Shoot Removal Induces Chloroplast Development in Roots via Cytokinin Signaling[1]

Koichi Kobayashi*, Ai Ohnishi, Daichi Sasaki, Sho Fujii, Akira Iwase, Keiko Sugimoto, Tatsuru Masuda, and Hajime Wada

Department of Life Sciences (K.K., A.O., D.S., S.F., H.W.) and Department of General Systems Studies (T.M.), Graduate School of Arts and Sciences, University of Tokyo, Tokyo 153-8902, Japan; and RIKEN Center for Sustainable Resource Science, Tsurumi, Yokohama, Kanagawa 230-0045, Japan (A.I., K.S.)

ORCID IDs: 0000-0003-3726-4790 (K.K.); 0000-0002-0766-3638 (A.O.); 0000-0003-3294-7939 (A.I.); 0000-0002-9209-8230 (K.S.); 0000-0002-1585-5832 (T.M.).

The development of plant chloroplasts is regulated by various developmental, environmental, and hormonal cues. In Arabidopsis (*Arabidopsis thaliana*), chloroplast development is repressed in roots via auxin signaling. However, roots develop chloroplasts when they are detached from the shoot. In contrast to auxin, cytokinin positively affects chloroplast development in roots, but the role and signaling pathway of cytokinin in the root greening response remain unclear. To understand the regulatory pathways of chloroplast development in the plant stress response, we examined the mechanisms underlying the conditional greening of detached roots. In wild-type Arabidopsis roots, shoot removal activates type B ARABIDOPSIS RESPONSE REGULATOR (ARR)-mediated cytokinin signaling and induces chlorophyll accumulation and photosynthetic remodeling. ARR1 and ARR12 are essential for up-regulating nucleus- and plastid-encoded genes associated with chloroplast development in detached roots. In this process, WOUND INDUCED DEDIFFERENTIATION1 and class B GATA transcription factors (B-GATAs) act upstream and downstream of ARRs, respectively. Overexpression of B-GATAs promotes root greening, as does shoot removal, dependent on a light signaling transcription factor, LONG HYPOCOTYL5. Auxin represses the root greening response independent of ARR signaling. *GNC-LIKE* (*GNL*), a B-GATA, is strongly up-regulated in detached roots via ARR1 and ARR12 but is repressed by auxin, so *GNL* may function at the point of convergence of cytokinin and auxin signaling in the root greening response.

Like embryonic cells of higher plants that differentiate into various cell types, embryonic proplastids are converted into different types of plastids within cells (e.g. chloroplasts in leaves, amyloplasts in seeds and roots, and chromoplasts in flowers and fruits). Differentiated plastids have specific functions associated with the host cell types in addition to common roles in fundamental metabolism, such as the synthesis of amino acids and fatty acids (Jarvis and López-Juez, 2013). Because plastids are the site of various metabolic reactions and are involved in plant growth regulation, tight coordination between plastid and cell differentiation is essential for plant development.

Chloroplasts, which accumulate chlorophyll (Chl) and develop photosynthetic machinery in thylakoid membranes, convert solar energy into chemical energy and fix carbon dioxide into carbohydrates by photosynthesis. The biogenesis of chloroplasts is strictly regulated in response to the functional state of photosynthetic cells under various growth conditions to avoid photooxidative damage from unbalanced photosynthetic reactions. Chloroplast biogenesis requires a coordinated synthesis of photosynthetic proteins with Chl and other photosynthetic components. Photosynthesis-associated nuclear genes form tight coexpression networks with each other (Kobayashi et al., 2012b), which suggests that transcriptional regulation plays a pivotal role in regulating chloroplast biogenesis. Transcription factors known as positive regulators of chloroplast differentiation include LONG-HYPOCOTYL5 (HY5), GOLDEN2-LIKE (GLK), and class B GATA transcription factors (B-GATAs), including GATA, NITRATE-INDUCIBLE CARBON METABOLISM INVOLVED (GNC) and GNC-LIKE/CYTOKININ-RESPONSIVE GATA1 (GNL/CGA1). HY5 functions in photomorphogenesis downstream of light signaling pathways (Gangappa and Botto, 2016) and directly targets many of the light-inducible photosynthesis-associated genes (Lee et al., 2007; Toledo-Ortiz et al., 2014). Deficiency of HY5 causes down-regulation of the target photosynthetic genes in deetiolated seedlings

and roots (McCormac and Terry, 2002; Kobayashi et al., 2012a, 2014). GLK transcription factors also directly up-regulate photosynthesis-associated genes, presumably by binding to their promoter regions (Waters et al., 2009).

Arabidopsis (*Arabidopsis thaliana*) has two GLK isoforms, GLK1 and GLK2, which function redundantly, and only the double mutant *glk1 glk2* causes reduced expression of photosynthesis-associated genes with decreased Chl content in leaves (Fitter et al., 2002). Overexpression of Arabidopsis *GLKs* and their orthologs in rice (*Oryza sativa*) and tomato (*Solanum lycopersicum*) causes ectopic chloroplast development in nonphotosynthetic organs such as roots, fruits, and callus cells (Nakamura et al., 2009; Kobayashi et al., 2012a; Powell et al., 2012), so this family is one of the key factors in chloroplast differentiation.

Overexpression of *GNC* and *GNL* induces ectopic chloroplast development in roots, hypocotyls, and leaf epidermis of Arabidopsis (Chiang et al., 2012) and in leaf sheath epidermis of rice (Hudson et al., 2013). These factors can increase the number of chloroplasts in photosynthetic and nonphotosynthetic cells with their overexpression (Hudson et al., 2011; Chiang et al., 2012). The expression of *GNC* and *GNL* is induced by cytokinin via type B ARABIDOPSIS RESPONSE REGULATOR1 (ARR1) and ARR12 and is repressed by GA and auxin (Mara and Irish, 2008; Richter et al., 2010, 2013). Together with four other B-GATA paralogs, GNC and GNL are implicated in the regulation of chloroplast development in addition to various developmental processes related to light and several hormonal responses (Behringer and Schwechheimer, 2015). However, GNC and GNL up-regulate Chl biosynthesis genes indirectly and do not affect the expression of genes involved in chloroplast division (Hudson et al., 2011). How these factors participate in regulating chloroplast differentiation remains unclear.

