The Plant Cell, Vol. 24: 1081–1095, March 2012, www.plantcell.org

Regulation of Root Greening by Light and Auxin/Cytokinin Signaling in *Arabidopsis* [W]

Koichi Kobayashi,[a,b,1] Shinsuke Baba,[c] Takeshi Obayashi,[d] Mayuko Sato,[b] Kiminori Toyooka,[b] Mika Keränen,[e] Eva-Mari Aro,[e] Hidehiro Fukaki,[f] Hiroyuki Ohta,[c] Keiko Sugimoto,[b] and Tatsuru Masuda[a]

[a] Graduate School of Arts and Sciences, University of Tokyo, Meguro-ku, Tokyo 153-8902, Japan
[b] RIKEN Plant Science Center, Tsurumi-ku, Yokohama 230-0045, Japan
[c] Center for Biological Resources and Informatics, Tokyo Institute of Technology, Midori-ku, Yokohama 226-8501, Japan
[d] Graduate School of Information Sciences, Tohoku University, Aoba-ku, Sendai 980-8579, Japan
[e] Department of Biochemistry and Food Chemistry, Molecular Plant Biology, University of Turku, FI20014 Turku, Finland
[f] Department of Biology, Graduate School of Science, Kobe University, Kobe 657-8501, Japan

Tight coordination between plastid differentiation and plant development is best evidenced by the synchronized development of photosynthetic tissues and the biogenesis of chloroplasts. Here, we show that *Arabidopsis thaliana* roots demonstrate accelerated chlorophyll accumulation and chloroplast development when they are detached from shoots. However, this phenomenon is repressed by auxin treatment. Mutant analyses suggest that auxin transported from the shoot represses root greening via the function of INDOLE-3-ACETIC ACID14, AUXIN RESPONSE FACTOR7 (ARF7), and ARF19. Cytokinin signaling, on the contrary, is required for chlorophyll biosynthesis in roots. The regulation by auxin/cytokinin is dependent on the transcription factor LONG HYPOCOTYL5 (HY5), which is required for the expression of key chlorophyll biosynthesis genes in roots. The expression of yet another root greening transcription factor, GOLDEN2-LIKE2 (GLK2), was found to be regulated in opposing directions by auxin and cytokinin. Furthermore, both the hormone signaling and the GLK transcription factors modified the accumulation of HY5 in roots. Overexpression of *GLK*s in the *hy5* mutant provided evidence that GLKs require HY5 to maximize their activities in root greening. We conclude that the combination of HY5 and GLKs, functioning downstream of light and auxin/cytokinin signaling pathways, is responsible for coordinated expression of the key genes in chloroplast biogenesis.

INTRODUCTION

In higher plants, plastids undergo profound morphological changes upon acquiring different functions typical of the organ type. Chloroplasts, which develop the thylakoid membrane network and photosynthetic machineries, are the most noticeable feature of green cells in leaves. Biogenesis of chloroplasts from proplastids occurs coordinately with leaf development from primordia, resulting in the activation of photosynthesis (Lopez-Juez and Pyke, 2005). By contrast, plant roots, which develop mainly nonphotosynthetic plastids, such as amyloplasts, usually grow underground as heterotrophic organs that are dependent on leaves as their energy source. However, reflecting the plasticity of plant tissues, roots of several plant species have the potential to turn green when exposed to light. In fact, photosynthesis in roots of the epiphytic *Orchidaceae* (Benzing et al., 1983) and in aerial roots of mangroves (Gill and Tomlinson, 1977) does contribute to the carbon economy of the whole plant. Root cultures derived from evolutionarily distant taxa (e.g., *Asteraceae* and *Solanaceae*) exhibit the ability to develop chloroplasts and to grow photoautotrophically (Flores et al., 1993). Therefore, roots generally appear to have the capacity to assemble a functional photosynthetic apparatus. Although roots are heterotrophic organs in *Arabidopsis thaliana*, they partially turn green in the presence of light. It is known that this response is enhanced in *de-etiolated1* (*det1*) (Chory and Peto, 1990) and *constitutive photomorphogenic1* (*cop1*) mutants (Deng and Quail, 1992), whereas it is absent in *long hypocotyl5* (*hy5*) mutants (Oyama et al., 1997; Usami et al., 2004). The data accumulated so far suggest that *Arabidopsis* roots also have the potential to develop chloroplasts. Nevertheless, their ability to do so is repressed by a COP1/DET1-mediating signaling pathway. The system for repressing chloroplast biogenesis, which remains unclear at the molecular level, could be an important mechanism for regulating plastid development during the differentiation of plant organs.

One of the most important events during greening is the accumulation of chlorophyll, which functions as a pigment for light energy harvesting and charge separation in photosystem I (PSI) and photosystem II (PSII). Because chlorophyll and its intermediates are strong photosensitizers, plant cells must strictly regulate their metabolism to coincide with the construction of the photosynthetic machinery (Tanaka et al., 2011). Indeed, the expression profiles of genes involved in chlorophyll biosynthesis suggest that *HEMA1* (encoding glutamyl-tRNA reductase), *CHLH* (encoding the H subunit of Mg-chelatase),

[1] Address correspondence to kkobayashi@bio.c.u-tokyo.ac.jp.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Koichi Kobayashi (kkobayashi@bio.c.u-tokyo.ac.jp).
[W] Online version contains Web-only data.
www.plantcell.org/cgi/doi/10.1105/tpc.111.092254

GENOME UNCOUPLED4, *CHL27* (encoding the membrane subunit of Mg-protoporphyrin IX monomethyl ester cyclase), *CHLOROPHYLL A OXYGENASE* (*CAO*), and *CHLP* (encoding geranylgeranyl pyrophosphate reductase) are highly coexpressed with nuclear-encoded photosynthesis-related genes and comprise key genes for the regulation of chlorophyll biosynthesis (Matsumoto et al., 2004; Masuda and Fujita, 2008). The tight coexpression observed between chlorophyll biosynthetic and photosynthetic genes suggests that there is a central transcriptional regulation system governing chlorophyll metabolism as well as photosynthetic proteins essential for the assembly of the photosynthetic machinery.

For chloroplast biogenesis, light is apparently a major regulator with various photoreceptors involved. Responses to blue light are mediated mainly by cryptochrome and phototropin, whereas phytochromes are responsible for regulating growth and development in response to red and far-red light (Chen et al., 2004). After red light absorption, phytochromes relocate to the nucleus where they regulate the response to light through two main mechanisms. First, they act to exclude the E3-ubiquitin ligase COP1 from the nucleus, thereby preventing the degradation of the positive signaling factors HY5, LONG HYPOCOTYL IN FAR-RED1, and LONG AFTER FAR-RED LIGHT1 (Bae and Choi, 2008). Second, phytochromes bind and target a family of basic helix-loop-helix proteins for degradation, thus relieving repression of light responses such as inhibition of hypocotyl elongation and germination (Duek and Fankhauser, 2005). Among those basic helix-loop-helix transcription factors, phytochrome-interacting proteins are responsible for the negative regulation of chloroplast development and chlorophyll biosynthesis in the dark (Leivar et al., 2009; Shin et al., 2009; Stephenson et al., 2009). Meanwhile, the GOLDEN2-LIKE (GLK) transcription factors were identified from *Arabidopsis* as positive regulators that coregulate and synchronize the expression of nuclear photosynthetic and chlorophyll biosynthetic genes (Waters et al., 2009). Interestingly, *Arabidopsis* GLKs strongly upregulate genes involved in the chlorophyll biosynthetic pathway, in particular *HEMA1*, *CHLH*, *CHL27*, and *CAO* (Waters et al., 2009), which are members of the coexpression network (Matsumoto et al., 2004; Masuda and Fujita, 2008). It has been proposed that GLKs help to optimize photosynthetic capacity in varying environmental and developmental conditions independently of the phyB-mediated light signaling pathway (Waters et al., 2009). Thus, both light and GLK signals may be necessary for the coordinated expression of photosynthetic genes.

In this study, to unravel the mechanism that regulates chloroplast development in conjunction with organ type development, we focused on chlorophyll biosynthesis and its regulation in *Arabidopsis* roots. We found that detached roots activate chlorophyll biosynthesis through a reduction in auxin signaling, reflecting the repressive effect of auxin on root greening. By contrast, chlorophyll biosynthesis in roots is positively regulated by cytokinin signaling, suggesting that auxin/cytokinin signaling is involved in the determination of chloroplast biogenesis in roots. We show that the concerted function of at least two transcription factors, HY5 and GLK2, acting downstream of the light and auxin/cytokinin signaling pathways, is responsible for the coordinated expression of the key genes involved in chlorophyll biosynthesis and photosynthesis upon greening of *Arabidopsis* roots.

RESULTS

Chlorophyll Accumulation in Roots Is Regulated in Opposing Directions by Auxin and Cytokinin

Although *Arabidopsis* roots are heterotrophic organs that are dependent on the shoot as their energy source, we found that they display chlorophyll accumulation when detached from the shoot. In fact, the amount of chlorophyll in detached wild-type Columbia-0 (Col) roots was five times higher than that in intact roots (Figure 1A). It is well known that auxins synthesized in the shoot undergo polar transport to the root via a specific transport system that includes the AUXIN RESISTANT1 (AUX1) and PIN-FORMED families of auxin influx and efflux carrier proteins (Robert and Friml, 2009). To examine the involvement of auxin transport from the shoot in root greening, we applied 1 μM auxin indole-3-acetic acid (IAA) to detached roots. Whereas the IAA treatment had no effect on chlorophyll accumulation in intact roots, it significantly decreased the chlorophyll content in detached roots (Figure 1A). Increased accumulation of chlorophyll

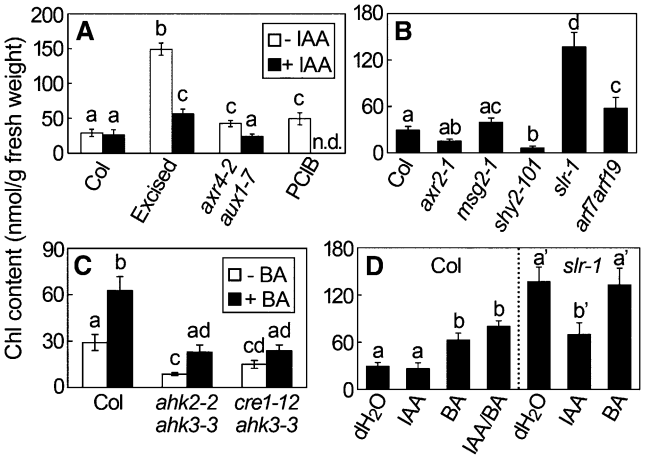

Figure 1. Involvement of Auxin and Cytokinin in the Accumulation of Chlorophyll in the Root.

(A) Chlorophyll (Chl) contents in several root samples treated with (+) or without (–) 1 μM IAA. "Excised" means excised roots grown for 7 d with or without IAA after detachment from 14-d-old Col seedlings. "PCIB" corresponds to the roots of 21-d-old Col seedlings treated with 10 μM PCIB for 7 d. n.d., not determined.

(B) Chlorophyll content in the roots of 21-d-old auxin signaling mutants.

(C) Chlorophyll content in the roots of 21-d-old wild-type Col and the double mutants of cytokinin receptors (*ahk2-2 ahk3-3* and *cre1-12 ahk3-3*) treated with (+) or without (–) 1 μM BA for 7 d.

(D) Chlorophyll content in the roots of 21-d-old Col or *slr-1* seedlings treated with deionized water (dH_2O), 1 μM IAA, 1 μM BA, or both IAA and BA for 7 d.

