

Light Quality Regulates Lateral Root Development in Tobacco Seedlings by Shifting Auxin Distributions

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Abstract Light is an important environmental regulator of diverse growth and developmental processes in plants. However, the mechanisms by which light quality regulates root growth are poorly understood. We analyzed lateral root (LR) growth of tobacco seedlings in response to three kinds of light qualities (red, white, and blue). Primary (1°) LR number and secondary (2°) LR density were elevated under red light (on days 9 and 12 of treatment) in comparison with white and blue lights. Higher IAA concentrations measured in roots and lower in leaves of plants treated with red light suggest that red light accelerated auxin transport from the leaves to roots (in comparison with other light qualities). Corroborative evidence for this suggestion was provided by elevated *DR5::GUS* expression levels at the shoot/root junction and in the 2° LR

region. Applications of N-1-naphthylphthalamic acid (NPA) to red light-treated seedlings reduced both 1° LR number and 2° LR density to levels similar to those measured under white light; *DR5::GUS* expression levels were also similar between these light qualities after NPA application. Results were similar following exogenous auxin (NAA) application to blue light-treated seedlings. Direct [³H]IAA transport measurement indicated that the polar auxin transport from shoot to root was increased by red light. Red light promoted *PIN3* expression levels and blue light reduced *PIN1*, *3–4* expression levels in the shoot/root junction and in the root, indicating that these genes play key roles in auxin transport regulation by red and blue lights. Overall, our findings suggest that three kinds of light qualities regulate LR formation in tobacco seedlings through modification of auxin polar transport.

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Introduction

Plants have diverse adaptive mechanisms for adapting to environmental changes. The developmental plasticity of root systems is a crucial adaptive trait of terrestrial plants. Growing roots continually branch and form lateral roots (LRs). LRs develop from founder cells in the pericycle, the outermost layer of the root vascular cylinder (stele) (De Smet and others 2006). Auxin has a dominant role in both the specification of founder cells that initiate LRs and the later stages of LR development (Forde 2002; De Smet and others 2006). A differentiated distribution of auxin is required for LR organogenesis.

Diverse endogenous and environmental signals are integrated to mediate changes in auxin distribution by altering the hormones polar transport, which regulates LR formation (Vanneste and Friml 2009; Lavenus and others 2013). Auxin is synthesized mostly in aboveground tissues (for example, shoot apices and young leaves) (Ljung and others 2001) and redistributed through the interaction of auxin influx carriers, such as the AUX1/LAX family proteins, and auxin efflux carriers, including PIN and the ABCB/PGP family proteins (Friml 2003; Blakeslee and others 2005; Zazimalova and others 2010; Peret and others 2012). Among these auxin transporters, the PIN efflux carrier proteins have polar localization at the plasma membrane; their polarity determines the direction of auxin flux (Wisniewska and others 2006).

Light is an important environmental signal that regulates diverse growth and developmental processes in plants. Multiple hormonal pathways in these processes are often modulated by light to mediate developmental changes (Lau and Deng 2010). It has become increasingly clear that light conditions may alter auxin homeostasis by modifying its biosynthesis and degradation; light also regulates auxin distribution, which coordinates plant developmental responses to shifting light environments (Lau and Deng 2010; Sassi and others 2013).

Plants are able to adapt to a varying light environment, including changes in photoperiod, light intensity, and light quality. Among wavelengths, red and blue spectral components are not only the most effective for photosynthesis (McCree 1972) but they also markedly affect plant morphogenesis; for example, by influencing the growth of lateral and adventitious roots (Thomas and Dickinson 1979; Cosgrove 1981; Mortensen and Strømme 1987; Reed and others 1994; Poudel and others 2008; Lin and others 2013). Light signals are detected in the model plant *Arabidopsis* by three types of sensory photoreceptor: phytochrome, phototropin, and cryptochrome (Chen and others 2004; Christie 2007). Root-localized photoreceptors

regulate root growth; in addition, shoot-localized photoreceptors respond to intercepted light signals by transmitting long-distance signals to regulate root development (Salisbury and others 2007). Furthermore, LR formation shifts in photoreceptor mutants are linked to changes in shoot–root transport of auxin mediated by several *PIN* expression changes at the transcriptional level (Salisbury and others 2007; Zeng and others 2010; Moni and others 2014). Thus, auxin polar transport appears to participate in light quality-regulated root growth in *Arabidopsis*. However, the literature on root growth regulation by light quality in diverse plant species and experimental conditions is contradictory. For example, red light is more effective than blue light in promoting cherry explant adventitious root elongation and less effective in adventitious root formation (Iacona and Muleo 2010); however, red light induces twice as many adventitious roots in bean explants than blue light (Fletcher and others 1965). Hence, the mechanisms by which light quality regulates root growth remain poorly understood.