Because chloroplasts are developmentally linked with photosynthetic tissues, various hormonal and environmental cues affect chloroplast differentiation. We reported that auxin transported from shoots represses chloroplast differentiation in Arabidopsis roots (Kobayashi et al., 2012a). When roots were detached from the shoot, they increased greening via reduced auxin signaling from the shoot. Indeed, roots deficient in auxin signaling showed an up-regulation of photosynthesis-associated genes along with increased *GLK2* expression and HY5 protein level. In contrast to auxin, cytokinin positively affects chloroplast differentiation, because cytokinin deficiency down-regulates chloroplast-related genes in green tissues, whereas excess cytokinin up-regulates the genes in roots (Brenner and Schmülling, 2012). In fact, cytokinin treatment up-regulates photosynthesis-associated genes in Arabidopsis roots, whereas mutation in the cytokinin receptors ARABIDOPSIS HISTIDINE KINASE2 (AHK2) and AHK3 down-regulated such genes (Kobayashi et al., 2012a). The expression levels of key Chl biosynthesis genes were correlated with Chl content in roots, which

suggests that transcriptional regulation plays a central role in controlling chloroplast development in roots. In addition, a recent study revealed that cytokinin up-regulates genes involved in Chl biosynthesis and light harvesting and enhances cotyledon greening during the etioplast-chloroplast transition in Arabidopsis (Cortleven et al., 2016). In this process, B-type ARRs (ARR1, ARR10, and ARR12) play an important role in up-regulating genes involved in Chl biosynthesis.

The conditional greening of detached roots indicates that plastids in roots can plastically differentiate into chloroplasts by light and hormone signaling as needed (Kobayashi et al., 2012a). This greening response may function to compensate for the loss of photosynthetic tissues. Indeed, we reported that photosynthesis in detached Arabidopsis roots contributed to carbon assimilation and the maintenance of biomass (Kobayashi et al., 2013). However, the underlying molecular mechanisms are largely unknown. To gain insight into regulatory mechanisms controlling chloroplast development in plants, we examined the signaling pathway involved in root greening after shoot removal in Arabidopsis. Our data suggest that shoot removal induces the quantitative and qualitative development of chloroplasts in roots, with cytokinin signaling via ARR1 and ARR12 functioning downstream of wounding signaling to up-regulate genes associated with chloroplast development, including chloroplast-related transcription factors and plastid-encoded genes.

RESULTS

Type B ARR-Mediated Cytokinin Signaling Promotes the Greening of Detached Roots Downstream of Wounding Signaling

To assess the involvement of cytokinin signaling in the greening of detached roots, first we examined whether cytokinin signaling is activated in roots in response to shoot removal. Cytokinin signaling was monitored in transgenic plants carrying a synthetic cytokinin reporter, *TWO-COMPONENT-OUTPUT-SENSOR* (*TCS*)::*GFP* (Müller and Sheen, 2008), and its improved version, *TCSn*::*GFP* (Zürcher et al., 2013), which harbored the concatemerized type B ARR-binding motifs. To induce root greening, shoots of 14-d-old seedlings were removed at the root-hypocotyl junction and detached roots were incubated for 7 d on agar medium under light. For the control, intact roots of 21-d-old seedlings continuously grown on agar medium were detached from shoots just before analysis.

In *TCSn*::*GFP* transgenic plants, green fluorescence was brighter in detached roots than in intact roots (Fig. 1A). The same results were observed in the *TCS*::*GFP* line (Supplemental Fig. S1A). GFP fluorescence in detached roots was broadly observed, with that at the cutting site most prominent. Some detached roots produced callus at the cutting site, and in these cases, callus showed prominent *TCS(n)*::*GFP* fluorescence with relatively weak Chl fluorescence (Supplemental
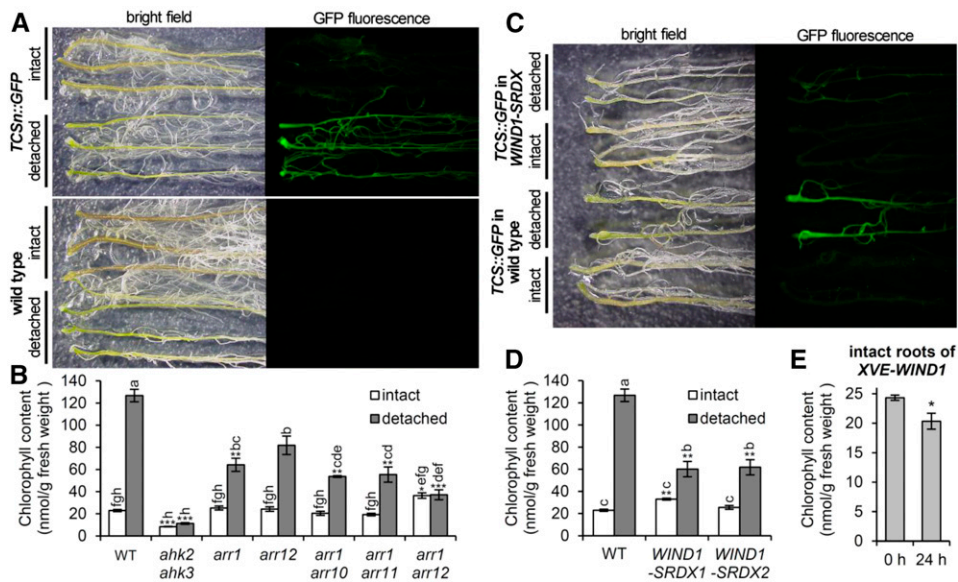
Figure 1. Cytokinin signaling functions in the greening of detached roots downstream of wounding signaling. A, GFP fluorescence in roots of the *TCSn::GFP* line and the wild-type control. B, Chl content in intact and detached roots of the wild type (WT) and mutants involved in cytokinin signaling. C, GFP fluorescence in roots of the *TCS::GFP* lines with the wild-type and *WIND1-SRDX* background. D, Chl content in intact and detached roots of the wild type and two independent lines of *WIND1-SRDX* plants. In A to D, for the detached root sample, roots were excised from 14-d-old seedlings and grown for 7 d, whereas for the intact root control, the shoots of 21-d-old seedlings were removed just before observation. E, Chl content in intact roots of the *XVE-WIND1* line. Root Chl content is shown for 21-d-old seedlings treated with 17β-estradiol for 24 h compared with the untreated control (0 h). Student's *t* test: *, $P < 0.05$. For B, D, and E, data are means ± SE from three or more independent experiments. Different letters indicate significant differences by the Tukey-Kramer multiple comparison test ($P < 0.05$). Asterisks indicate significant differences from the wild type of each condition (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, Student's *t* test after a Bonferroni correction for multiple comparisons).