In all panels, values are the means ± SD from several independent experiments ($n > 3$). Different letters indicate statistically significant differences by Tukey-Kramer multiple comparison test ($P < 0.05$).

was also observed in untreated, intact roots of an auxin-resistant mutant *axr4-2 aux1-7*, which is defective in auxin influx transport (Hobbie and Estelle, 1995; Yamamoto and Yamamoto, 1999; Dharmasiri et al., 2006). However, the enhanced chlorophyll accumulation in the mutant roots was canceled when exogenous IAA was applied to the roots. These data imply that the endogenous auxin transported from the shoot acts as a negative signal for chlorophyll accumulation in roots. To assess further an involvement of auxin in repression of chlorophyll accumulation in roots, we evaluated the effect of *p*-chlorophenoxyisobutyric acid (PCIB), a compound widely used to inhibit auxin action, on the accumulation of chlorophyll in Col roots. As shown in Figure 1A, PCIB treatment increased chlorophyll accumulation in roots 1.7 times compared with the untreated control, suggesting that auxin action is required for the repression of chlorophyll accumulation.

To dissect the auxin signaling pathway involved in the regulation of chlorophyll biosynthesis in *Arabidopsis* roots, we analyzed several auxin signaling mutants. Among the *Aux/IAA* genes, various gain-of-function mutations that increase the stability of the gene products have been characterized. Loss-of-function mutations in auxin response factor (*ARF*) genes have likewise been reported. Various aberrant phenotypes of auxin-mediated growth and development, as well as altered gene expression in response to auxin, were recorded in these mutants (Liscum and Reed, 2002). In this study, we analyzed the *shy2-101*, *axr2-1*, *msg2-1*, *slr-1*, and *arf7 arf19* (*nph4-1 arf19-1*) mutants to gain insight into the regulation of chlorophyll accumulation in *Arabidopsis* roots. *shy2* mutants (*shy2-1* to *shy2-3*) were originally isolated as dominant photomorphogenic mutants in which *hy2* or *phyB* mutations were suppressed (Kim et al., 1996; Reed et al., 1998); thereafter, they were determined to have mutations in *IAA3* (Tian and Reed, 1999; Liscum and Reed, 2002). Here, we used *shy2-101*, which is a gain-of-function mutant of *IAA3* in the Col background (T. Goh, T. Mimura, and H. Fukaki, unpublished data). *axr2-1*, *msg2-1*, and *slr-1* are gain-of-function mutants of *IAA7* (Nagpal et al., 2000), *IAA19* (Tatematsu et al., 2004), and *IAA14* (Fukaki et al., 2002), respectively. *arf7 arf19* is a double loss-of-function mutant of *ARF7* and *ARF19* (Okushima et al., 2005; Wilmoth et al., 2005), the activities of which were proposed to be negatively regulated by IAA14 (Fukaki et al., 2005).

Among these mutants, the roots of *shy2-101* showed a large reduction in chlorophyll content, whereas the chlorophyll content of *slr-1* and *arf7 arf19* roots was significantly higher than in Col roots (Figure 1B). In particular, *slr-1* showed an approximately fivefold increase in root chlorophyll content. These mutants, by contrast, did not show large differences in chlorophyll content in the shoot when compared with the Col shoot (see Supplemental Figure 1 online). Therefore, it is likely that the auxin signaling pathway, mediated by IAA14 and ARF7/19, is responsible for the repression of chlorophyll biosynthetic genes in roots and that the perturbation of the pathway causes enhanced chlorophyll accumulation in roots.

The effect of cytokinin on root greening was examined next because cytokinin is a potent promoter of greening in leaves and also has several antagonistic effects against auxin (Moubayidin et al., 2009). As shown in Figure 1C, when Col wild type was treated with 1 μM 6-benzyladenine (BA), a synthetic cytokinin, chlorophyll accumulation in the root was doubled compared with the untreated control. To test whether the promoting effect of BA occurred via activation of the cytokinin signaling pathway, the chlorophyll contents in roots of cytokinin receptor double mutants, *ahk2 ahk3* (*ahk2-2 ahk3-3*) and *cre1 ahk3* (*cre1-12 ahk3-3*) (Higuchi et al., 2004), were examined (Figure 1C). In the absence of exogenous cytokinin, the chlorophyll content in the roots of these double mutants was less than half that in the Col wild type. When the mutants were treated with 1 μM BA, cytokinin-enhanced chlorophyll accumulation was observed in the roots, even though the effect was rather modest. These results demonstrated that both endogenous and exogenous cytokinin act as a positive signal enhancing chlorophyll accumulation in the root.

Subsequently, we also examined the effects of auxin on the activation of chlorophyll biosynthesis by cytokinin. As shown in Figure 1D, the enhanced chlorophyll accumulation in roots caused by BA treatment was not reduced by cotreatment with the same amount of IAA, indicating that auxin cannot antagonistically suppress the effect of cytokinin on chlorophyll accumulation. In *slr-1*, however, the exogenously applied IAA significantly reduced chlorophyll accumulation (Figure 1D), confirming that the enhanced chlorophyll accumulation found in untreated *slr-1* roots is caused by a perturbation of auxin signaling. When BA was exogenously applied to the *slr-1* mutant, chlorophyll accumulation in the roots did not significantly increase (Figure 1D), which may indicate that the BA treatment and the *slr-1* mutation activate root greening via the same signaling pathway.

Regiospecific Regulation of Chlorophyll Accumulation in Roots

To determine the site of chlorophyll accumulation in the root, we investigated chlorophyll autofluorescence in a transgenic *Arabidopsis* line (*SCR_{pro}:GFP*) that expresses green fluorescent protein (*GFP*) in the endodermal layer via *SCARECROW* (*SCR*) promoter activity (Wysocka-Diller et al., 2000). In roots of 14-d-old *SCR_{pro}:GFP* seedlings, chlorophyll fluorescence was observed mainly in the mature region of the primary root and was hardly detectable near root tip regions (around 6 cm from the root-hypocotyl junction) (see Supplemental Figure 2 online). In the mature primary root, chlorophyll fluorescence was detected in the inner area of the GFP-expressing endodermis layer, demonstrating that chlorophyll accumulation occurs predominantly in the stele. This regiospecific accumulation of chlorophyll was further confirmed in more developed root tissues of 21-d-old seedlings (Figures 2A and 2B). In the primary root of the wild type, the chlorophyll fluorescence was detected circularly in the thickened stele, whereas no fluorescence was observed in the outer cell layers, composed of endodermis, cortex, and epidermis cells. A similar distribution of chlorophyll-containing cells was observed in the roots of the *slr-1* mutant and the BA-treated wild type, but the chlorophyll autofluorescence in these roots was stronger than that in the untreated Col wild type (Figure 2A). In *slr-1*, chlorophyll fluorescence was also observed in the central region of the primary root (Figure 2B).

To assess the effect of regiospecific inhibition of IAA14-mediated auxin signaling on root greening, we compared chlorophyll

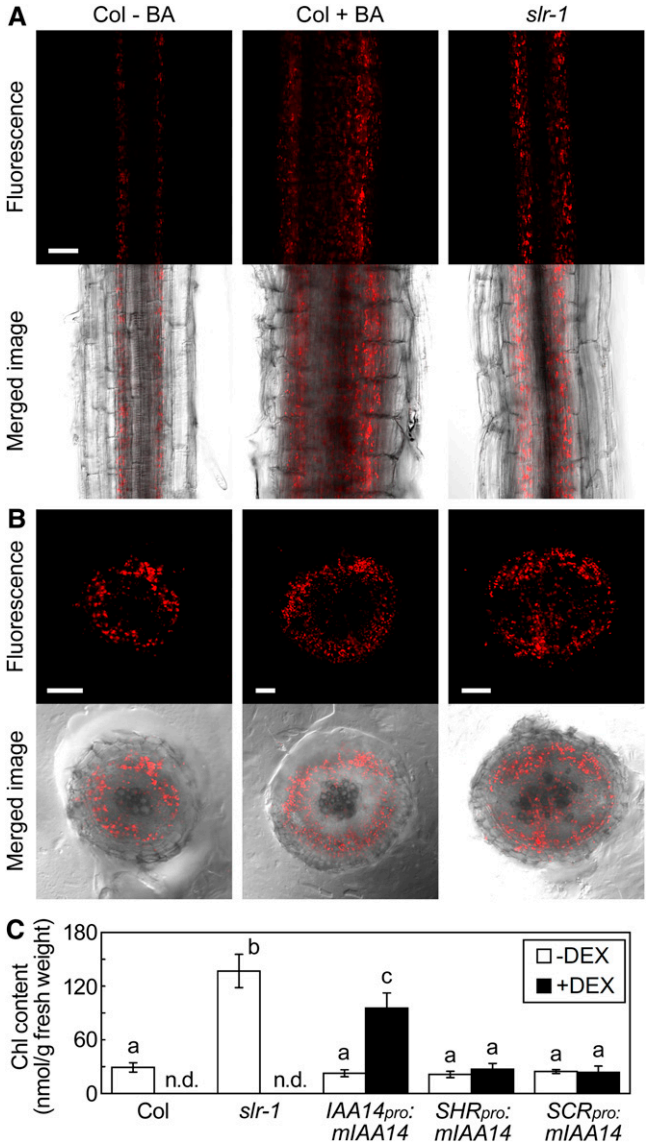

Figure 2. Regiospecific Accumulation of Chlorophyll in the Root.

(A) and **(B)** Confocal microscopy of chlorophyll fluorescence in the primary root of 21-d-old *slr-1* and wild-type Col seedlings treated with (+) or without (–) 1 μM BA for 7 d. Chlorophyll fluorescence was detected in intact roots **(A)** or cross sections of the roots **(B)**. Merged images represent differential interference contrast images merged with the fluorescence micrographs. Bars = 50 μm.

(C) Chlorophyll (Chl) content in the roots of 21-d-old seedlings expressing the stabilized mutant IAA14 protein (mIAA14) driven by the indicated promoter in the presence (+) or absence (–) of 1 μM Dex. Values are the means ± SD from three independent experiments ($n > 3$). Different letters indicate statistically significant differences by Tukey-Kramer multiple comparison test ($P < 0.05$). n.d., not determined.

accumulation in transgenic plants expressing the stabilized mutant IAA14 (mIAA14) protein, which was identified originally as a causative protein of the *slr-1* mutant, under the control of tissue-specific promoters (Fukaki et al., 2005). In the root tip region, the 2.0-kb *IAA14* promoter ($IAA14_{pro}$) is activated in the epidermal tissue (Fukaki et al., 2002; Vanneste et al., 2005; Swarup et al., 2008), whereas the 2.5-kb *SHR* promoter (SHR_{pro}) is active in the stele (Helariutta et al., 2000). The activity of the 2.5-kb *SCR* promoter (SCR_{pro}) is limited to the endodermis and the meristematic region, such as the quiescent center and cortex/endodermal initial (Helariutta et al., 2000). As previously reported by Fukaki et al. (2002), strong $IAA14_{pro}$ activities were detected in the stele from the mature region of the primary root (see Supplemental Figure 3 online). By contrast, the SHR_{pro} (Helariutta et al., 2000) and SCR_{pro} activities (Di Laurenzio et al., 1996; Malamy and Benfey, 1997) were low in the mature region and strong in the root tip region (see Supplemental Figure 3 online). In fact, GFP expression in SCR_{pro}:*GFP* roots decreased as the tissues matured (see Supplemental Figure 2 online). Because *mIAA14* is constructed as a glucocorticoid receptor (GR)–fused cDNA, the expressed protein can function in the nucleus only in the presence of dexamethasone (Dex) (Fukaki et al., 2005). Indeed, almost all transgenic plants having these constructs failed to develop lateral roots in the presence of Dex due to the inhibitory action of mIAA14 in auxin signaling, while they appeared identical to the wild type in the absence of Dex (Fukaki et al., 2005). As shown in Supplemental Figure 4 online, when Dex was applied to these lines, intense chlorophyll autofluorescence was observed in the roots of $IAA14_{pro}$:*mIAA14-GR*, whereas only weak fluorescence was detected in the roots of SHR_{pro}:*mIAA14-GR* and SCR_{pro}:*mIAA14-GR*. In fact, the chlorophyll content of $IAA14_{pro}$:*mIAA14-GR* roots was substantially increased by the Dex treatment, while the chlorophyll levels in the roots of SHR_{pro}:*mIAA14-GR* and SCR_{pro}:*mIAA14-GR* were equivalent to that of the wild type, even in the presence of Dex (Figure 2C). These results show that although SCR_{pro}- and SHR_{pro}-driven mIAA14 expression is sufficient to block lateral root formation (Fukaki et al., 2005), it does not promote chlorophyll accumulation in the root. The strong $IAA14_{pro}$ activity in the stele of the mature root region (see Supplemental Figure 3 online), where chlorophyll accumulation actively occurs (see Supplemental Figure 2 online), would be important to regulate root greening in response to various hormonal conditions.