Tobacco (*Nicotiana tabacum* L.) is an important economic plant in China. Lateral roots make up the bulk of the root system of its seedlings, which lack the obvious tap root found in most dicotyledonous plants. Artificial light sources are often used in spring to provide supplementary illumination for industrial-scale tobacco seedling production when the sky is continually overcast. The effect of light quality on tobacco seedling growth, including LR development, has not been fully explored even though a well-developed root system in the early stages of development is crucial for seedling survival after transplantation and for promoting optimal root system formation throughout the life of the plant. The extent to which light quality mediates auxin polar transport in the modulation of LR growth remains obscure. In this study, we monitored LR development in tobacco seedlings under red, blue, and white lights over 12 days. We determined auxin concentrations in tobacco seedling leaves and roots by high-pressure liquid chromatography (HPLC). Transgenic plants carrying the auxin reporter construct *DR5::GUS* were used to identify the pattern of auxin distribution and determine the way in which this pattern is affected by light quality. In addition, we examined the effects of (i) the auxin polar transport inhibitor N-1-naphthylphthalamic acid (NPA) on seedlings held under red light and (ii) exogenous auxin NAA on seedlings held under blue light, respectively. The expression levels of the auxin efflux carrier *PIN* were also analyzed by real-time quantitative reverse transcription (qRT) PCR. We determined that light quality regulates tobacco LR development, at least in part, by shifting auxin distribution within the plant.

Materials and Methods

We used the tobacco cultivar K326 in this study. Seeds were germinated in trays filled with a mixture of peat and vermiculite (v:v, 1:1), and were held in a greenhouse under natural light with day/night temperatures of 28/22 °C. We transferred individual 25-day-old seedlings of uniform size and vigor through holes in the lids into plastic boxes (ten holes per pot with one seedling per hole) filled with one-quarter strength Hoagland's nutrient solution (Hoagland and Arnon 1950). The nutrient solution was renewed daily and adjusted to pH 6. After transplantation to the nutrient solution, tobacco seedlings were moved into three closed cabinets equipped with light-emitting photodiodes (LEDs; Qingdao Caled Photoelectric Technology Co., Qingdao, China) that generated white, blue (maximum at 448 nm), and red (maximum at 660 nm) illumination, respectively. LEDs' spectral energy distribution (Fig. S1) was determined with a calibrated spectroradiometer (ASD FieldSpec HandHeld, Boulder, CO, USA). Before starting the experiment, we tightly wrapped the bottoms and sides of the pots in aluminum foil to block light penetration. All the treatments were kept on a 16/8-h light/dark photoperiod with an above-canopy irradiance of about 108 W m^{-2} (measured daily), maintained by adjusting the heights of the lamps. Each treatment included 12 replicates arranged in a randomized design to avoid edge effects. Tobacco plants were harvested at different times in the 12-day period following treatment. We flash-frozen plant samples and held them in a freezer at -40 °C prior to determining IAA and the relative expression levels of genes.

Treatments with exogenous auxin NAA (α -naphthylacetic acid, dissolved in 1 M NaOH) and the auxin transport inhibitor NPA (N-1-naphthylphthalamic acid, dissolved in dimethylsulfoxide [DMSO]) (Thomson and others 1973; Reed and others 1998) were applied via the plant growth media. We grew 25-day-old tobacco seedlings for 12 days in hydroponic medium containing NAA or NPA.

Measurements of Root System Architecture

Although tobacco is dicotyledonous, the taproot is not well developed. We observed significant development in primary (1°) and secondary (2°) LR but no changes in taproots after treatments in hydroponic culture. We therefore chose to measure LR responses to light quality. We measured the lengths of 1° LRs with a ruler. Average lengths of 2° LRs were determined with a WinRhizo scanner-based image analysis system (Regent Instruments, Montreal, QC, Canada). We counted the number of LRs. Secondary LR density was obtained as the quotient of 2° LR number divided by the 1° LR length.

Measurements of Indole-3-acetic Acid (IAA)

IAA content was determined by HPLC. We obtained fresh weights of samples, which were then immediately frozen in liquid nitrogen. Free IAA was purified and quantified following the procedure of Song and others (2013). A standard IAA sample was obtained from Sigma-Aldrich (St Louis, MO, USA).

GUS Histochemical Assay

Wild-type (cv. SR1) and *DR5::GUS* transgenic seeds were kindly provided by Alice Y. Cheung (University of Massachusetts, Amherst, MA, USA) and Tao Lizhen (South China Agricultural University, Guangzhou, China). *DR5* and the natural *GH3* promoter in tobacco have similarly strong auxin responsiveness (Tao and others 2002). We treated tissues with ethanol prior to observation to remove chlorophyll pigmentation. Stained tissues were photographed under an Olympus SZX2-ILLK stereomicroscope equipped with a color CCD camera (Olympus, Tokyo, Japan).