Fig. S1B). Under the same conditions, wild-type plants showed no green fluorescence in intact or detached roots (Fig. 1A), so the green fluorescence detected in detached *TCSn::GFP* roots was not autofluorescence from greenish cells but specifically reflected the type B ARR-mediated GFP expression.

Next, we evaluated the contribution of type B ARRs to the greening of detached roots by measuring Chl content in roots of several *arr* mutants (Fig. 1B). Consistent with a previous report (Kobayashi et al., 2012a), a double mutant for cytokinin receptors (*ahk2 ahk3*) showed a reduced Chl level in intact roots compared with the wild type. None of the *arr* mutants showed decreased Chl content in intact roots. Of note, Chl accumulation was slightly higher in the *arr1 arr12* double mutant than in the wild type. As reported previously (Kobayashi et al., 2012a), Chl content in wild-type roots was increased substantially on shoot removal. By contrast, *ahk2 ahk3* roots showed no noticeable increase in Chl content on shoot removal. Although Chl content was increased in detached roots of *arr1*, *arr12*, *arr1 arr10*, and *arr1 arr11*, the level was lower than in detached wild-type roots. Moreover, the *arr1 arr12* double mutant showed no increase in root Chl content on shoot removal. Therefore, these type B ARRs are not essential for constitutive Chl accumulation in roots but are required for conditional root greening in response to shoot removal.

Type B ARR-mediated cytokinin signaling is activated in response to wounding stress via a transcription factor, WOUND INDUCED DEDIFFERENTIATION1 (WIND1; Iwase et al., 2011a). WIND1 cooperates with its close homologs (WIND2, WIND3, and WIND4) in a redundant manner to regulate wound-induced cellular reprogramming. Expression of WIND1-SUPERMAN REPRESSION DOMAIN (SRDX) chimeric proteins under the control of the *WIND1* promoter dominantly repressed the function of WIND1 and presumably its homologs, which resulted in the strong impairment of reprogramming processes after wounding (Iwase et al., 2011a, 2015). Because *WIND1-SRDX* lines showed strongly suppressed activation of type B ARR-mediated cytokinin signaling at wounding sites (Iwase et al., 2011a), we tested whether WIND1 signaling is involved in the activation of type B ARR signaling in detached Arabidopsis roots. In contrast to the strong green fluorescence in detached roots of *TCS::GFP* plants in the wild-type background, green fluorescence was not remarkably enhanced by shoot removal in *Pro_{WIND1}*: *WIND1-SRDX* lines carrying the *TCS::GFP* gene. Moreover, we confirmed lower Chl accumulation in detached roots of two independent *WIND1-SRDX* lines than in detached wild-type roots (Fig. 1D), which suggests that WIND1 is involved in the root greening response.

To address whether *WIND1* expression can solely activate Chl accumulation in roots, we tested β-estradiol-triggered overexpression of *WIND1* in 21-d-old LexA-VP16-estrogen receptor (*XVE*)-*WIND1* lines (Iwase et al., 2011a). A previous report showed that mature *XVE-WIND1* seedlings treated with β-estradiol for long periods formed callus-like cell masses in shoots and roots, as did the stable *WIND1* overexpression line (*35S:WIND1*; Iwase et al., 2011a). Moreover, induction of *WIND1* in *XVE-WIND1* seedlings for 1 d greatly enhanced a competence for shoot regeneration from roots (Iwase et al., 2015), which indicates that transient *WIND1* induction strongly affects cellular differentiation states. In this study, WIND1 induction by β-estradiol for 24 h did not increase root Chl content and even decreased it slightly (Fig. 1E). These data are consistent with the report that ectopic *WIND1* overexpression induced cell dedifferentiation and callus formation, thereby resulting in the degreening of green tissues (Iwase et al., 2011a). Therefore, although WIND1 is conditionally required for the greening of detached roots by activating type B ARR signaling in response to wounding, its ectopic overexpression would cause excessive reprograming effects to cells and degreening.

Type B ARR-Mediated Cytokinin Signaling Up-Regulates Genes Associated with Chloroplast Differentiation in Detached Roots

To assess whether shoot removal induces chloroplast-related gene expression in roots, we analyzed the expression of photosynthesis-associated nuclear genes in wild-type roots. *LHCA4* and *LHCB6* encode the light-harvesting complex subunits of PSI and PSII, respectively. *CHLH* encodes the H subunit of magnesium chelatase involved in Chl biosynthesis, and *RBCS* encodes the small subunit of Rubisco. All of these genes were strongly up-regulated in detached wild-type roots compared with intact roots (Fig. 2A). In addition, *HEMA1*, which encodes the major isoform of glutamyl-tRNA reductase involved in Chl biosynthesis, was up-regulated in roots on shoot removal, whereas the expression of *HEMA2* encoding the minor isoform was not induced in detached roots (Supplemental Fig. S2). We also examined the expression of plastid-encoded photosynthetic genes. The genes *psaA* and *psbA* encode the core proteins of PSI and PSII, respectively, whereas *rbcL* encodes the Rubisco large subunit. In detached wild-type roots, the expression of *psaA*, *psbA*, and *rbcL* was increased 8- to 10-fold compared with intact roots (Fig. 2A). Therefore, shoot removal activates the formation of the photosynthetic machinery at the transcriptional level in roots.