A Partially Active Photosynthetic Apparatus Is Assembled in Greening Roots

To evaluate whether a functional photosynthetic apparatus is assembled in root plastids, the photosynthetic activity of PSII in the roots of *slr-1*, BA-treated, and untreated wild type were compared with that in the shoots of wild-type seedlings. As shown in Figure 3A, the maximum quantum yield of PSII (Fv/Fm) was ~0.72 in all root samples, whereas that in wild-type shoots was 0.79, showing that PSII is active in the root plastids although the efficiency is lower than in the shoot chloroplasts. When we examined the spatial pattern of Fv/Fm in Col roots, the activity was detected at the basal area near the hypocotyl junction (see Supplemental Figure 5 online). The activity was observed more

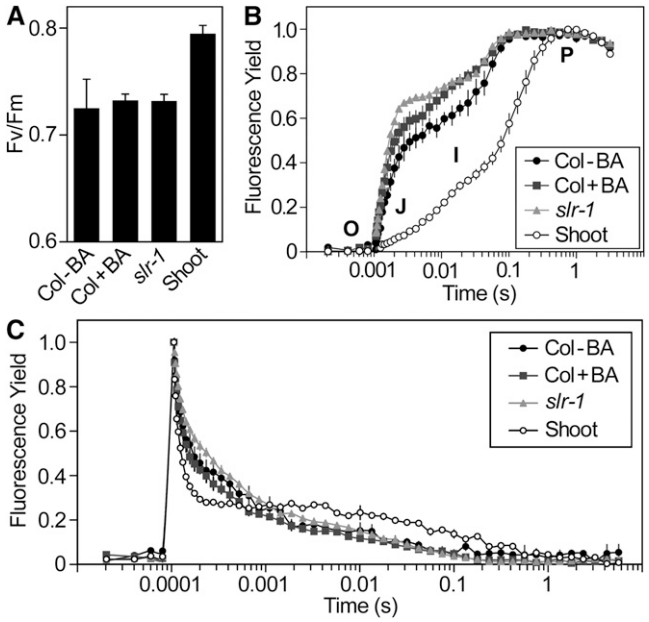

Figure 3. Electron Transport in PSII of Root Samples.

Chlorophyll fluorescence in roots of 21-d-old *slr-1* and Col seedlings treated with (+) or without (–) 1 μM BA for 7 d was compared with that of shoots of untreated Col seedlings (Shoot). Maximum quantum yield of PSII (Fv/Fm) **(A)**, transient fluorescence induction kinetics **(B)**, and fluorescence decay kinetics of chlorophyll **(C)** were determined in these samples. Two inflections, labeled J and I, are observed between the levels O (origin) and P (peak). Values are the means ± SD (n = 5).

broadly in the roots of *slr-1* and BA-treated Col, whereas it was scarcely detected in the roots of *ahk2 ahk3*, consistent with the quantitative chlorophyll content results (Figure 1). In addition, the roots of *hy5-215*, in which no chlorophyll accumulation was reported (Oyama et al., 1997; Usami et al., 2004), showed no PSII activity.

Next, we analyzed the transient kinetics of chlorophyll fluorescence in roots using a logarithmic timing series (Figure 3B). As reported previously (Govindjee, 1995), a typical polyphasic fluorescence rise exhibiting the origin inflection-intermediary peak-peak (O-J-I-P) transients was observed in wild-type shoots. The O-J-I-P transients were also observed in all root samples, indicating the presence of functional electron transport in PSII. In wild-type roots, however, the O-J transition occurred with a higher fluorescence yield than in the shoots, indicating that electron transport from Q_A^- to Q_B is slowed down in the root plastids. Moreover, high chlorophyll fluorescence at the O-J transition was also observed in roots of *slr-1* and BA-treated wild type, suggesting that hormone signaling does not improve the efficiency of photosynthetic electron transport in the root. We also measured Q_A^- reoxidation kinetics in these samples (Figure 3C). In wild-type shoots, a rapid decay of chlorophyll fluorescence was observed, reflecting fast electron transport from Q_A^- to Q_B. Q_A^- reoxidation was also observed in all root samples, although the decay of fluorescence occurred more slowly. The data confirm that electron transport in PSII is functional even in root plastids, although the transport from Q_A^- to Q_B is retarded.

Ultrastructure of Root Plastid

Functional assembly of photosystems in roots (Figure 3) suggests that roots can develop photosynthetically active plastids. Consistently, transmission electron microscopy revealed plastids with thylakoid membranes in the wild-type primary root, even in the absence of cytokinin, although the membranes of these plastids were less developed than those of leaf chloroplasts (Figure 4). Moreover, in the *slr-1* root, many plastids developed starch grains in addition to thylakoid membranes (Figure 4C), reflecting a high chloroplast-developing activity in the mutant root. These developed plastids were detected primarily in a population of small cells surrounding xylem cells in the stele and they were undetectable in outer vacuolated cells (see Supplemental Figure 6 online). In addition, we found several developed plastids in the central vascular region of the *slr-1* root, consistent with the observation of chlorophyll fluorescence (Figure 2B).

Gene Expression Analysis of Roots from BA-Treated and *slr-1* Seedlings

To reveal regulatory steps involved in root greening by phytohormones, gene expression analyses were performed on greenish roots. First, we used the microarray expression analysis as a preliminary screen to identify candidate genes. Expression profiles from 14-d-old *slr-1* and BA-treated Col roots were compared with those from the untreated Col control. These data show that 10 of the 20 most upregulated genes in both greenish roots were photosynthetic genes encoding light-harvesting chlorophyll binding proteins (LHCI and LHCII), subunits of the reaction centers of PSI and PSII, and the small subunit of ribulose 1,5-bisphosphate carboxylase (see Supplemental Data Set 1 online), suggesting that auxin/cytokinin signaling can affect the biogenesis of photosynthetic machineries at the transcriptional

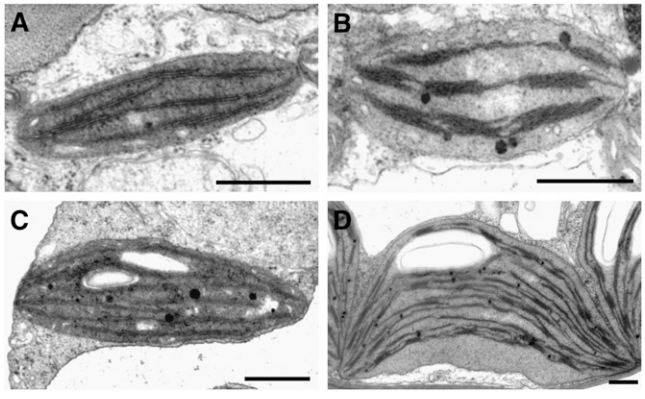

Figure 4. Plastid Development in the Root.

Plastid ultrastructure in the primary root cells of untreated wild-type Col **(A)**, BA-treated Col **(B)**, and untreated *slr-1* **(C)**. A typical chloroplast in mature *Arabidopsis* leaf cells is shown in **(D)** for structure comparison. Bars = 0.5 μm.

level. In fact, the transcript profiles indicate that most of the nuclear genes encoding subunits of the PS-LHC complexes are upregulated in both types of greenish roots (see Supplemental Data Set 2 online) with key genes involved in the chlorophyll biosynthesis pathway (*CHLH*, *CHL27*, *PORB*, and *CHLP*) (see Supplemental Data Set 3 online). The upregulation of genes associated with chlorophyll biosynthesis and light harvesting was verified by quantitative RT-PCR (qRT-PCR) analysis. As shown in Figure 5A, strong upregulation of *CHLH*, *CHL27*, and *CHLP* was observed in roots of BA-treated or *slr-1* seedlings, in addition to the increased expression of *LHCA4* and *LHCB4.1*. By contrast, the expression levels of these genes were significantly reduced in the roots of *ahk2 ahk3* double mutants and also severely impaired in *hy5-215* roots. As shown in Figure 5B, the accumulation of chlorophyll in roots corresponded with the expression of *CHLH*, *CHL27*, and *CHLP* (Figure 5A), suggesting that the expression of these genes is one of the determining steps of chlorophyll biosynthesis in the root and that phytohormones play a role in this regulation.

HY5 Is a Prerequisite for Chlorophyll Accumulation in Roots

We found that chlorophyll accumulation in the root increased as seedlings grew. In fact, the amount of chlorophyll in the root of a 21-d-old wild-type plant (≈30 nmol/g fresh weight) was 3 times higher than that of a 14-d-old wild-type plant (≈10 nmol/g fresh weight) (Figures 1 and 5B). A similar increase was also observed in the *slr-1* mutant, suggesting that chlorophyll accumulation in the root is linked with tissue development. By contrast, the amount of chlorophyll in the BA-treated roots was comparable in 14-d-old and 21-d-old seedlings on a fresh weight basis, maybe because both samples were subjected to BA treatment for the same period of time (7 d). In *hy5-215* roots, the amount of chlorophyll stayed at low levels during seedling growth (Figures 5B and 6A), showing that HY5 has an essential role in root greening.

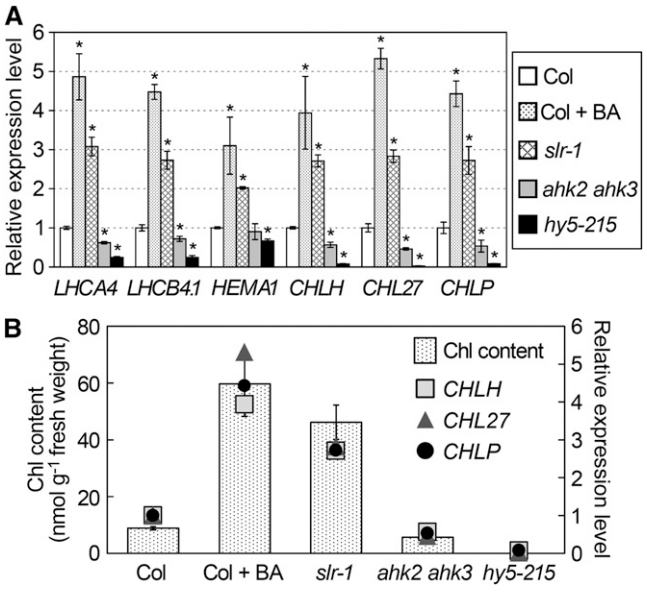

Figure 5. Expression of Light-Harvesting Genes and Chlorophyll Biosynthesis Genes in the Root.

(A) qRT-PCR analysis in roots of untreated Col seedlings, Col seedlings treated with 1 μM BA (Col + BA), and *slr-1*, *ahk2 ahk3*, and *hy5-215* seedlings grown for 14 d. Gene expression levels were normalized to untreated Col levels. Values are the means ± SD from three independent experiments. An asterisk indicates significant difference from untreated Col ($P < 0.05$, Student's *t* test).

(B) Correspondence between expression of chlorophyll biosynthesis genes and chlorophyll content in roots of 14-d-old seedlings. Chlorophyll (Chl) content in the root of each plant was plotted together with the expression profiles of chlorophyll biosynthesis genes shown in **(A)**. Values are the means ± SD ($n > 3$).