[^3H]IAA Transport Assay

[^3H]IAA polar transport was assayed after light treatment as described by Song and others (2013). The [^3H]IAA solution contained $0.5 \mu\text{M}$ [^3H]IAA (20 Ci mmol^{-1}) in 2 % dimethylsulfoxide (DMSO), 25 mM MES (pH 5.2), and 0.25 % agar. [^3H]IAA solution ($20 \mu\text{L}$) was applied to the cut surface after tobacco shoots were removed at 2 cm above the root–shoot junction. After an 18 h (overnight) incubation in darkness, ten replicate roots were sampled and weighed. Root samples were incubated in scintillation solution (4 mL) for 18 h. [^3H]IAA radioactivity was detected using a Beckman Coulter LS6500 Multipurpose Scintillation Counter.

qRT-PCR Analysis of Gene Expression

Transcript levels of the tobacco auxin carriers *NtPIN1* (KC347302.1), *NtPIN1b* (KC460399.1), *NtPIN3* (KC425459.1), *NtPIN3b* (KC438370.1), *NtPIN4* (KC433529.1), and *NtPIN9* (KC433528.1) were compared to that of *NtL25* (L18908.1), a stable reference gene (Schmidt and Delaney 2010). We extracted total RNA from the root–shoot junctions and roots of tobacco seedlings after exposure to different light qualities for 12 days. RNA extraction, reverse transcription, and qRT-PCR followed procedures of Chen and others (2012). Primer sets for *PIN* genes and the reference gene are listed in Supplementary Data Table S1. Because some *PIN* genes are closely similar, we first designed these primers manually, then tested them using the Primer 5 software, and validated them experimentally. We used a threefold replication.

Data Analysis

Data were pooled for calculation of means and standard errors (SE) and then subjected to one-way analysis of variance, followed by LSD multiple comparisons tests to detect significant pairwise differences ($p < 0.05$) between means. We used the SAS 9.2 software (SAS Institute; Cary, NC, USA) for all statistical analyses.

Results

Plant Growth and Root Morphology

Plant growth responses were distinctly different between the three light quality treatments (Fig. 1). Fresh weights of shoots and roots were higher under red light and lower under blue light in comparison with weights under white light. However, shoot to root ratios were not significantly different among treatments. LR growth differed markedly among light conditions (Fig. 1d–g). Compared to white light, 1° LR number was elevated by 17 % under red light and reduced by 23 % under blue light. However, average 1° LR length was reduced by 13 % under red light and elevated by 18 % under blue light. Trends were similar for 2° LR formation and elongation. For example, 2° LR density was markedly enhanced by red light and reduced by blue light (compared to white light). Average 2° LR length was reduced by red light and enhanced by blue light. Based on the simplicity and precision of measurement, we selected LR formation (including 1° LR number and 2° LR density) as an index of LR growth in subsequent experiments.

Time-course Trends in LR Formation

We examined LR time-course trends under three light quality treatments over 12 days following transplantation into hydroponic media (Fig. 2). 1° LR number increased most rapidly under red light; on days 9 and 12, values under red light were elevated by about 17 % over values under white light (Fig. 2a). Conversely, blue light treatment reduced 1° LR number by about 22 % on days 9 and 12 in comparison to white light (Fig. 2a). 2° LR density responses tracked similar trends (Fig. 2b).

Endogenous IAA Concentration and Distribution in the Shoot/Root Junction and Root

The phytohormone auxin acts as a common integrator that regulates LR formation in response to varying light conditions (Salisbury and others 2007; Sassi and others 2013).

To determine whether auxin is involved in light quality effects on LR formation, we first analyzed IAA concentration in the leaves and roots of tobacco seedlings under red, blue, and white lights (Fig. 3). Values in leaves were significantly reduced (by 20 %) and markedly increased (by 15 %) under red and blue lights, respectively, in comparison with white light treatment (Fig. 3a). These relationships were reversed in roots: concentrations under red light were elevated by 32 % and reduced by 33 % under blue light relative to the values under white light (Fig. 3b). Thus, red and blue lights appear to promote and inhibit auxin polar transport from the leaves to roots, respectively.

We subsequently used transgenic tobacco plants expressing the *DR5::GUS* construct to examine the effect of light quality on the distribution of auxin (Fig. 4). Results were congruent with those in Fig. 3: red and blue lights promoted and inhibited the expression of *DR5::GUS* in the shoot/root junction and in the 2° LR region, respectively (in comparison with white light), suggesting once more that the red and blue lights promote and inhibit auxin polar transport from the leaves to roots, respectively.