To elucidate the regulatory pathways involved in the greening of detached roots, we analyzed the expression of genes encoding nuclear transcription factors associated with chloroplast differentiation (*GNC*, *GNL*, *GLK1*, and *GLK2*; Fig. 2B). *GNL* was greatly up-regulated in wild-type roots on shoot removal, whereas the expression

of *GNC* was increased only moderately. The expression of *GLK1* and *GLK2* was not increased significantly in detached roots.

Plastid-encoded photosynthetic genes such as *psaA*, *psbA*, and *rbcL* are transcribed mainly by plastid-encoded RNA polymerase (PEP). PEP genes, including *rpoB*, which encodes the β-chain of PEP, are transcribed by nucleus-encoded plastid RNA polymerase (NEP; De Santis-MacIossek et al., 1999). In PEP components, only sigma factors (SIGs), transcription initiation factors that are required for binding PEP to specific promoters of plastid genes, are encoded in the nuclear genome (Schweer et al., 2010). To address the mechanism involved in the up-regulation of plastid-encoded photosynthetic genes in detached roots, we investigated the expression of genes associated with plastid transcription (Fig. 2C). In addition to plastid-encoded *rpoB*, many *SIG* genes, particularly *SIG4*, were up-regulated in wild-type roots on shoot removal. These results were consistent with the increased expression of PEP-dependent photosynthetic genes (*psaA*, *psbA*, and *rbcL*) in detached roots. Meanwhile, the expression of the NEP genes *RPOTp* and *RPOTmp* was not increased significantly by shoot removal compared with intact roots.

Because peroxisomes and mitochondria transform their functions in concert with chloroplast differentiation during the development of photosynthetic tissues (Hayashi and Nishimura, 2006), we examined whether the expression of genes related to these organelles changes along with the expression of chloroplast-related genes during root greening (Fig. 2D). *GOX1* encodes glycolate oxidase in mitochondria, and *GLDP1* encodes the P protein of the Gly decarboxylase complex in peroxisomes. These genes are both involved in photorespiratory pathways and are expressed mainly in photosynthetic tissues (Kamada et al., 2003; Engel et al., 2007). *CuAOx*, which encodes putative copper amine oxidase localized in peroxisomes, is expressed specifically in roots (Kamada et al., 2003) and, thus, was used as a marker for nonphotosynthetic expression related to organelle functions. In wild-type detached roots, the expression of photorespiratory genes, particularly *GOX1*, was increased, whereas that of *CuAOx* was decreased (Fig. 2D).

To assess whether the shoot-like gene expression occurs globally in detached roots, we examined the expression of various root-specific genes (*At3g16450*, *At5g14020*, *At1g66280*, *At1g28290*, and *At5g65790*; Obayashi et al., 2004) that are not specifically responsive to cytokinin or wounding (Kilian et al., 2007; Winter et al., 2007). We found no general tendency in expression patterns of root-specific genes in response to shoot removal (Fig. 2E).

To ascertain whether type B ARR-mediated cytokinin signaling is involved in the transcriptional modification in detached roots, we examined gene expression in roots of the *arr1 arr12* double mutant. In intact roots, the expression of some photosynthetic genes appeared to be increased in *arr1 arr12* compared with the wild type (Fig. 2A), which is consistent with
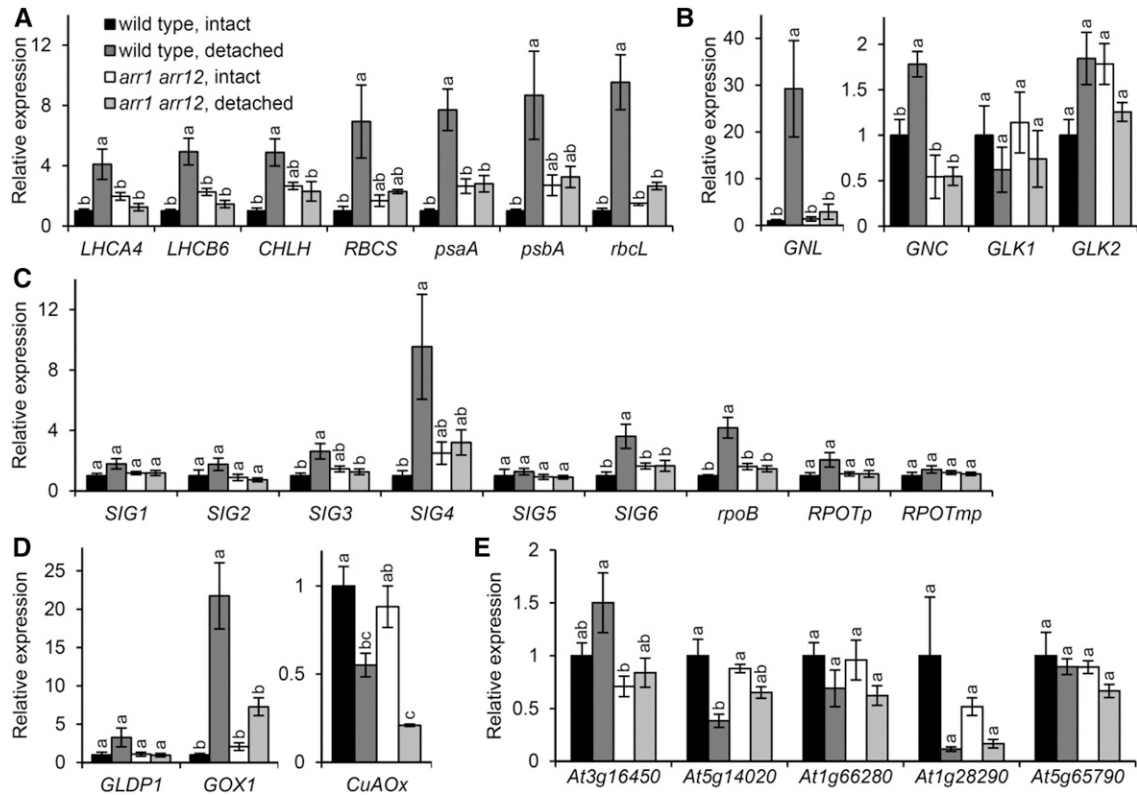
Figure 2. Expression of genes associated with chloroplast differentiation in intact and detached roots. Quantitative RT-PCR analysis of mRNA expression is shown for genes associated with photosynthesis (A), nuclear transcription (B), plastid transcription (C), mitochondrial and peroxisomal functions (D), and root tissues (E) in roots of the wild type and the *arr1 arr12* double mutant. Data are means ± SE fold difference from the untreated wild type after normalizing to *ACTIN8* ($n > 3$). The sample preparation was as in Figure 1. Different letters indicate significant differences for each gene ($P < 0.05$, Tukey-Kramer multiple comparison test).