It has been shown that light signaling is required for chlorophyll accumulation in roots. Using monochromatic lights, Usami et al. (2004) reported that *Arabidopsis* root greening is most effectively promoted by blue light, which may be perceived by phytochrome A or B in addition to cryptochrome. To examine the crosstalk between cytokinin and light signaling, we analyzed the effect of BA on chlorophyll accumulation in the roots of photoreceptor mutants. As shown in Figure 6A, accumulation of chlorophyll in the roots of *cry1* (*hy4-1*) and *phyA phyB* (*phyA-201 phyB-5*) mutants was much lower than in the corresponding ecotype background (Landsberg *erecta*), a result consistent with a previous analysis (Usami et al., 2004). Interestingly, BA treatment increased the chlorophyll content in the root of these photoreceptor mutants, suggesting that cytokinin can act independently from or downstream of these photoreceptors. HY5 is a transcription factor that acts downstream of COP1 as a positive regulator of photomorphogenesis (Bae and Choi, 2008) and is required for both phytochrome and cryptochrome responses for the induction of root greening (Usami et al., 2004). Thus, we examined whether exogenous BA can complement HY5 deficiency in roots. As shown in Figure 6A, chlorophyll was almost undetectable in roots of *hy5-215*, and levels remained low upon BA application, showing that cytokinin signaling requires HY5 to induce root greening.

To examine the crosstalk between auxin and light, we then tested *hy2 slr-1* (*hy2-101 slr-1*) and *hy5 slr-1* (*hy5-215 slr-1*) double mutants for chlorophyll accumulation in the root. *HY2* encodes a phytochromobilin synthase, which catalyzes the production of the phytochromobilin chromophore of phytochromes (Kohchi et al., 2001). In *hy2-101*, the root chlorophyll content was ~20% that of the wild type (Figure 6B). In the *hy2 slr-1* double mutant, however, the chlorophyll content of the roots was restored to almost wild-type levels. By contrast, chlorophyll accumulation in roots was almost undetectable in the *hy5 slr-1* mutant, demonstrating that the effect of the *slr-1* mutation on root greening is also HY5 dependent.

Accumulation of HY5 Is Important but Not Sufficient for Root Greening

It has been reported that cytokinin can promote the expression of anthocyanin biosynthetic genes in the presence of light via the reduction of COP1-dependent degradation of HY5 (Vandenbussche et al., 2007). To examine whether this is also the case in root greening, we performed immunoblot analysis of proteins extracted from roots. Consistent with the transcriptome analysis, the

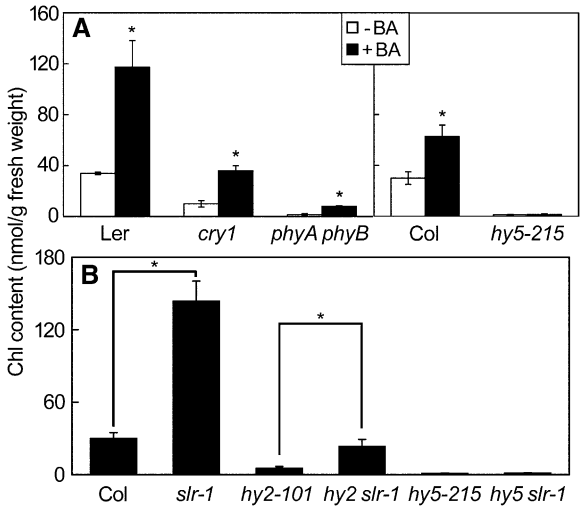

Figure 6. Chlorophyll Accumulation in Roots of Light Signaling Mutants.

(A) Chlorophyll (Chl) contents in the roots of 21-d-old light-signaling mutants were determined in the presence (+) or absence (–) of 1 μM BA. Values are the means ± SD ($n > 3$). An asterisk indicates significant difference from each untreated plant ($P < 0.05$, Student's *t* test).

(B) Chlorophyll contents in the roots of 21-d-old Col plants and *hy2-101* and *hy5-215* mutants with or without the *slr-1* mutation. Values are the means ± SD ($n > 3$). An asterisk indicates significant difference between plants with and without the *slr-1* mutation ($P < 0.05$, Student's *t* test).

amounts of the large subunit of ribulose 1,5-bisphosphate carboxylase and LHCII increased in the roots of BA-treated and *slr-1* seedlings compared with untreated wild type, whereas they were undetectable in *hy5-215* roots (Figure 7A). The amount of HY5 also increased in *slr-1* roots, although we did not observe an obvious increase in HY5 protein induced by BA application to wild-type roots.

To investigate the correlation between HY5 accumulation and chlorophyll biosynthesis, we analyzed the localization and accumulation of HY5 in roots using the *hy5-1/HY5*$_{pro}$*:HY5-YFP* (for yellow fluorescent protein) transgenic line (Oravecz et al., 2006). The *HY5* promoter-driven HY5-YFP fusion protein accumulated widely in the primary root and lateral roots, whereas chlorophyll fluorescence was limited to the primary root (Figure 7B). Moreover, although chlorophyll fluorescence gradually decreased toward the tip from the base of the primary root, HY5 accumulation in the nucleus was observed constantly over a wide range of the root (Figure 7C). These results suggest that although HY5 is required for chlorophyll biosynthesis in the root, other factors are also necessary for root greening.

Involvement of GLK Transcription Factors in Root Greening

Because the expression of photosynthetic genes in the root was upregulated by BA treatment and the *slr-1* mutation (Figure 5; see Supplemental Data Set 1 online), we tested the responses of several genes involved in the regulation of photomorphogenesis and chloroplast development (see Supplemental Data Set 4 online). Among the genes listed in Supplemental Data Set 4, only the expression of *GLK2* appeared to be upregulated by BA treatment (1.94-fold) and also weakly in the *slr-1* mutant (1.41-fold). By contrast, the expression level of *GLK1*, a homolog of *GLK2*, did not change in these roots. We confirmed the induction of *GLK2* in the root of 14-d-old *slr-1* and BA-treated Col seedlings by qRT-PCR analysis (Figure 8A). The expression of *GLK1* was too low to be quantified in our experimental conditions, consistent with the report that *GLK1* is scarcely expressed in *Arabidopsis* roots (Fitter et al., 2002), but the expression of *GLK2* in the root was upregulated 2.84-fold by BA treatment and 1.55-fold in the *slr-1* mutant compared with the untreated wild-type control, thereby supporting the microarray data. In *ahk2 ahk3* and *hy5-215* roots, *GLK2* transcript levels did not change significantly as compared with the untreated wild type.

Considering the function of GLKs in upregulating their target photosynthetic genes by binding to their promoters directly (Waters et al., 2009), it is likely that the induction of *GLK2* expression by BA treatment and the *slr-1* mutation plays a role in

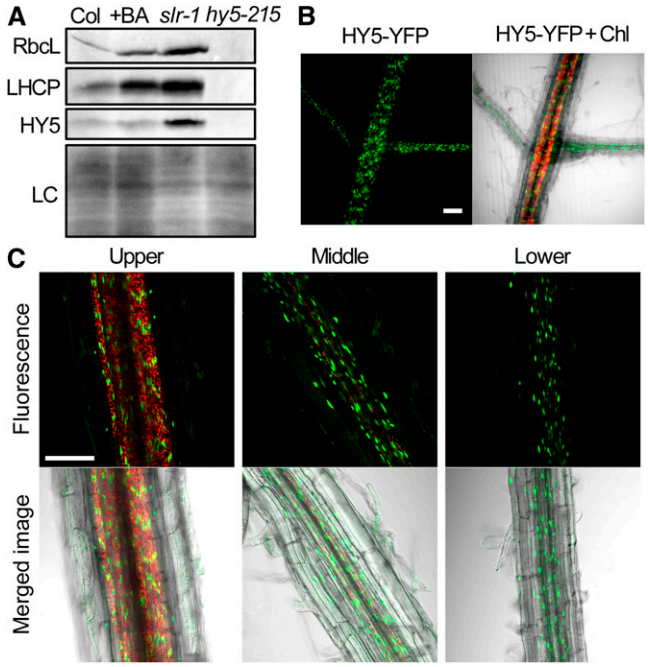

Figure 7. HY5 Accumulation in the Root.

(A) Immunoblot analysis of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RbcL), light-harvesting chlorophyll *a/b* binding protein (LHCP), and HY5 in roots of untreated Col, BA-treated Col (+BA), *slr-1*, and *hy5-215*. Staining of total proteins approximately between 20 and 35 kD is shown as a loading control (LC).

(B) Fluorescence of HY5-YFP proteins in the root of the *hy5-1/HY5*$_{pro}$*:HY5-YFP* transgenic plant. Green fluorescence corresponds to HY5-YFP, whereas the red fluorescence in the merged image (right) shows accumulation of chlorophyll (chlorophyll).

(C) Fluorescence of HY5-YFP (green) and chlorophyll autofluorescence (red) was detected in the primary root of *HY5-YFP* seedlings at 4.0 cm (Upper), 6.0 cm (Middle), and 8.0 cm (Lower) from the root-hypocotyl junction.

Bars = 100 μm.

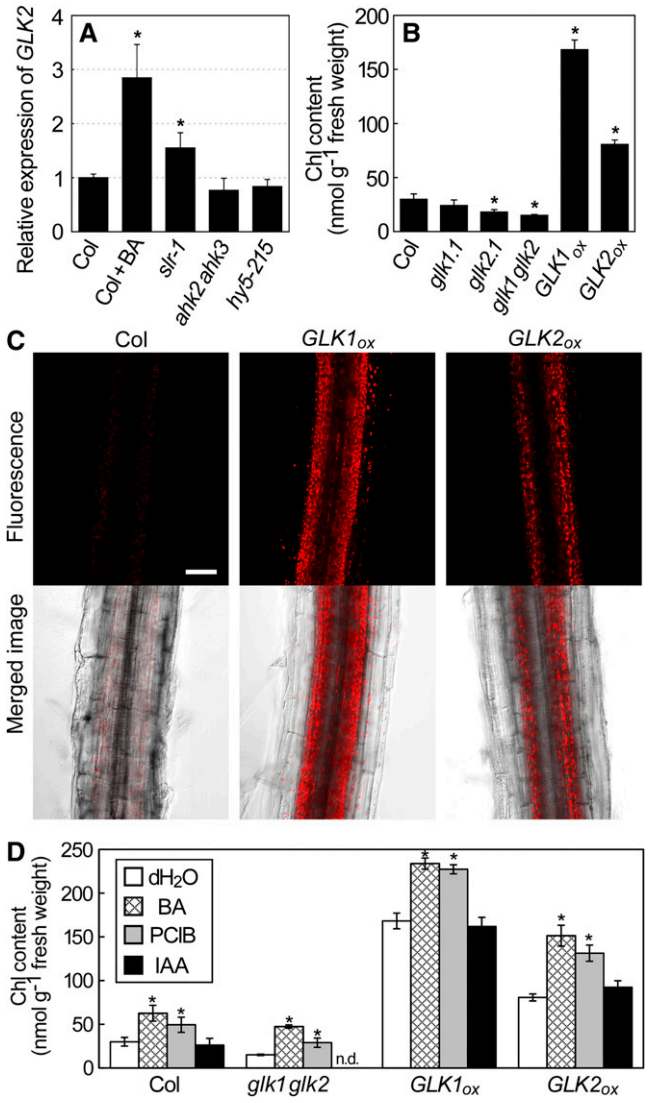

Figure 8. Involvement of GLK Transcription Factors in Root Greening.

(A) Expression levels of *GLK2* in roots of untreated Col, BA-treated Col (Col + BA), *slr-1*, *ahk2 ahk3*, and *hy5-215* grown for 14 d in the light. Gene expression levels were normalized to untreated Col levels. Values are the means ± SD from three independent experiments. An asterisk indicates significant difference from untreated Col control ($P < 0.05$, Student's *t* test).