Effects of NAA and NPA Application on LR Formation

We examined LR formation responses to the application of (i) exogenous NAA and (ii) the auxin polar transport inhibitor NPA to determine whether auxin distribution (regulated by light quality) was responsible for below-ground changes in tobacco seedling morphology (Fig. S2, Fig. 5). 1° LR number and 2° LR density in red light-treated tobacco seedlings decreased significantly with increasing concentration of applied NPA (range tested: 0–160 nM) (Fig. S2). Values of 1° LR number and 2° LR density in seedlings held under red light and treated with 20 nM NPA fell to those of seedlings under white light. LR formation was promoted and inhibited in seedlings under blue light and treated with NAA concentrations of (i) 5–10 nM and (ii) 20 and 40 nM, respectively (Fig. S2). Application of 10 nM NAA to blue light-treated seedlings increased 1° LR number and 2° LR density values to those of seedlings under white light. Moreover, the expression levels of *DR5::GUS* in the shoot/root junction and in the 2° LR region of seedlings (i) under red light and treated with NPA and (ii) under blue light and treated with NAA were similar to those of seedlings under white light; these observations are in agreement with the changes in LR formation. Thus, light quality appears to regulate LR growth, at least in part by shifting auxin distributions within tobacco seedlings.

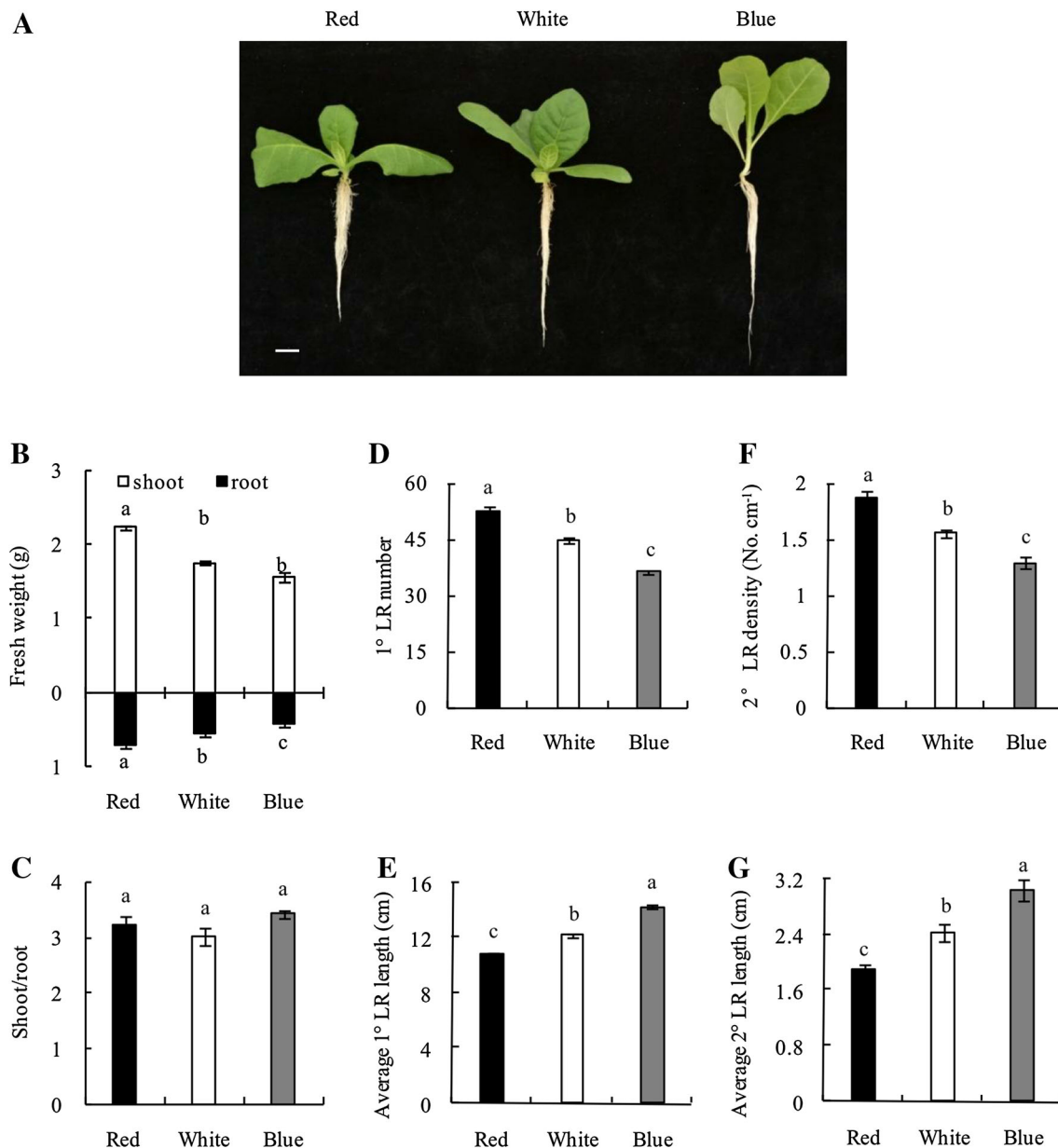


Fig. 1 Phenotypes of tobacco seedlings in response to three kinds of light qualities. Seedlings were grown for 12 days in hydroponic media under *red*, *white*, and *blue* lights. Scale = 2 cm. Values are