the slightly higher Chl accumulation in intact *arr1 arr12* roots. However, in detached roots, the expression of these genes was substantially lower in *arr1 arr12* than in the wild type. In addition, genes associated with plastid transcription were not up-regulated in mutant roots on shoot removal (Fig. 2C). In *arr1 arr12*, the expression of *GLDP1*, *GOX1*, *GNC*, and *GNL* was similar in detached and intact roots, whereas that of *CuAOx* was decreased by shoot removal as in the wild type (Fig. 2, B and D). Thus, ARR1 and ARR12 are required for the transcriptional induction of chloroplast differentiation in detached roots. Meanwhile, as in the wild type, *arr1 arr12* showed no general tendency in expression patterns of root-specific genes on shoot removal (Fig. 2E).

Shoot Removal Changes the Quality of Photosynthetic Complexes in Root Chloroplasts

To evaluate the effect of shoot removal on the quality of the photosynthetic apparatus in root chloroplasts, we analyzed the transient kinetics of Chl fluorescence using a logarithmic timing series in the wild type (Fig. 3A). In a typical polyphasic fluorescence increase called

the origin-inflection-intermediary peak-peak (OJIP) transient, the O-J phase and the J-I phase reflect the photochemical process in PSII and the reduction in the plastoquinone pool, respectively, whereas the I-P phase is related to the process in PSI (Schansker et al., 2005). The O-J transition occurred with higher fluorescence yield in both intact and detached roots than in leaves, which indicates an impaired electron transfer within PSII. However, unlike intact roots, detached roots showed enhanced inflection at the I transient, which resulted in lower Chl fluorescence at the I transient than in intact roots. Electron transport around PSI may somehow change in root chloroplasts on shoot removal, which improves electron transport in PSII. When the electron transport between the primary and secondary plastoquinones in PSII was inhibited with 3-(3,4-dichlorophenyl)-1,1-dimethylurea, both intact and detached roots showed a faster fluorescence increase than leaves. This result suggests that the size of the PSII antenna relative to the reaction center is larger in roots than in leaves, as described (Kobayashi et al., 2013), even after shoot removal.

To address whether the state of Chl-protein complexes is changed in root chloroplasts in response to
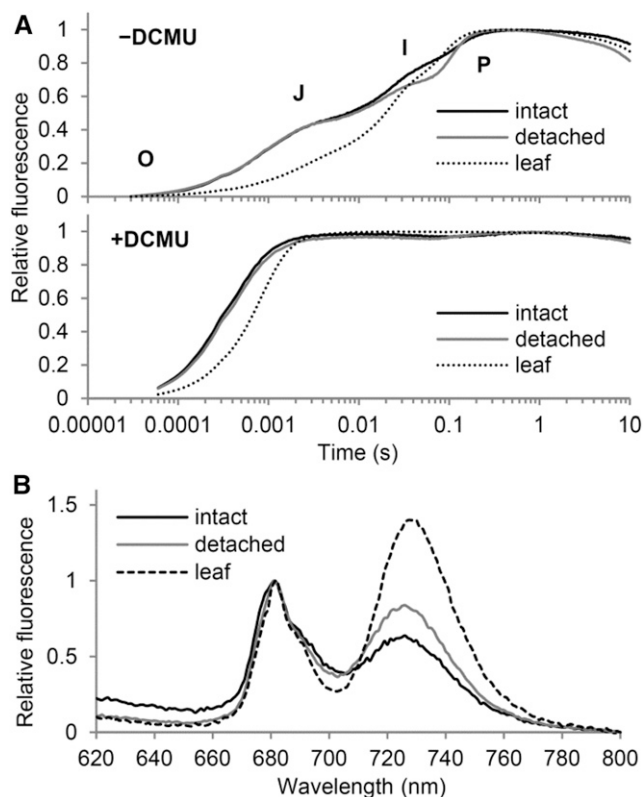
Figure 3. Comparison of photosystems in detached and intact roots and in leaves. A, Transient fluorescence induction kinetics of Chl in the absence (−) or presence (+) of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Data are means from three independent experiments. Two inflections, J and I, are seen between the levels O (origin) and P (peak) only in the −DCMU samples. B, The 77K Chl fluorescence spectra in 2 μg mL^{-1} Chl fractions. Representative spectra from three independent experiments are shown. Data were normalized to the emission peak from PSII at 682 nm with background correction at 800 nm.