(B) Chlorophyll content in the roots of 21-d-old *glk* mutants and GLK overexpressors ($GLK1_{OX}$ and $GLK2_{OX}$). Values are the means ± SD ($n > 3$). An asterisk indicates significant difference from Col ($P < 0.05$, Student's *t* test).

(C) Confocal microscopy of chlorophyll fluorescence in the primary root of 21-d-old Col, $GLK1_{OX}$, and $GLK2_{OX}$. Bar = 50 μm.

(D) Effects of phytohormones on chlorophyll accumulation in roots of *glk* mutants and overexpressors. Chlorophyll (Chl) contents were determined in roots of 21-d-old seedlings treated with deionized water (dH_2O), 1 μM BA, 10 μM PCIB, or 1 μM IAA for 7 d. Values are the means ± SD ($n > 3$). An asterisk indicates significant difference from each untreated (dH_2O) line ($P < 0.05$, Student's *t* test). n.d., not determined.

chlorophyll accumulation in the greenish roots. To assess the involvement of GLKs in hormonal regulation of root greening, we examined knockout mutants (*glk1.1*, *glk2.1*, and *glk1 glk2*) and overexpression lines ($GLK1_{OX}$ and $GLK2_{OX}$ in the *glk1 glk2* background) of GLK isoforms in *Arabidopsis* (Fitter et al., 2002; Waters et al., 2008). In $GLK1_{OX}$ plants, the chlorophyll content of roots increased substantially (Figure 8B), which is consistent with a recent report in rice (*Oryza sativa*), demonstrating that overexpression of GLK1 results in excess accumulation of chlorophyll in the callus and roots (Nakamura et al., 2009). Moreover, $GLK2_{OX}$ also showed high accumulation of chlorophyll in roots, although the level was lower than that in $GLK1_{OX}$. In fact, prominent chlorophyll fluorescence was detected in roots of these overexpression lines mainly in the stele (Figure 8C). By contrast, the chlorophyll content was significantly reduced, by 60%, in roots of the *glk2.1* mutant compared with wild-type Col, while that in *glk1.1* was not largely different from the wild type (Figure 8B). This result is consistent with the fact that the expression of only *GLK2* is detectable in the root (Fitter et al., 2002). Although the reduction in chlorophyll content in the root was more pronounced in the *glk1 glk2* double mutant than in the *glk2.1* single mutant, half of the chlorophyll amount present in wild-type roots was still detected in the roots of the double mutant. Furthermore, application of both BA and PCIB to the double mutant increased the chlorophyll content in the root (Figure 8D). These results contrast with those from *hy5-215*; thus, it is apparent that GLK1 and GLK2 are not essential transcription factors for root greening and that phytohormones can regulate chlorophyll biosynthesis in the root in a manner at least partially independent of GLKs. Enhancement of chlorophyll accumulation by treatment with BA or PCIB was also observed in both overexpression lines. The maximum chlorophyll accumulation in the root (BA-treated $GLK1_{OX}$) reached ~10% of chlorophyll amounts observed in wild-type *Arabidopsis* leaves (~2000 nmol/g fresh weight). On the other hand, IAA treatment did not affect excess accumulation of chlorophyll in the roots of *GLK* overexpressors (Figure 8D), suggesting that the suppressive auxin signaling does not function downstream of GLKs in the regulation of root greening.

Genetic Interaction between HY5 and GLK Transcription Factors

Because several lines of evidence imply that the accumulation of HY5 is a critical factor for root greening (Figure 7A) (Deng and Quail, 1992; Chory et al., 1994; Usami et al., 2004), we investigated HY5 levels in roots of $GLK1_{OX}$ and $GLK2_{OX}$. In the roots of both overexpression lines, the amount of HY5 increased together with LHCP, whereas it did not in the wild-type root (Figure 9A). In particular, the accumulation of HY5 in $GLK1_{OX}$ roots is higher than that in $GLK2_{OX}$ roots, which is consistent with the levels of chlorophyll and LHCP in these roots.

To reveal a genetic interaction between HY5 and GLKs, we generated *hy5* mutants overexpressing *GLK1* or *GLK2* (*hy5*/$GLK1_{OX}$ and *hy5*/$GLK2_{OX}$) by crossing *hy5-215* with each GLK_{OX} line. Both transgenic plants demonstrated high expression of each *GLK* gene in the *hy5-215* background (Figure 9B). Intriguingly, although chlorophyll levels in the roots of both *hy5*/$GLK1_{OX}$

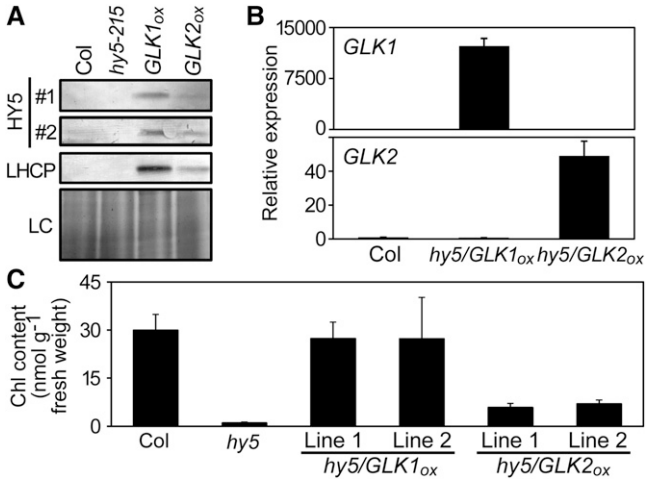

Figure 9. Genetic Interaction between HY5 and GLK Factors.

(A) Immunoblot analysis of HY5 and light-harvesting chlorophyll *a*/*b* binding proteins (LHCP) in wild-type Col, *hy5-215*, $GLK1_{OX}$, and $GLK2_{OX}$. A biological duplication (#1 and #2) for HY5 detection is shown. Staining of total proteins approximately between 20 and 35 kD is shown as a loading control (LC).

(B) qRT-PCR analysis of *GLK1* and *GLK2* expression in roots of 21-d-old Col, *hy5*/$GLK1_{OX}$, and *hy5*/$GLK2_{OX}$. Gene expression levels were normalized to Col levels. Values are the means ± SD from three independent experiments.

(C) Chlorophyll (Chl) contents in roots of Col, *hy5-215*, *hy5*/$GLK1_{OX}$, and *hy5*/$GLK2_{OX}$ grown for 21 d under continuous light. For each *hy5*/$GLK1_{OX}$ and *hy5*/$GLK2_{OX}$ plant, two distinct lines were used for the analysis. Values are the means ± SD from three independent experiments.

and *hy5*/$GLK2_{OX}$ plants were higher than those in the parental *hy5-215* mutant, they were still much lower than those in the $GLK1_{OX}$ and $GLK2_{OX}$ parental lines (Figure 9C). These data suggest that HY5 plays a crucial role for GLK functions in the root. Nonetheless, the slight accumulation of chlorophyll in *hy5*/GLK_{OX} roots indicates that the excessive expression of GLK can partly induce root greening in the absence of HY5.

DISCUSSION

Opposing Effects of Cytokinin and Auxin on Chlorophyll Biosynthesis in *Arabidopsis* Roots

Here, we demonstrated that in *Arabidopsis* roots, both chlorophyll biosynthesis and chloroplast biogenesis are positively regulated by cytokinin signaling and negatively regulated by auxin signaling (Figure 10). Since these phytohormones are pivotal regulators of organogenesis in plants, it is conceivable that the cytokinin/auxin signaling pathway is involved in the coordination of plastid development and organ development. Cytokinin is perceived by three His kinases, CRE1 (AHK4), AHK2, and AHK3, which initiate intracellular phosphotransfer to subsequent response regulators (Higuchi et al., 2004). The reduction in chlorophyll accumulation and the expression of chlorophyll biosynthetic genes in the roots of cytokinin receptor mutants demonstrate that cytokinin signaling plays an important role in root greening (Figures 1C and 5). Because the *cre1 ahk3* and *ahk2 ahk3* double mutants retain BA-enhanced root greening (Figure 1C), it is likely that three cytokinin receptors redundantly perceive and pass on the cytokinin signal to subsequent steps for root greening, a result consistent with a previous report (Higuchi et al., 2004).

In contrast with the positive effects of cytokinin, we found that auxin, which is transported to the root from the shoot by a specific polar transport system (Robert and Friml, 2009), negatively regulates chlorophyll biosynthesis in roots (Figure 1A). In the auxin signaling pathway, Aux/IAAs and ARFs modulate auxin-responsive transcription, thereby regulating many growth and developmental processes (Guilfoyle and Hagen, 2007). Different sets of the 29 Aux/IAA and 23 ARF proteins found in *Arabidopsis* possess different functions (Liscum and Reed, 2002; Guilfoyle and Hagen, 2007). In fact, the action of the auxin signal in root greening was found to be rather complex. While *slr-1* and *arf7 arf19* showed significant enhancement of chlorophyll accumulation in the root, *shy2-101* roots showed a reduction in chlorophyll content (Figure 1B). It has been reported that gain-of-function mutations in different Aux/IAA genes have contrasting effects on the same auxin response (Liscum and Reed, 2002). Likewise, opposite effects of *slr-1* and *shy2-101* on root greening may be attributed to differences in expression patterns and/or target factors between SLR/IAA14 and SHY2/IAA3. Meanwhile, the similarity in chlorophyll accumulation between *slr-1* and *arf7 arf19* roots is reasonable because both ARF7 and ARF19 are molecular targets of IAA14 (Fukaki et al.,

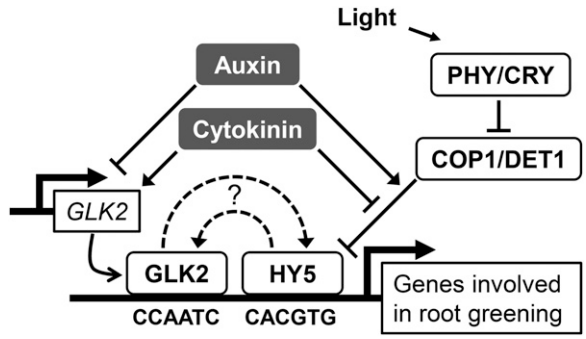

Figure 10. Model of Crosstalk between Auxin/Cytokinin and Light Signaling.

Light signals through phytochromes (PHY) and cryptochromes (CRY) repress COP1/DET1-dependent degradation of HY5. Cytokinin can also function to stabilize the HY5 protein, whereas auxin acts in opposing directions. The stabilized HY5 protein induces the expression of genes involved in root greening by binding to G-box elements (CACGTG) in their promoter regions. The expression of yet another root greening transcription factor, *GLK2*, is also regulated in opposing directions by auxin and cytokinin at the transcriptional level. The upregulated GLK2 induces gene expression related to root greening directly by binding to their promoter regions containing a putative *cis*-element (CCAATC). GLK2 can also induce accumulation of HY5, whereas HY5 maximizes the GLK2 activity on root greening.

2005, 2006; Okushima et al., 2005). Considering that auxin is required for repression of root greening (Figure 1A), the auxin signaling mediated by IAA14 and ARF7/19 is responsible for the repression of chlorophyll biosynthesis in the root; conversely, perturbation of the pathway causes enhanced chlorophyll accumulation in roots. Because the enhancement of chlorophyll accumulation in the roots of *arf7 arf19* was less pronounced than that in *slr-1* (Figure 1B), other ARFs potentially targeted by IAA14 may negatively regulate chlorophyll biosynthesis in the root. On the other hand, exogenous IAA treatment of *slr-1* seedlings led to a significant reduction in chlorophyll accumulation (Figure 1D), suggesting that other Aux/IAA proteins may also function in root greening, as proposed previously with respect to root development (Tatematsu et al., 2004; Weijers et al., 2005; Fukaki et al., 2007).