mean \pm SE; different *lower case letters* indicate significant differences between means ($p < 0.05$; ANOVA)

[³H]IAA Transport

To monitor changes in auxin levels in tobacco roots, we measured direct auxin transport using radiolabeled IAA in response to three light color treatments (Fig. 6). [³H]IAA solution was applied to the cut surface after 12 days of light treatment. After an 18 h incubation in darkness, [³H]IAA radioactivity was detected in the roots. Values in roots were significantly increased (by 54.5 %) and markedly reduced (by 40.4 %) under red and blue lights,

respectively, in comparison with white light treatment. This result confirmed the increased polar auxin transport from shoot to root by red light (Fig. 3).

Expression of *PIN* Genes in the Shoot/Root Junction and Root

We demonstrated that LR formation in tobacco seedlings is highly dependent on auxin transport from the shoot (largely through the polar transport stream), a mechanism that is

Fig. 2 Time courses of change in **a** primary lateral root (1° LR) number and **b** secondary (2°) LR density in response to three kinds of light qualities. Seedlings were grown for 12 days in hydroponic media under *red*, *white*, and *blue* lights. Values are mean \pm SE

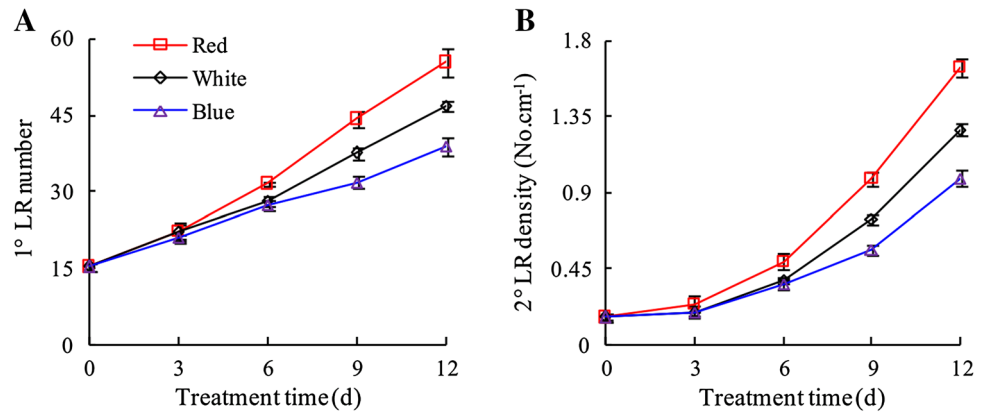


Fig. 3 Effects of three kinds of light qualities on IAA concentrations in the shoot (a) and root (b) of tobacco seedlings. Seedlings were grown for 12 days in hydroponic media under *red*, *white*, and *blue* lights. Values are mean \pm SE; different *lower case letters* indicate significant differences between means ($p < 0.05$; ANOVA)