shoot removal, we measured Chl fluorescence spectra at 77K in membrane fractions containing 2 μg mL^{-1} Chl (Fig. 3B). Fluorescence emitted from PSI (F_{PSI}), observed at 732 nm in the leaf sample, was slightly blue shifted in intact wild-type roots, as was found previously (Kobayashi et al., 2013). The same shift was observed in detached roots, so the coupling state between PSI and LHCI in roots was not changed by shoot removal. We observed maximal fluorescence emission from PSII (F_{PSII}) at 682 nm in both leaf and root samples. The mean F_{PSI}/F_{PSII} ratio was lower in intact roots than in leaves (0.66 ± 0.03 versus 1.52 ± 0.20), which suggests less spillover of excitation energy from the PSII to the PSI antenna (Krause, 1991) or simply a lower abundance of PSI relative to PSII, as was suggested previously (Kobayashi et al., 2013). However, the F_{PSI}/F_{PSII} ratio was greater in detached roots than in intact roots (0.80 ± 0.04 versus 0.66 ± 0.03), which suggests that, with shoot removal, the energy distribution between PSII and PSI in root chloroplasts is partially improved to close to the leaf chloroplast status.

Cytokinin Signaling Improves Photosynthetic Efficiency in Detached Roots

Because shoot removal affected photosynthetic components in roots (Fig. 3), we analyzed photosynthetic efficiency in detached roots by measuring Chl fluorescence with a fiber-optic pulse amplitude modulation (PAM) fluorometer. As reported previously (Kobayashi et al., 2013), the actual PSII efficiency (Y_{II}) under light was lower in intact roots than in leaves of the wild type. However, the Y_{II} value in roots was increased to close to the leaf level on shoot removal, although the induction profiles of Y_{II} differed between leaves and detached roots (Fig. 4A). Specifically, the detached roots showed a fast and transient increase in Y_{II} after actinic light illumination, which was not observed in leaves and intact roots, through an unknown mechanism. Meanwhile, the slightly lower F_v/F_m in intact roots than in leaves was not improved by shoot removal.

Y_{II} can be considered a product of two components: the qP, which represents the primary redox status and, thus, the openness of PSII, and F_v'/F_m'. Both of these values were lower in intact roots than in leaves, with qP more strongly decreased than F_v'/F_m'. However, these values in wild-type roots were increased to leaf levels after shoot removal, which suggests that electron transport downstream of PSII is ameliorated in detached roots. We also evaluated Y_{NPQ} and Y_{NO}, which represent the proportion of regulated and nonregulated dissipation of light energy in PSII, respectively (Kramer et al., 2004). As reported (Kobayashi et al., 2013), Y_{NPQ} was higher in intact roots than in leaves, whereas Y_{NO} was similar between roots and leaves. However, in detached roots, Y_{NPQ} decreased to a level similar to that in leaves. Thus, chloroplasts in detached roots may have photosynthetic characteristics close to those in leaves.

Next, we evaluated the involvement of cytokinin signaling in the regulation of photosynthetic activity in roots. Treatment of intact roots with a synthetic cytokinin, BA, induced changes in photosynthetic parameters similar to those in detached roots; that is, it increased Y_{II}, qP, and F_v'/F_m' and decreased Y_{NPQ} compared with untreated roots (Fig. 4A). To further ascertain the role of cytokinin signaling, we analyzed photosynthetic activities in *arr1 arr12* roots (Fig. 4B). Unlike the wild type, in *arr1 arr12*, neither shoot removal nor BA treatment greatly changed photosynthetic variables in roots. Thus, cytokinin signaling via ARR1 and ARR12 plays a central role in photosynthetic remodeling in detached roots.

Transcription Factors Involved in the Greening of Detached Roots

The removal of shoots induced a prominent expression of *GNL* in roots along with a slight increase in *GNC* expression via type B ARR signaling (Fig. 2B). To elucidate the role of these transcription factors in the
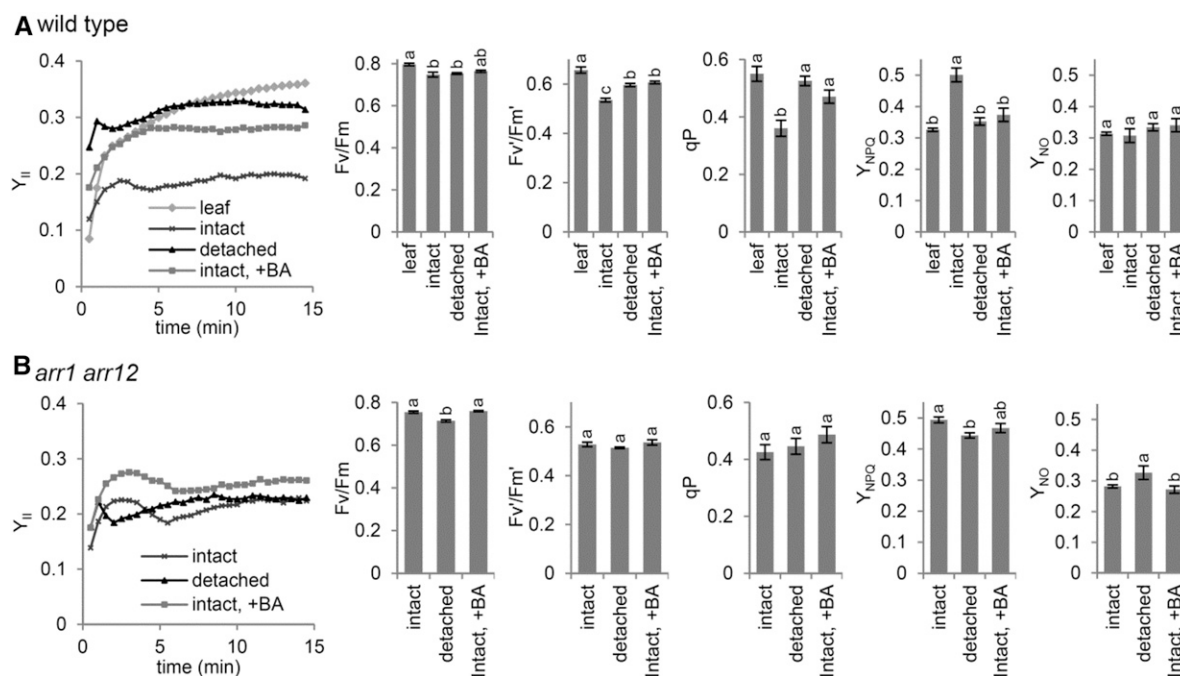
Figure 4. Improvement of photosynthetic efficiency in detached roots via cytokinin signaling. Photosynthetic parameters are shown for 21-d-old detached roots and intact roots treated with 6-benzyladenine (BA) of the wild type (A) and the *arr1 arr12* double mutant (B) compared with untreated intact roots. In A, wild-type leaves were used as a reference of the photosynthetically competent organ. Data are means ± SE ($n > 4$). F_v/F_m, Maximum quantum yield of PSII; F_v'/F_m', maximum quantum yield of PSII under light conditions; qP, coefficient of photochemical quenching; Y_{NPQ}, quantum yield of regulated energy dissipation; Y_{NO}, quantum yield of nonregulated energy dissipation. Slow induction kinetics under actinic light (intensity, 420 μmol photons m^{-2} s^{-1}) are shown for Y_{II}, and other data were obtained after actinic light exposure for 15 min. Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer multiple comparison test).