It is known that auxin and cytokinin act antagonistically to regulate various developmental events, such as shoot/root formation from callus, apical dominance, and lateral root formation, and that complex crosstalk exists between the signaling networks of these two hormones (Moubayidin et al., 2009). In this study, cytokinin and auxin were found to function in opposite directions in the regulation of chlorophyll accumulation in *Arabidopsis* roots. Since exogenously added IAA did not reduce the chlorophyll content in wild-type roots and auxin-dependent alterations of chlorophyll levels were observed only when auxin signaling was perturbed by physical, chemical, or genetic manipulations (Figures 1A and 1B), it is likely that endogenous auxin is enough to fully repress the chlorophyll biosynthesis in roots. In addition, even in the presence of endogenous or exogenous auxin, there was no diminution of the positive effect of exogenous BA on chlorophyll accumulation (Figure 1D). Thus, the promoting effect of cytokinin may override the negative effect of auxin.

Chlorophyll accumulation and chloroplast biogenesis are most pronounced in the stele of the mature primary root (Figures 2A and 2B; see Supplemental Figure 2 online). This spatial pattern was essentially unchanged by BA application, suggesting that chlorophyll accumulation in the root is dependent on the cell type and that cytokinin does not change the pattern, but only enhances chlorophyll accumulation. Although these cells are also the main sites of chloroplast development in the *slr-1* root, weak accumulation of chlorophyll and partial thylakoid membrane formation were additionally observed in plastids developed in the central vascular region (Figure 2B; see Supplemental Figure 6C online), inferring that the change in cell types or modification of cell type–dependent chlorophyll accumulation also occurs in the root by disruption of auxin signaling. Because strong $IAA14_{pro}$ activity was observed in the vascular tissues of the primary roots (see Supplemental Figure 3 online) (Fukaki et al., 2002), we suspect that auxin signaling in these tissues is important for the regulation of chloroplast development in the root.

Crosstalk between Auxin/Cytokinin and Light Signaling in Root Greening

Usami et al. (2004) reported that root greening is regulated by a strong synergistic interaction between phytochromes A and B and cryptochromes in *Arabidopsis*. Consistent with this report, our analysis showed a significant reduction in chlorophyll accumulation in the roots of *phyA phyB*, *cry1*, and *hy2-101* (Figure 6). BA treatment or introduction of the *slr-1* mutation, however, increased chlorophyll accumulation in these photoreceptor mutants, suggesting that the effects of cytokinin and auxin occur independently or downstream of these photoreceptors. By contrast, the complete loss of chlorophyll in *hy5-215* roots was not rescued by BA treatment or the *slr-1* mutation. Therefore, HY5 is a prerequisite for root greening, and the hormonal regulation is HY5 dependent. Indeed, cytokinin promotes the expression of anthocyanin biosynthetic genes in the presence of light by reducing the COP1-dependent degradation of HY5 (Vandenbussche et al., 2007). Furthermore, root greening has been observed in *cop1* and *det1* mutants (Chory and Peto, 1990; Deng and Quail, 1992), indicating the importance of HY5 stability for chlorophyll accumulation in the root. Although increased accumulation of HY5 was not clearly observed in the BA-treated roots, the HY5 level increased in *slr-1* roots (Figure 7A), a possible indication that these phytohormones regulate chloroplast development in the root by modulating the stability of HY5 (Figure 10). This idea is consistent with our data indicating that both BA treatment and the *slr-1* mutation upregulate the expression of the key chlorophyll biosynthesis genes and photosynthetic genes in the root (Figure 5), many of which are in vivo targets of HY5 (see Supplemental Data Sets 2 and 3 online) (Lee et al., 2007).

Crosstalk between Auxin/Cytokinin and GLK Signaling in Root Greening

Waters et al. (2009) reported that potent transcription factors, GLK1 and GLK2, coordinate the expression of nuclear-encoded photosynthesis-related genes, including chlorophyll biosynthesis genes in *Arabidopsis*. Consistent with the fact that *GLK2* transcript is more abundant than that of *GLK1* in the root (Fitter et al., 2002), the *glk2.1* mutant, but not the *glk1.1* mutant, showed a significant reduction in chlorophyll accumulation (Figure 8B). Although these data demonstrate that GLK2 is the main isoform responsible for root greening in *Arabidopsis*, both $GLK1_{OX}$ and $GLK2_{OX}$ showed dramatic enhancement of chlorophyll accumulation in the root. Because similar functions have been indicated for GLK1 and GLK2 in aerial organs (Waters et al., 2009), it is likely that the functions of GLK1 and GLK2 are differentiated by their expression levels, as proposed by Fitter et al. (2002). Although our data demonstrate an involvement of GLKs in root greening, the *glk1 glk2* double mutant still accumulated a certain level of chlorophyll in its roots, showing that GLKs are not indispensable for root greening.

Consistent with the identification of GLKs as *Arabidopsis* RESPONSE REGULATOR-B proteins involved in the cytokinin signaling pathway (Imamura et al., 1999; Fitter et al., 2002), the expression of *GLK2* was found to be upregulated in the greening roots of BA-treated and *slr-1* seedlings (Figure 8A). Considering the high levels of chlorophyll accumulation in $GLK1_{OX}$ and $GLK2_{OX}$ (Figure 8B), it is likely that GLK2 is one of the hormone signaling–responsive factors that regulates root greening. However, chlorophyll content was still increased in the root of the *glk1 glk2* double mutant in response to BA and PCIB (Figure 8D), indicating that other factors are also involved in hormonal regulation of chlorophyll accumulation. Because high accumulation

of HY5 was observed in *slr-1* roots (Figure 7A) and cytokinin-treated *Arabidopsis* seedlings (Vandenbussche et al., 2007), the stabilization and/or modification of HY5 may be involved in hormonal regulation of root greening independently of GLK signaling.

HY5 and GLK Transcription Factors in Root Greening

Our data demonstrate that two types of transcription factors, HY5 and GLKs, are involved in the regulation of root greening; the former is a pivotal factor for chlorophyll accumulation in roots and the latter, while not essential, is a strong activator of root greening. Although *GLK2* is found in a list of putative targets of HY5 (Lee et al., 2007) and the possibility of HY5-dependent regulation of *GLK2* expression has been discussed (Waters et al., 2009), our expression data show that HY5 is not essential for *GLK2* expression in roots (Figure 8A). Thus, *GLK2* is unlikely to act downstream of HY5, at least in root greening. Meanwhile, a genetic interaction between *HY5* and *GLK*s was revealed by the analysis of *hy5* mutants overexpressing *GLK1* or *GLK2* (Figure 9C). Intriguingly, the high expression of these factors in the *hy5* background triggered only partial accumulation of chlorophyll in roots, suggesting that GLK factors require HY5 to maximize their function in the root. It was previously reported that GLK proteins can interact with G-box binding factors (Tamai et al., 2002). Thus, slight accumulation of chlorophyll in the root of *hy5*/*GLK*$_{OX}$ plants may be explained by partial compensation of the HY5 deficiency by other G-box binding factors, such as a HY5-HOMOLOG (HYH), in combination with the excessive accumulation of GLK factors. On the other hand, high accumulation of HY5 was observed in the roots of *GLK* overexpressors (Figure 9A), implying that GLKs can also act positively on HY5. The positive relationship between HY5 and GLKs is consistent with the fact that most key chlorophyll biosynthetic genes are targets of both HY5 and GLKs (see Supplemental Data Set 3 online) (Lee et al., 2007; Waters et al., 2009). Although further analyses are required to elucidate the molecular interactions between GLKs and G-box binding factors, including HY5, we conclude that the combination of at least two transcription factors, HY5 and GLK2, is involved in the coordinated expression of nuclear-encoded photosynthesis-related genes in the root (Figure 10).

Plastic Differentiation of Root Plastids

The regulatory mechanism for chloroplast differentiation in roots is certainly distinct from that in aerial organs, such as cotyledons and leaves, because of their developmental differences. Also, the composition of interacting factors for root greening seems far simpler than that in aerial organs, in which numerous activators and repressors are involved in distinct complexes. Thus, the simple root system is likely to be a good model for elucidation of the coordinated expression of photosynthesis-related genes, although the results cannot be applied directly to the aerial organs. Although the chlorophyll level in *hy5-215* roots is almost negligible (Figure 6), chlorophyll accumulation in the leaves is not strongly impaired even in the *hy5 hyh* double mutant (Sibout et al., 2006). In addition, there are no notable differences in chlorophyll contents in leaves between wild-type and auxin signaling mutants that have greenish roots (see Supplemental Figure 1 online). However, cytokinins promote photomorphogenesis at the early stage of greening in cotyledons (Chory et al., 1994) and induce chlorophyll biosynthesis (Lew and Tsuji, 1982) via transcriptional activation of regulatory genes (Masuda et al., 1995). In addition, some auxin signals have been proposed to be involved in the repression of photomorphogenesis (Kim et al., 1996, 1998; Nagpal et al., 2000). Our recent data in *Arabidopsis* also suggest that cytokinin and auxin signaling play important roles in chlorophyll biosynthesis in cotyledons during photomorphogenesis (Hedtke et al., 2012). Thus, it is possible that the regulation of an early stage of chloroplast differentiation in deetiolated cotyledons resembles that in roots, in which cytokinin and auxin have opposing effects, although the factors involved might be significantly different. Therefore, our idea that the combination of auxin/cytokinin- and light-dependent transcription factors coordinates the expression of photosynthesis-related genes can be extended to aerial organs and merits further analysis. In addition to cytokinin and auxin, many other phytohormones, such as ethylene, gibberellin, and brassinosteroid, are known to affect expression of photosynthesis-related genes during skotomorphogenesis to photomorphogenesis (Tanaka et al., 2011). Thus, intricate interactions across light and hormone signaling pathways should be unraveled to understand the regulatory mechanism for chloroplast development.

In conclusion, we show that, together with light signaling, auxin/cytokinin signaling is involved in the biosynthesis of chlorophyll and photosynthetic machineries in root plastids via the combination of at least two transcription factors, HY5 and GLK2. Although roots usually develop nonphotosynthetic plastids, our results show that root plastids can plastically differentiate into photosynthetically active chloroplasts by modulating light signaling and phytohormone signaling. Considering that photosynthesis by roots actually contributes to the carbon economy in some species, our findings are relevant to the production of photosynthetically efficient crops that are cultivated hydroponically in plant factories where the roots can also be illuminated. The effects of increased photosynthesis by "green roots" for the carbon economy of the whole plant should be evaluated in the future.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana plants were grown vertically on solid medium (1× Murashige and Skoog medium, 1% [w/v] Suc, and 0.8% [w/v] agar, pH 5.7) at 23°C under continuous light. The double mutants of cytokinin receptors (*ahk2-2 ahk3-3* and *cre1-12 ahk3-3*) were described by Higuchi et al. (2004). The auxin mutants *axr4-2 aux1-7* (Hobbie and Estelle, 1995), *axr2-1* (Timpte et al., 1994), *msg2-1* (Tatematsu et al., 2004), *slr-1* (Fukaki et al., 2002), and *arf7 arf19* (*nph4-1 arf19-1*) (Okushima et al., 2005) were previously described. The *IAA14*$_{pro}$*:mIAA14-GR*, *SHR*$_{pro}$*:mIAA14-GR*, and *SCR*$_{pro}$*:mIAA14-GR* lines were described by Fukaki et al. (2005). *IAA14*$_{pro}$*:GUS* was described by Fukaki et al. (2002). *SCR*$_{pro}$*:GFP* and *SHR*$_{pro}$*:GUS* are described by Helariutta et al. (2000). End199 was described by Di Laurenzio (1996) and Malamy and Benfey (1997). *hy2-101* and *hy5-215* were described by Kohchi et al. (2001) and Oyama et al. (1997), respectively. *glk1.1*, *glk2.1*, *glk1 glk2* (Fitter et al., 2002), *GLK1*$_{OX}$

(*35S*:*GLK1*), and *GLK2*$_{OX}$ (*35S*:*GLK2*) (Waters et al., 2008) were provided by Jane A. Langdale, whereas *hy5-1*/*HY5*$_{pro}$:*HY5-YFP* (Oravecz et al., 2006) was provided by Roman Ulm. *cry1* (*hy4-1*) and *phyA-201 phyB-5* were obtained from The Arabidopsis Information Resource. For microarray analysis, qRT-PCR analysis, and the chlorophyll determination in Figure 5B, plants were grown for 7 d in the presence or absence of 1 μM BA following growth on Murashige and Skoog medium for 7 d. For all other analyses here, plants were grown for 14 d and then grown for another 7 d in the presence or absence of various growth factors (BA, IAA, and PCIB).