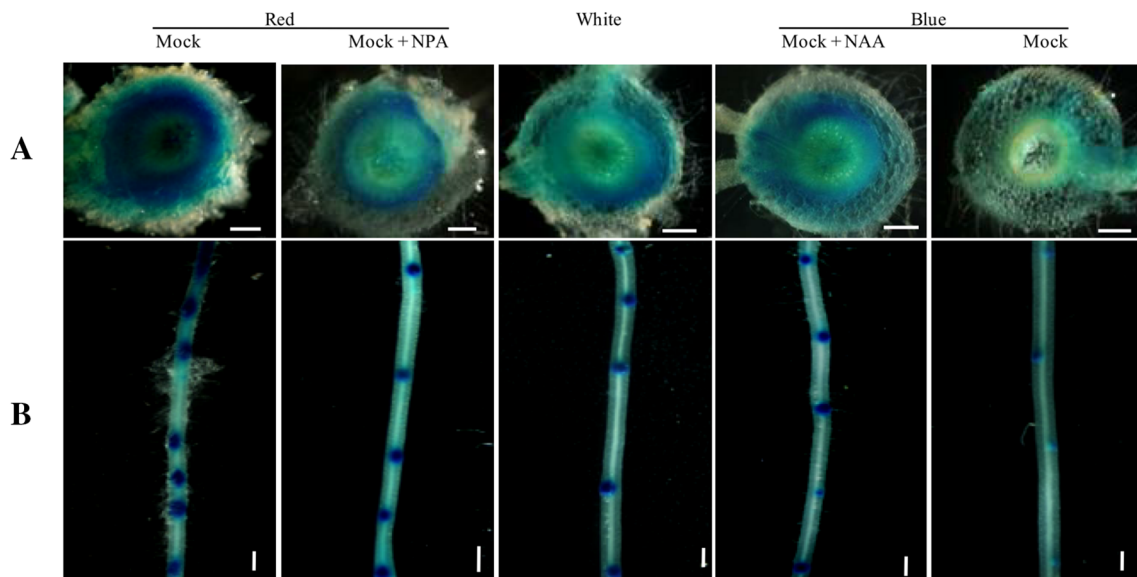
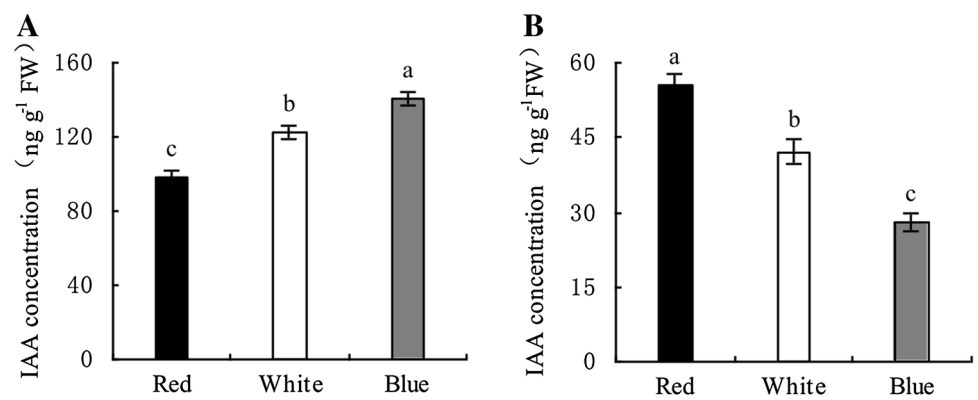


Fig. 4 Effects of synthetic NAA and NPA on DR5::GUS expression in the shoot/root junction (a) and secondary (2°) lateral root region (b) of tobacco seedlings grown under *red*, *white*, and *blue* lights.

Seedlings were grown in hydroponic media for 12 days under three light colors with or without 10 nM NAA or 20 nM NPA. Scale = 1 mm

Fig. 5 Effect of exogenous NAA and NPA on primary lateral root (1° LR) number (a) and secondary (2°) LR density (b) of tobacco seedlings under three colors of light. Seedlings were grown in hydroponic media for 12 days under red, white, or blue lights with or without 10 nM NAA or 20 nM NPA. Values are mean \pm SE; different lower case letters indicate significant differences between means ($p < 0.05$; ANOVA)

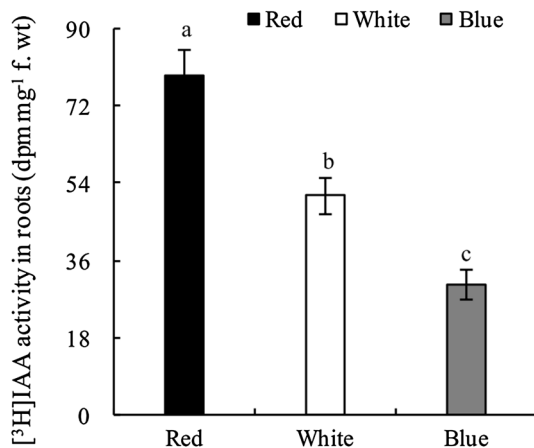
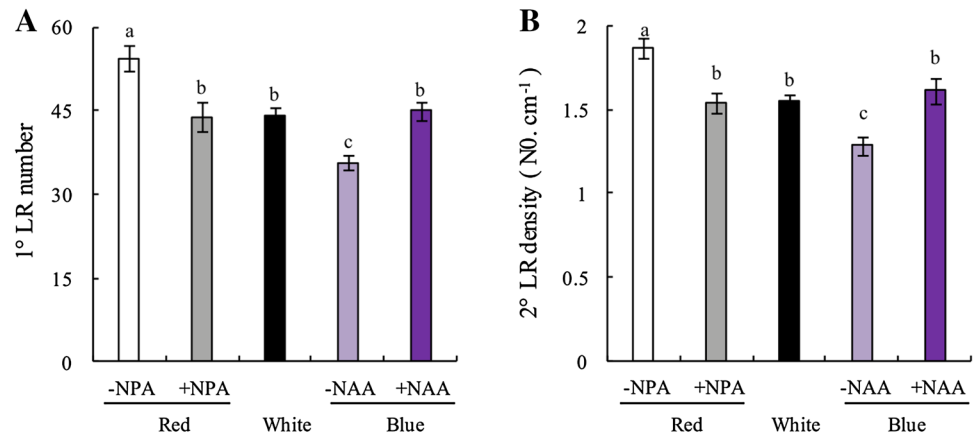


Fig. 6 [3 H]IAA transport from the junction to lateral roots (LRs) in tobacco seedlings. Radiolabeled IAA was given after 12 days of light treatments, as described in the materials and methods. Plants were kept in the dark for 18 h before being assayed for radioactivity in the roots. Values are mean \pm SE; different lower case letters indicate significant differences between means ($p < 0.05$; ANOVA)

facilitated by proteins of the PIN family. We quantified the transcript levels of *PIN* genes in the shoot/root junctions and roots of the seedlings treated with light of three colors (Fig. 7). qRT-PCR showed that the expression levels of *PIN1*, *PIN1b*, *PIN3*, and *PIN4* in the shoot/root junction of blue light-treated seedlings were significantly reduced in comparison with values under white light, whereas expression levels of *PIN3b* were elevated and those of *PIN9* were unaffected by the treatment. Compared with white light, the expression levels of *PIN1*, *PIN1b*, *PIN3*, and *PIN3b* were elevated under red light, but those of *PIN4* and *PIN9* were unaffected. The expression level of *PIN9* was unaffected by red and blue light treatments. *PIN3b* expression was enhanced by both monochromatic light treatments (relative to white light treatment).

The expressions of *PIN* genes in roots were also affected by light quality (Fig. 7b). Compared with white light, the

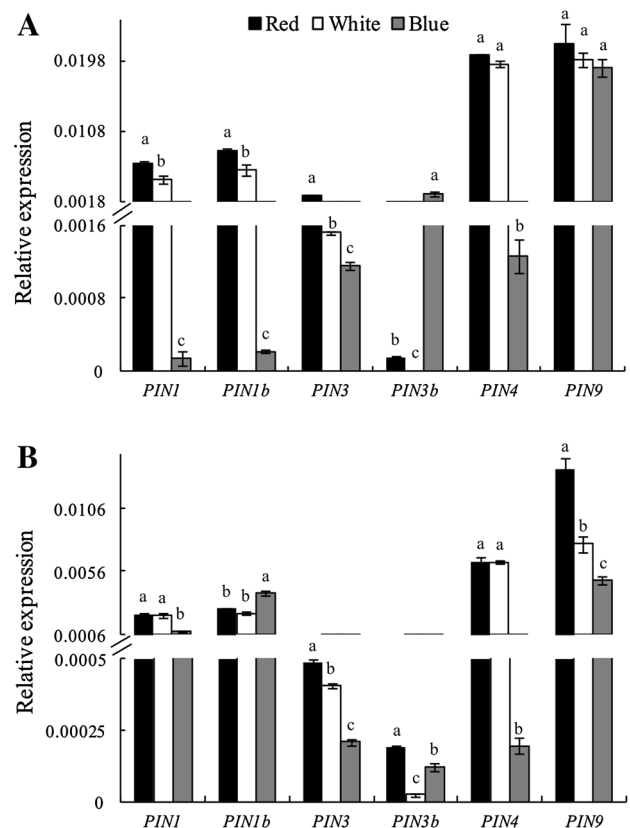


Fig. 7 qRT-PCR analysis of PIN family genes in the shoot/root junction (a) and root (b) of tobacco seedlings under red, blue, and white lights; 25-day-old seedlings were transplanted from soil to hydroponic media and exposed for 12 days to light of three colors at the same irradiance. Values are mean \pm SE; different lower case letters indicate significant differences between means ($p < 0.05$; ANOVA)

expression levels of *PIN3*, *PIN3b*, and *PIN9* were elevated under red light, but those of *PIN1*, *PIN1b*, and *PIN4* were unaffected. Blue light promoted *PIN1b* and *PIN3b* expressions in roots compared to white light, but the expressions of *PIN1*, *PIN3*, *PIN4*, and *PIN9* were inhibited.

Discussion

The development of the optimal root morphology, including formation of LRs, is crucial for the successful growth of transplants. In addition to providing anchorage, LRs contribute to water-use efficiency and facilitate the extraction of micro- and macronutrients from the soil (Casimiro and others 2001; Peret and others 2009). The growth of LRs varies greatly with environmental conditions, such as fluctuations in the light climate. Light is a critical environmental signal that controls plant growth and development, especially during continually overcast and rainy spring conditions when the tobacco seedlings are in nurseries. The effects of different types of supplementary illumination on tobacco seedling LR formation are largely unexplored. Here we show that red light promotes LR formation (in comparison with other wavelengths) and auxin polar transport from the shoot to root.

Light gradients in the soil have been largely overlooked in tests of plant responses to the environment. Nevertheless, data accumulated over the past 50 years have shown how wavelength affects adventitious roots formation on explants and cuttings exposed to light (Fletcher and others 1965; Fuernkranz and others 1990; Gabryszewska and Rudnicki 1994; Niemi and others 2005; Iacona and Muleo 2010; Ruedell and others 2013). Reports on root growth regulation by light quality in diverse plant species and experimental conditions are contradictory. Iacona and Muleo (2010) reported that red light inhibits adventitious root formation but is more effective in the promotion of adventitious root elongation in cherry explants than blue light. However, Fletcher and others (1965) found that red light promotes a doubling of adventitious root numbers in bean explants (relative to blue light). To date, the effects of light qualities supplied to aboveground organs (of intact plants) on the development of belowground roots (in darkness) have been explored only in *Arabidopsis* (Salisbury and others 2007). Salisbury and others (*loc. cit.*) reported that phytochrome regulates the emergence of LRs with or without exposure to light, suggesting that the shoot-localized phytochromes responding to aboveground light signals modulate root growth via a long-distance transmission mechanism. Furthermore, Galen and others (2007) demonstrated linkages between root-localized production of the photoreceptor phototropin-1, root growth efficiency, and drought tolerance, indicating that root plasticity in response to light signals contributes to the ecological adaptability of *Arabidopsis*. These data suggest that roots contain photoreceptors, which may receive ambient light through the soil or by transmission downward through the vascular cylinder.

In our study, we analyzed LR formation and elongation in tobacco seedlings responding to three light colors. 1° LR number and 2° LR density were promoted by red light. On days 9 and 12 of the experiment, values for these parameters were elevated in red light treatments in comparison with white and blue light treatments. Conversely, LR length was shortest under red light. All of these effects suggest that red light should be considered as a component of supplementary illumination for the industrial production of tobacco seedlings.

Auxin is often modulated by light in a mechanism that regulates these root developmental processes. Sassi and others (2013) have demonstrated that the control of auxin fluxes plays a central role in regulating and coordinating LR developmental responses to changing light environments. *Phytochrome A* and *B* mutants of *Arabidopsis* have low rates of LR formation associated with reduced auxin polar transport from shoot to root, suggesting that seedlings control LR formation by altering auxin distribution between the shoot and root (Salisbury and others 2007). Canamero and others (2006) provided evidence for a cryptochrome regulatory role (via modulations of auxin transport) in diverse aspects of root growth. The transcript abundances of the auxin efflux carriers *PIN3* and *PIN7* are phytochrome regulated (Devlin and others 2003). Components of the *PIN3* mutant phenotype are reportedly light specific (Friml and others 2002). Thus, phytochrome may control auxin transport by altering the levels and/or the cellular locations of PIN proteins. The elevated IAA concentration in roots, lower in leaves, and increased auxin transport from shoot to root of red light-treated seedlings in our experiments suggest that red light accelerated auxin transport from leaves to roots in comparison to other light colors (Figs. 3, 6); corroborative evidence was provided by the elevated expression levels of *DR5::GUS* in the shoot/root junction and in the 2° LR region (Fig. 4). Furthermore, application of NPA reduced both 1° LR numbers and 2° LR density in red light-treated seedlings to levels observed in seedlings held under white light; expression levels of *DR5::GUS* were also similar between these two treatments. Similar results were obtained when we applied NAA to blue light-treated seedlings. Our qPCR analyses showed that red light promoted *PIN3* expression in both the shoot/root junction and root, indicating that *PIN3* had a key role in auxin transport regulation by red light. Blue light reduced the expression level of *PIN1,3–4* in both the shoot/root junction and the root, thereby demonstrating a pivotal role of *PIN1,3–4* in auxin transport regulation by blue light. Thus, three colors of light regulated LR formation in tobacco seedlings, largely through mediation of auxin polar transport.

This finding will contribute to the design of supplementary lighting to aid the industrial production of high-quality tobacco seedlings.

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