greening of detached roots, we analyzed Chl content in roots of the *gnc gnl* mutant along with the *glk1 glk2* mutant (Fig. 5A). Chl content was lower in intact roots of *glk1 glk2* than in the wild type, as reported previously (Kobayashi et al., 2012a), whereas the content in intact *gnc gnl* roots was almost equivalent to the wild-type level. However, in detached roots, Chl content was lower in *gnc gnl* than in the wild type. Although Chl content increased 5.5-fold with shoot removal in wild-type roots, that in *gnc gnl* roots increased only 3.4-fold from the level in intact roots. Hence, GNC and GNL are conditionally required for Chl accumulation in roots in response to shoot removal. Chl content also was lower in detached *glk1 glk2* roots than in wild-type roots. However, the magnitude of the increase in root Chl content on shoot removal was similar between *glk1 glk2* and the wild type, which suggests that GLKs constitutively function in Chl accumulation in both intact and detached roots.

We also investigated the effect of the overexpression of *GNC* (*GNCox*) and *GNL* (*GNLox*) on root greening. As reported previously (Chiang et al., 2012), overexpression of *GNC* and *GNL* greatly enhanced Chl accumulation in intact roots (Fig. 5B). Because enhanced Chl accumulation was already observed in roots of 10-d-old *GNCox* seedlings (Chiang et al., 2012),

overexpression of these genes would increase the rate of Chl accumulation during root development. In these overexpression lines, the size and number of green plastids were increased in cells of the mature region of the primary root (Fig. 5, C and D; Supplemental Fig. S3A), which indicates the ectopic activation of chloroplast development in intact roots of these lines. In fact, Chiang et al. (2012) revealed that overexpression of *GNC* or *GNL* caused ectopic chloroplast development in the cortex in addition to increased chloroplast number in the pericycle cells in roots. Similarly, cells of detached wild-type roots showed increased size and number of green plastids.

We next examined whether GNC and GNL are involved in the transcriptional regulation of photosynthesis-associated genes in roots. Intact roots of the *gnc gnl* double mutant showed no or only slightly decreased expression of photosynthesis-associated genes compared with intact wild-type roots (Fig. 5E). After shoot removal, the *gnc gnl* mutant showed increased expression of these genes (Fig. 5E), but to a lesser extent than the wild type (Fig. 2). By contrast, overexpression of these GATA factors, particularly *GNLox*, substantially increased the expression of photosynthesis-associated genes in intact roots (Fig. 5F). As an exception, *HEMA1* was not up-regulated in *GNCox* and *GNLox* roots, although
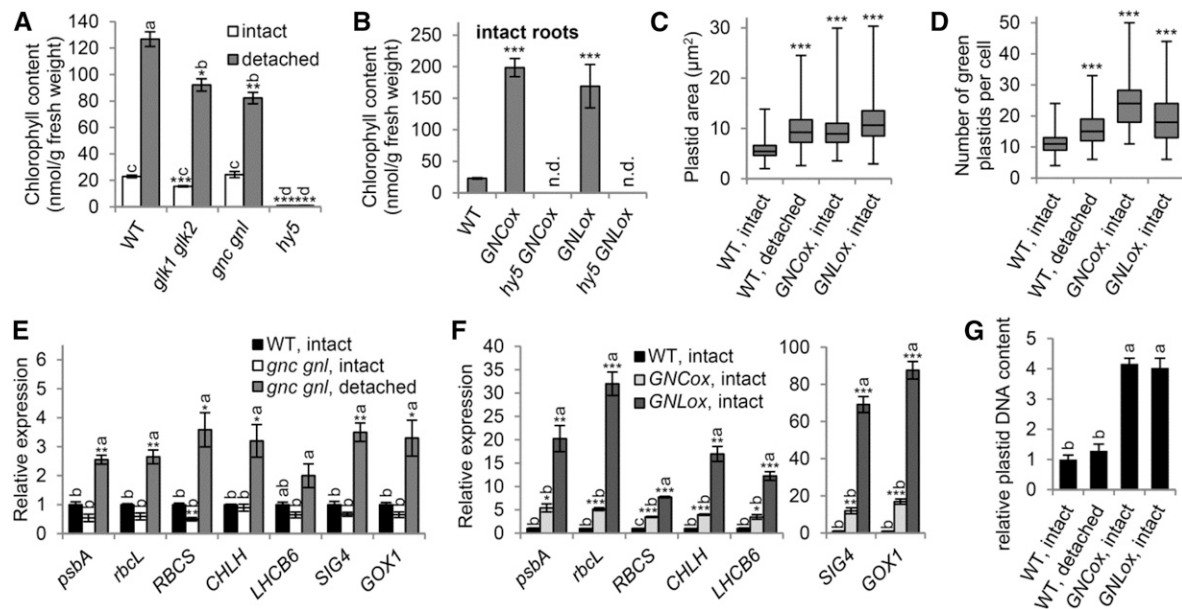
Figure 5. Involvement of transcription factors in the root greening response after shoot removal. A and B, Chl content in intact and detached roots of each mutant line (A) and intact roots with *GNC* and *GNL* overexpression (*GNCox* and *GNLox*, respectively) in the wild type (WT) and *hy5* mutant background (B). Data are means ± SE from three or more independent experiments. n.d., Not determined. C and D, Size (C) and number (D) of green plastids in primary root cells. The horizontal line represents the median; the top and bottom of boxes represent the upper and lower quartiles, respectively; and whiskers represent the range (C, *n* > 900; D, *n* > 80). ***, $P < 0.001$ by Student's *t* test. E and F, Expression of photosynthesis-associated genes in intact and detached roots of the *gnc gnl* double mutant (E) and intact roots of *GNCox* and *GNLox* (F). Data are means ± SE fold difference from the intact wild-type root samples after normalizing to *ACTIN8* (*n* = 3). G, Plastid DNA content in intact and detached root cells. The content of plastid DNA relative to nuclear DNA was determined by real-time PCR-based quantification of DNA content with *rpoB*-specific (plastid-encoded) and *ACTIN8*-specific (nucleus-encoded) primers. Data are means ± SE fold difference from intact wild-type root samples (*n* = 3). Samples at 21 d old were used for all experiments. Different letters indicate significant differences by the Tukey-Kramer multiple comparison test (*P* < 0.05). Asterisks indicate significant differences from the wild type of each condition (*, *P* < 0.05; **, *P* < 0.01; and ***, *P* < 0.001, Student's *t* test after a Bonferroni correction for multiple comparisons).

its expression was induced by shoot removal via ARR1- and ARR12-mediated cytokinin signaling (Supplemental Fig. S2).

Because transcript levels of plastid-encoded genes may be linked with plastid DNA abundance in cells, we analyzed plastid DNA content in roots by quantitative PCR analysis. In the wild type, plastid DNA content did not differ largely in detached and intact roots (Fig. 5G; Supplemental Fig. S3B). These data suggest that the increased expression of plastid-encoded genes in detached wild-type roots is not attributed to the increased number of plastid genome copies but reflects a specific activation of plastid gene expression. Meanwhile, plastid DNA content was increased greatly in intact roots of *GNCox* and *GNLox* lines. The plastid DNA content was similar between these two lines (Fig. 5G), although *GNLox* roots showed much stronger expression of plastid-encoded genes than *GNCox* roots (Fig. 5F).

In contrast to GLKs and GATA factors, which are important but not essential for root greening, HY5 is indispensable for Chl accumulation in roots, as illustrated by a complete albino phenotype of *hy5* mutant roots (Oyama et al., 1997; Usami et al., 2004; Kobayashi

et al., 2012a). Moreover, we reported previously that overexpression of GLKs in the *hy5* mutant background only slightly increased Chl accumulation in roots (Kobayashi et al., 2012a), which suggests that GLK factors require HY5 to maximize their function in the root. Thus, we evaluated the role of HY5 in root greening after shoot removal. The *hy5* mutant did not accumulate Chl even in detached roots (Fig. 5A). Moreover, overexpression of *GNC* or *GNL* in the *hy5* background (*hy5 GNCox* and *hy5 GNLox*) did not increase Chl content in roots (Fig. 5B). These data demonstrate that HY5 also is essential for the conditional root greening mediated by GATA factors.

B-GATAs Improve Photosynthesis in Roots

To examine the involvement of GLK and B-GATAs in regulating root photosynthesis, we analyzed the photosynthetic parameters of roots in *glk1 glk2* and *gnc gnl* mutants along with the wild type and the *arr1 arr12* mutant using an imaging PAM fluorometer. In this experiment, 28-d-old root samples were used to obtain sufficient Chl fluorescence signals for the PAM analysis.

In intact roots, Y_{II} values were similar for all mutants and the wild type (Fig. 6A). In detached roots grown for 7 d without shoots, the wild type showed increased Y_{II}, whereas *arr1 arr12* showed no remarkable increase in Y_{II} (Fig. 6B), which confirms the results in Figure 4. The *glk1 glk2* double mutant showed a similar Y_{II} profile to the wild type in detached roots, so GLKs are not required for the remodeling of root photosynthesis on shoot removal. The *gnc gnl* roots also showed increased Y_{II} on shoot removal, although the values appeared slightly lower than that for the wild type. We also evaluated the effect of BA treatment on root photosynthesis. Consistent with the data in Figure 4, BA treatment increased the Y_{II} of roots in the wild type but not in *arr1 arr12* (Fig. 6C). In both the *glk1 glk2* and *gnc gnl* mutants, root Y_{II} was increased only moderately by BA treatment, so these factors are not essential but are partially important for the cytokinin-induced remodeling of root photosynthesis.

We also analyzed photosynthetic activity in roots of *GNCox* and *GNLox* lines and the *GLK1* overexpression line (*GLK1ox*). As reported previously (Kobayashi et al., 2013), in *GLK1ox*, Chl accumulation in roots was not accompanied by improved photosynthetic efficiency and even decreased F_v/F_m and F_v'/F_m' (Fig. 6, D–F). By contrast, in intact roots, Y_{II} was higher for *GNCox* and *GNLox* than for the wild type (Fig. 6D). