the use of D-Root to study root development and physiological responses will give

more reliable and realistic results that will be helpful to discriminate the effect of light on root responses. Based on our results, the D-Root system could also be applied to shoot studies. Our results also highlight the fact that illumination of normally underground parts of the plant is a condition that influences growth and responses of the whole plant. Finally, we consider that light can be a stress for root development and recommend the use of D-Root to prevent illumination of roots in future studies.

## EXPERIMENTAL PROCEDURES

### Materials

The SKP2Bp::GUS marker was used to count emerged LRs and LRP as described in Manzano *et al.* (2012). All the mutants, *phyA-211* (Reed *et al.*, 1994), *phyB-9* (Reed *et al.*, 1993), *cry1-104/cry2-1* (Guo *et al.*, 1998), *phot1/phot2* (Jarillo *et al.*, 2001) and PIN2: PIN2-GFP (Bililou *et al.*, 2005) are in Columbia ecotype. All seedlings were sown under sterile conditions on vertically oriented 12 cm × 12 cm plates containing half-strength Murashige and Skoog (MS1/2) with 0.05% 2-(*N*-morpholone)-ethanesulfonic acid (MES), 1% sucrose and 1% plant-agar (Duchefa Biochemie B.V., <https://www.duchefa-biochemie.com/>) under a 16-h light/8-h dark photoperiod at 21/18°C. To prepare MS without nitrogen we used MS1/2 modified basal salt mixture (PhytoTechnology, <http://phyto-techlab.com/>) minus nitrogen.

### The D-Root device

The D-root device was developed to grow *Arabidopsis* seedlings with the roots in darkness and the shoot in the light. The device is a methacrylate box in which a square plastic plate is inserted (Figure S4). The dimension of the D-Root depends on the size of the square plastic plate used. For this work, we designed a box using 3 mm thick black methacrylate and a comb using 8 mm thick black methacrylate. The square plates were filled with 60 ml of the corresponding medium. The manipulation and assembly of the D-Root is shown in Video S1.

### Light-grown and dark-grown roots

**Light-grown roots.** *Arabidopsis* seedlings were grown in vertically square plates with white light illumination ( $75 \mu\text{mol m}^{-2} \text{sec}^{-1}$ ) under a 16-h light/8-h dark photoperiod at 21/18°C without using the methacrylate box. To reproduce the experimental conditions in the D-Root, the methacrylate comb was inserted into the agar, similar to the DGR setup. To increase the light intensity in the root (by three times), three fluorescents were positioned laterally to the aperture of the methacrylate box to illuminate the roots alone with  $280 \mu\text{mol m}^{-2} \text{sec}^{-1}$ .

**Dark-grown roots.** The square plate was introduced into the methacrylate box and the methacrylate comb was inserted into the agar to block the light from the top fluorescents. Using a maximum–minimum thermometer we checked the temperature in both LGR and DGR, which was similar in both devices.

**Red-, green- or blue-grown roots.** The roots were illuminated with a red, green or blue LED (intensity,  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ : red, 28; green, 30; blue, 35; red/green/blue, 50) under a 16-h light/8-h dark/photoperiod.

**Ultraviolet-B-grown roots.** *Arabidopsis* seedlings were germinated in half-strength MS medium for 3 days, and then the plates were transferred to UV-B lamp illumination for nine extra days (B-1000 UVP 100 W, 365-nm long wave, intensity  $2 \text{ mW cm}^{-2}$ ). Roots were UV-illuminated from 9:00 a.m. to 9 p.m. with 20-min pulses every 3 h.

### Root growth assays and microscopic analysis

Primary root length was determined as described previously by Lucas *et al.* (2011). To count LRP we used the SKP2Bp::GUS marker lines (Manzano *et al.*, 2012). This line was stained for GUS activity as described and GUS-positive LRP were counted. Confocal images were taken using a Leica SP8 system (<http://www.leica.com/>). To quantify the GFP levels, the Leica AF lite was used. Roots were stained with  $1 \mu\text{g ml}^{-1}$  of propidium iodide.

### Hormone, salt and mannitol treatment

*Arabidopsis* seedlings were grown in MS1/2 for 4 days in LGR or DGR devices. Afterwards, seedlings were transferred to fresh medium without any hormone (MS) or containing  $1 \mu\text{M}$  2,4-D (auxin),  $10 \mu\text{M}$  ABA,  $5 \mu\text{M}$  1-aminocyclopropane-1-carboxylic acid (ACC, ethylene precursor),  $1 \mu\text{M}$  brassinolide (BL),  $5 \mu\text{M}$  gibberellic acid ( $\text{GA}_3$ ),  $5 \mu\text{M}$  JA,  $1 \mu\text{M}$  6-benzylaminopurine (BAP, cytokinin) and  $10 \mu\text{M}$  NPA for an extra 8 days. The transferred seedlings were grown in LGR or DGR devices. For the salt treatment and mannitol treatments, *Arabidopsis* seedlings were grown in MS1/2 for 4 days in LGR or DGR devices and transferred to 100 mM or 140 mM of NaCl or 250 mM or 300 mM of mannitol for an extra 8 days in LGR or DGR devices.

### Determination of ROS

*Arabidopsis* plants were submerged in an aqueous solution containing 0.01% Triton X-100 and  $2.52 \text{ mg ml}^{-1}$  of fluorescein diacetate for 7 min. Afterwards, roots were washed in three volumes of deionized water for 7 min and then mounted in deionized water and a picture was taken using a DFC380C camera (Leica) coupled to a Leica 2000 fluorescence microscope. To determine the ROS levels, the fluorescence was quantified using a Photoshop 7 application. Intensity in the green channel was measured in 20 independent roots and represented as an average and deviation of the average.

### Anthocyanin determination

*Arabidopsis* plants were grown MS1/2 medium for 12 days. Anthocyanin was extracted and quantified as described by Swain and Hillis (1959) using 10–15 leaves per replicate. The amounts of anthocyanin were calculated as absorbance at 530 per mg of tissue used. The results correspond to the average of five different biological replicates.

### Ion detection

Wild-type *Arabidopsis* seedlings were grown in MS1/2 with (+N) or without nitrogen (–N) in the LGR or DGR devices for 12 days. In the –N case, potassium was corrected by adding KCl. Then, roots and shoot were collected independently and dried at 100°C for 24–48 h in an air-oven. Sample digestions were carried out with 3 ml of  $\text{HNO}_3$  (Sigma-Aldrich Trace Metal grade, <http://www.sigmaaldrich.com/>) at 100°C in a thermostatically controlled bath for 4 h. Each sample was diluted to 10 ml with Milli-Q water. Subsequently, samples were analyzed by inductively coupled plasma-optical emission spectrometry (ICP-OES) on a Thermo Sci-

entific iCAP 6500 (<http://www.thermoscientific.com/>) using the following wavelengths for each element: B, 208.9 nm; Ca, 184.0 nm; Ca, 317.9 nm; Cd, 228.8 nm; Cr, 206.1 nm; Cu, 324.7 nm; Fe, 240.4 nm; Fe, 259.9 nm; K, 766.4 nm; Mg, 279.0 nm; Mg, 285.2 nm; Mn, 293.9 nm; Mo, 203.8 nm; Na, 589.5 nm; Ni, 231.6 nm; P, 177.4 nm; P, 178.2 nm; S, 182.0 nm; Sr, 216.5 nm; Zn, 206.2 nm.

### Statistical analyses

The data were statically analyzed by one-way ANOVA using the online tool [http://statistica.mooo.com/OneWay\\_Anova\\_with\\_TukeyHSD](http://statistica.mooo.com/OneWay_Anova_with_TukeyHSD). Univariate analyses were performed with Tukey's post hoc test.

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### SUPPORTING INFORMATION

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

**Figure S1.** Effect of light on auxin transport and signaling mutants.

**Figure S2.** Differential ion accumulation during nitrogen deprivation.

**Figure S3.** PIN2 level or distribution is not affected by light or darkness conditions.

**Figure S4.** Dimensions of the D-Root system.

**Video S1.** Assembling the D-Root system.

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