Chlorophyll Determination

Root samples crushed into powder in liquid nitrogen were homogenized in 80% acetone, and debris was removed by centrifugation at 10,000*g* for 5 min. The absorbance of the supernatant at 720, 663, 647, and 645 nm was measured with an Ultrospec 2100 *pro* spectrophotometer (GE Healthcare Biosciences). The chlorophyll (*a* and *b*) concentration of the samples was determined as described (Melis et al., 1987).

Microscopic Analyses

For Figures 2A and 8C, primary roots at ~4.0 cm from the root-hypocotyl junction were examined using a confocal laser scanning microscope (LSM 510 and LSM700; Carl Zeiss). To detect chlorophyll autofluorescence and fluorescence from the HY5-YFP protein for Figures 7B and 7C, primary roots at ~2.0 (Figure 7B), 4.0, 6.0, and 8.0 cm (Figure 7C) from the root-hypocotyl junction were examined using a confocal laser scanning microscope (LSM 510 and LSM700; Carl Zeiss). For these analyses, laser intensity and detection gain were fixed during a series of the analyses to compare fluorescence intensity among different samples. For cross-section analysis, primary roots ~2.0 cm from the root-hypocotyl junction were embedded in 5% (w/v) low-melting agar and sliced into rounds with razors. Chlorophyll autofluorescence was detected using another confocal laser scanning microscope (FV-1000; Olympus). For ultrastructure analyses, primary roots at ~4.0 cm from the root-hypocotyl junction were analyzed by transmission electron microscopy according to Toyooka et al. (2000) with modification. Tissues were fixed with 4% paraformaldehyde, 2% glutaraldehyde, and 150 mM Suc in 100 mM sodium cacodylate buffer, pH 7.4, for 2 h at room temperature. They were postfixed with 1% osmium tetroxide in 50 mM cacodylate buffer, pH 7.4, for 3 h. After dehydration in a graded methanol series, the samples were embedded in Epon812 resin. Images were recorded by JEM-1400 (JEOL) and merged by Photoshop CS5 (Adobe).

Chlorophyll Fluorescence Measurements

For chlorophyll fluorescence measurements in Figure 3, roots were detached from the shoots and incubated in the dark for 5 min before experiments. Chlorophyll fluorescence transients were measured with a fluorescence monitoring system (FL3000; Photon System Instruments). In the fluorescence induction experiments, data were collected in a logarithmic time series between 0.2 ms and 5 s after the onset of strong actinic light. The maximum quantum yield of PSII ([Fm − Fo]/Fm) was calculated from the minimum fluorescence yield (Fo) and the maximum fluorescence yield (Fm). In the fluorescence decay experiments, data were collected between 0.2 ms and 60 s following a single saturation flash.

Gene Expression Analysis

Total RNA was extracted using the RNeasy plant mini kit (Qiagen), with genomic DNA being digested by DNaseI according to the manufacturer's instructions. Global changes in gene expression were explored with spotted *Arabidopsis* 24k oligonucleotide arrays. Synthesis, labeling, and hybridization of cDNA and quantification of spot intensities were performed as described (Kangasjärvi et al., 2008). The expression data were normalized and analyzed using GeneSpring 7.2 in three independent biological replicates from RNA preparation to cDNA hybridization and spot quantification. Any given P values are derived from a paired Student's *t* test. The microarray data set has been deposited in ArrayExpress under accession number E-MEXP-3132 (http://www.ebi.ac.uk/arrayexpress/). For qRT-PCR analysis, reverse transcription was performed using an RT-PCR kit (RNA PCR kit, version 3.0; TaKaRa Bio). cDNA amplification was performed using the SYBR PreMix Ex Taq kit (TaKaRa) and 100 nM gene-specific primers (listed in Supplemental Table 1 online). Thermal cycling consisted of an initial denaturation step at 95°C for 10 s, followed by 40 cycles of 5 s at 95°C and 30 s at 62°C. Signal detection and quantification were performed in duplicate using MiniOpticon (Bio-Rad) for Figure 5 and Mx3000P (Agilent Technologies) for Figure 9B. The relative abundance of all transcripts amplified was normalized to the constitutive expression level of *ACTIN8* (Pfaffl, 2001). Three independent biological experiments were performed for each root sample.

Immunoblot Analysis

The immunoblot analysis was performed as described previously (Kobayashi et al., 2007) using 100 μg of total proteins for Figure 7A and 10 μg for Figure 9A. For the detection of HY5, the anti-HY5 antibody (Oyama et al., 1997) at a dilution of a 1:500 was used. For loading controls, total proteins blotted to nitrocellulose membranes were stained with 0.1% (w/v) Ponceau S (Nacalai Tesque) in 5% (v/v) acetic acid solution.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *LHCB4.1* (At5g01530), *LHCA4* (At3g47470), *HEMA1* (At1g58290), *CHLH* (At5g13630), *CHL27* (At3g56940), *CHLP* (At1g74470), *GLK1* (At2g20570), and *GLK2* (At5g44190). The microarray data set has been deposited in ArrayExpress under accession number E-MEXP-3132 (http://www.ebi.ac.uk/arrayexpress/).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Chlorophyll Content in Shoots of Auxin Signaling Mutants.

Supplemental Figure 2. Chlorophyll Accumulation in the Stele of the Primary Root.

Supplemental Figure 3. Promoter Activities of *IAA14*, *SHR*, and *SCR* in the Mature Root.

Supplemental Figure 4. Chlorophyll Autofluorescence in Roots Expressing the Stabilized Mutant IAA14 Protein.

Supplemental Figure 5. Spatial Images of Maximum Quantum Yield (Fv/Fm) in Roots.

Supplemental Figure 6. Plastid Distribution in the Primary Root of the Wild Type and *slr-1*.

Supplemental Table 1. Oligonucleotide Primers Used in This Study.

Supplemental Data Set 1. The 20 Most Upregulated Genes in Roots of *slr-1* and BA-Treated Wild Type.

Supplemental Data Set 2. Changes in Expression of Nuclear Genes Encoding Subunits of Photosystem I and II.

Supplemental Data Set 3. Changes in Expression of Genes Involved in Tetrapyrrole Biosynthesis.

Supplemental Data Set 4. Changes in Expression of Genes Involved in Regulation of Photomorphogenesis and Chloroplast Development.

ACKNOWLEDGMENTS

We thank Roman Ulm (University of Freiburg) for the supply of the *Arabidopsis* line *hy5-1/HY5$_{pro}$:HY5-YFP*; Jane A. Langdale (Oxford University) for *glk1.1*, *glk1.2*, *glk1 glk2*, *GLK1$_{OX}$*, and *GLK2$_{OX}$*; Philip N. Benfey (Duke University) for *SCR$_{pro}$:GFP*, *SHR$_{pro}$:GUS*, and End199; Tatsuo Kakimoto (Osaka University) for *ahk2-2 ahk3-3* and *cre1-12 ahk3-3*; Kiyotaka Okada (National Institute for Basic Biology) for *hy5-215*; and Takayuki Kohchi (Kyoto University) for *hy2-101*. We thank Kiyotaka Okada and Tokitaka Oyama (Kyoto University) for the supply of the anti-HY5 antibody. We also thank Nino A. Espinas (University of Tokyo) for English editing, Mayumi Wakazaki (RIKEN) for performing electron microscopy, and Chika Ikeda (RIKEN) for her technical assistance. This work was supported by Grants-in-Aid for Scientific Research (No. 21570035 to T.M. and No. 22119010 to K.S.) and in part by the Global Center of Excellence Program (K03) from the Ministry of Education, Culture, Sports and Technology, Japan, Center of Excellence Grant 118637 from the Academy of Finland, and a Research Fellowship for Young Scientists from the Japanese Society for the Promotion of Science to K.K. K.K is a recipient of a RIKEN postdoctoral fellowship.

AUTHOR CONTRIBUTIONS

K.K. conceived the project, designed the study, performed most of the experiments, analyzed data, and wrote the article. T.M. designed and directed the study, performed experiments, analyzed data, and wrote the article. S.B. and M.S. performed experiments. T.O., K.T., and M.K. analyzed data. E.-M.A., H.F., H.O., and K.S. directed the study and contributed to writing the article.

Received October 3, 2011; revised February 10, 2012; accepted February 25, 2012; published March 13, 2012.

REFERENCES

Bae, G., and Choi, G. (2008). Decoding of light signals by plant phytochromes and their interacting proteins. Annu. Rev. Plant Biol. **59:** 281–311.

Benzing, D.H., Friedman, W.E., Peterson, G., and Renfrow, A. (1983). Shootlessness, velamentous roots, and the pre-eminence of Orchidaceae in the epiphytic biotope. Am. J. Bot. **70:** 121–133.

Chen, M., Chory, J., and Fankhauser, C. (2004). Light signal transduction in higher plants. Annu. Rev. Genet. **38:** 87–117.

Chory, J., and Peto, C.A. (1990). Mutations in the DET1 gene affect cell-type-specific expression of light-regulated genes and chloroplast development in Arabidopsis. Proc. Natl. Acad. Sci. USA **87:** 8776–8780.

Chory, J., Reinecke, D., Sim, S., Washburn, T., and Brenner, M. (1994). A role for cytokinins in de-etiolation in Arabidopsis (det mutants have an altered response to cytokinins). Plant Physiol. **104:** 339–347.

Deng, X.W., and Quail, P.H. (1992). Genetic and phenotypic characterization of cop1 mutants of *Arabidopsis thaliana*. Plant J. **2:** 83–95.

Dharmasiri, S., Swarup, R., Mockaitis, K., Dharmasiri, N., Singh, S.K., Kowalchyk, M., Marchant, A., Mills, S., Sandberg, G., Bennett, M.J., and Estelle, M. (2006). AXR4 is required for localization of the auxin influx facilitator AUX1. Science **312:** 1218–1220.

Di Laurenzio, L., Wysocka-Diller, J., Malamy, J.E., Pysh, L., Helariutta, Y., Freshour, G., Hahn, M.G., Feldmann, K.A., and Benfey, P.N. (1996). The SCARECROW gene regulates an asymmetric cell division that is essential for generating the radial organization of the Arabidopsis root. Cell **86:** 423–433.

Duek, P.D., and Fankhauser, C. (2005). bHLH class transcription factors take centre stage in phytochrome signalling. Trends Plant Sci. **10:** 51–54.

Fitter, D.W., Martin, D.J., Copley, M.J., Scotland, R.W., and Langdale, J.A. (2002). GLK gene pairs regulate chloroplast development in diverse plant species. Plant J. **31:** 713–727.

Flores, H.E., Dai, Y., Cuello, J.L., Maldonado-Mendoza, I.E., and Loyola-Vargas, V.M. (1993). Green roots: Photosynthesis and photoautotrophy in an underground plant organ. Plant Physiol. **101:** 363–371.

Fukaki, H., Nakao, Y., Okushima, Y., Theologis, A., and Tasaka, M. (2005). Tissue-specific expression of stabilized SOLITARY-ROOT/IAA14 alters lateral root development in Arabidopsis. Plant J. **44:** 382–395.

Fukaki, H., Okushima, Y., and Tasaka, M. (2007). Auxin-mediated lateral root formation in higher plants. Int. Rev. Cytol. **256:** 111–137.

Fukaki, H., Tameda, S., Masuda, H., and Tasaka, M. (2002). Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of Arabidopsis. Plant J. **29:** 153–168.

Fukaki, H., Taniguchi, N., and Tasaka, M. (2006). PICKLE is required for SOLITARY-ROOT/IAA14-mediated repression of ARF7 and ARF19 activity during Arabidopsis lateral root initiation. Plant J. **48:** 380–389.

Gill, A.M., and Tomlinson, P.B. (1977). Studies of the growth of red mangrove (*Rhizophora mangle* L.) 4: The adult root system. Biotropica **9:** 145–155.

Govindjee, X. (1995). Sixty-three years since Kautsky: Chlorophyll a fluorescence. Aust. J. Plant Physiol. **22:** 131–160.

Guilfoyle, T.J., and Hagen, G. (2007). Auxin response factors. Curr. Opin. Plant Biol. **10:** 453–460.

Hedtke, B., Alawady, A., Albacete, A., Kobayashi, K., Melzer, M., Roitsch, T., Masuda, T., and Grimm, B. (2012). Deficiency in riboflavin biosynthesis affects tetrapyrrole biosynthesis in etiolated Arabidopsis tissue. Plant Mol. Biol. **78:** 77–93.

Helariutta, Y., Fukaki, H., Wysocka-Diller, J., Nakajima, K., Jung, J., Sena, G., Hauser, M.T., and Benfey, P.N. (2000). The SHORT-ROOT gene controls radial patterning of the Arabidopsis root through radial signaling. Cell **101:** 555–567.

Higuchi, M., et al. (2004). In planta functions of the Arabidopsis cytokinin receptor family. Proc. Natl. Acad. Sci. USA **101:** 8821–8826.

Hobbie, L., and Estelle, M. (1995). The axr4 auxin-resistant mutants of *Arabidopsis thaliana* define a gene important for root gravitropism and lateral root initiation. Plant J. **7:** 211–220.

Imamura, A., Hanaki, N., Nakamura, A., Suzuki, T., Taniguchi, M., Kiba, T., Ueguchi, C., Sugiyama, T., and Mizuno, T. (1999). Compilation and characterization of *Arabidopsis thaliana* response regulators implicated in His-Asp phosphorelay signal transduction. Plant Cell Physiol. **40:** 733–742.

Kangasjärvi, S., Lepistö, A., Hännikäinen, K., Piippo, M., Luomala, E.M., Aro, E.M., and Rintamäki, E. (2008). Diverse roles for chloroplast stromal and thylakoid-bound ascorbate peroxidases in plant stress responses. Biochem. J. **412:** 275–285.

Kim, B.C., Soh, M.C., Kang, B.J., Furuya, M., and Nam, H.G. (1996). Two dominant photomorphogenic mutations of *Arabidopsis thaliana* identified as suppressor mutations of hy2. Plant J. **9:** 441–456.

Kim, B.C., Soh, M.S., Hong, S.H., Furuya, M., and Nam, H.G. (1998).

Photomorphogenic development of the Arabidopsis shy2-1D mutation and its interaction with phytochromes in darkness. Plant J. **15:** 61–68.

Kobayashi, K., Kondo, M., Fukuda, H., Nishimura, M., and Ohta, H. (2007). Galactolipid synthesis in chloroplast inner envelope is essential for proper thylakoid biogenesis, photosynthesis, and embryogenesis. Proc. Natl. Acad. Sci. USA **104:** 17216–17221.

Kohchi, T., Mukougawa, K., Frankenberg, N., Masuda, M., Yokota, A., and Lagarias, J.C. (2001). The *Arabidopsis* HY2 gene encodes phytochromobilin synthase, a ferredoxin-dependent biliverdin reductase. Plant Cell **13:** 425–436.

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

Leivar, P., Tepperman, J.M., Monte, E., Calderon, R.H., Liu, T.L., and Quail, P.H. (2009). Definition of early transcriptional circuitry involved in light-induced reversal of PIF-imposed repression of photomorphogenesis in young *Arabidopsis* seedlings. Plant Cell **21:** 3535–3553.

Lew, R., and Tsuji, H. (1982). Effect of benzyladenine treatment duration on delta-aminolevulinic acid accumulation in the dark, chlorophyll lag phase abolition, and long-term chlorophyll production in excised cotyledons of dark-grown cucumber seedlings. Plant Physiol. **69:** 663–667.

Liscum, E., and Reed, J.W. (2002). Genetics of Aux/IAA and ARF action in plant growth and development. Plant Mol. Biol. **49:** 387–400.

Lopez-Juez, E., and Pyke, K.A. (2005). Plastids unleashed: Their development and their integration in plant development. Int. J. Dev. Biol. **49:** 557–577.

Malamy, J.E., and Benfey, P.N. (1997). Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. Development **124:** 33–44.

Masuda, T., and Fujita, Y. (2008). Regulation and evolution of chlorophyll metabolism. Photochem. Photobiol. Sci. **7:** 1131–1149.

Masuda, T., Ohta, H., Shioi, Y., Tsuji, H., and Takamiya, K. (1995). Gene expression profiling of the tetrapyrrole metabolic pathway in Arabidopsis with a mini-array system. Plant Cell Physiol. **36:** 1237–1243.

Matsumoto, F., Obayashi, T., Sasaki-Sekimoto, Y., Ohta, H., Takamiya, K., and Masuda, T. (2004). Gene expression profiling of the tetrapyrrole metabolic pathway in Arabidopsis with a mini-array system. Plant Physiol. **135:** 2379–2391.

Melis, A., Spangfort, M., and Andersson, B. (1987). Light-absorption and electron transport balance between photosystem-II and photosystem-I in spinach chloroplasts. Photochem. Photobiol. **45:** 129–136.

Moubayidin, L., Di Mambro, R., and Sabatini, S. (2009). Cytokinin-auxin crosstalk. Trends Plant Sci. **14:** 557–562.

Nagpal, P., Walker, L.M., Young, J.C., Sonawala, A., Timpte, C., Estelle, M., and Reed, J.W. (2000). AXR2 encodes a member of the Aux/IAA protein family. Plant Physiol. **123:** 563–574.

Nakamura, H., Muramatsu, M., Hakata, M., Ueno, O., Nagamura, Y., Hirochika, H., Takano, M., and Ichikawa, H. (2009). Ectopic overexpression of the transcription factor OsGLK1 induces chloroplast development in non-green rice cells. Plant Cell Physiol. **50:** 1933–1949.

Okushima, Y., et al. (2005). Functional genomic analysis of the AUXIN RESPONSE FACTOR gene family members in *Arabidopsis thaliana*: Unique and overlapping functions of ARF7 and ARF19. Plant Cell **17:** 444–463.

Oravecz, A., Baumann, A., Máté, Z., Brzezinska, A., Molinier, J., Oakeley, E.J., Adám, E., Schäfer, E., Nagy, F., and Ulm, R. (2006). CONSTITUTIVELY PHOTOMORPHOGENIC1 is required for the UV-B response in *Arabidopsis*. Plant Cell **18:** 1975–1990.

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

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29:** e45.

Reed, J.W., Elumalai, R.P., and Chory, J. (1998). Suppressors of an *Arabidopsis thaliana* phyB mutation identify genes that control light signaling and hypocotyl elongation. Genetics **148:** 1295–1310.

Robert, H.S., and Friml, J. (2009). Auxin and other signals on the move in plants. Nat. Chem. Biol. **5:** 325–332.

Shin, J., Kim, K., Kang, H., Zulfugarov, I.S., Bae, G., Lee, C.H., Lee, D., and Choi, G. (2009). Phytochromes promote seedling light responses by inhibiting four negatively-acting phytochrome-interacting factors. Proc. Natl. Acad. Sci. USA **106:** 7660–7665.

Sibout, R., Sukumar, P., Hettiarachchi, C., Holm, M., Muday, G.K., and Hardtke, C.S. (2006). Opposite root growth phenotypes of hy5 versus hy5 hyh mutants correlate with increased constitutive auxin signaling. PLoS Genet. **2:** e202.

Stephenson, P.G., Fankhauser, C., and Terry, M.J. (2009). PIF3 is a repressor of chloroplast development. Proc. Natl. Acad. Sci. USA **106:** 7654–7659.

Swarup, K., et al. (2008). The auxin influx carrier LAX3 promotes lateral root emergence. Nat. Cell Biol. **10:** 946–954.

Tamai, H., Iwabuchi, M., and Meshi, T. (2002). Arabidopsis GARP transcriptional activators interact with the Pro-rich activation domain shared by G-box-binding bZIP factors. Plant Cell Physiol. **43:** 99–107.

Tanaka, R., Kobayashi, K., and Masuda, T. (2011). Tetrapyrrole metabolism in *Arabidopsis thaliana*. The Arabidopsis Book **9:** e0145, doi/10.1199/tab.0145.

Tatematsu, K., Kumagai, S., Muto, H., Sato, A., Watahiki, M.K., Harper, R.M., Liscum, E., and Yamamoto, K.T. (2004). MASSUGU2 encodes Aux/IAA19, an auxin-regulated protein that functions together with the transcriptional activator NPH4/ARF7 to regulate differential growth responses of hypocotyl and formation of lateral roots in *Arabidopsis thaliana*. Plant Cell **16:** 379–393.

Tian, Q., and Reed, J.W. (1999). Control of auxin-regulated root development by the *Arabidopsis thaliana* SHY2/IAA3 gene. Development **126:** 711–721.

Timpte, C., Wilson, A.K., and Estelle, M. (1994). The axr2-1 mutation of *Arabidopsis thaliana* is a gain-of-function mutation that disrupts an early step in auxin response. Genetics **138:** 1239–1249.

Toyooka, K., Okamoto, T., and Minamikawa, T. (2000). Mass transport of proform of a KDEL-tailed cysteine proteinase (SH-EP) to protein storage vacuoles by endoplasmic reticulum-derived vesicle is involved in protein mobilization in germinating seeds. J. Cell Biol. **148:** 453–464.

Usami, T., Mochizuki, N., Kondo, M., Nishimura, M., and Nagatani, A. (2004). Cryptochromes and phytochromes synergistically regulate Arabidopsis root greening under blue light. Plant Cell Physiol. **45:** 1798–1808.

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

Vanneste, S., et al. (2005). Cell cycle progression in the pericycle is not sufficient for SOLITARY ROOT/IAA14-mediated lateral root initiation in *Arabidopsis thaliana*. Plant Cell **17:** 3035–3050.

Waters, M.T., Moylan, E.C., and Langdale, J.A. (2008). GLK transcription factors regulate chloroplast development in a cell-autonomous manner. Plant J. **56:** 432–444.

Waters, M.T., Wang, P., Korkaric, M., Capper, R.G., Saunders, N.J., and Langdale, J.A. (2009). GLK transcription factors coordinate expression of the photosynthetic apparatus in *Arabidopsis*. Plant Cell **21:** 1109–1128.

Weijers, D., Benkova, E., Jäger, K.E., Schlereth, A., Hamann, T., Kientz, M., Wilmoth, J.C., Reed, J.W., and Jürgens, G. (2005). Developmental specificity of auxin response by pairs of ARF and Aux/IAA transcriptional regulators. EMBO J. **24:** 1874–1885.

Wilmoth, J.C., Wang, S., Tiwari, S.B., Joshi, A.D., Hagen, G., Guilfoyle, T.J., Alonso, J.M., Ecker, J.R., and Reed, J.W. (2005). NPH4/ARF7 and ARF19 promote leaf expansion and auxin-induced lateral root formation. Plant J. **43:** 118–130.

Wysocka-Diller, J.W., Helariutta, Y., Fukaki, H., Malamy, J.E., and Benfey, P.N. (2000). Molecular analysis of SCARECROW function reveals a radial patterning mechanism common to root and shoot. Development **127:** 595–603.

Yamamoto, M., and Yamamoto, K.T. (1999). Effects of natural and synthetic auxins on the gravitropic growth habit of roots in two auxin-resistant mutants of Arabidopsis, axr1 and axr4: evidence for defects in the auxin influx mechanism of axr4. J. Plant Res. **112:** 391–396.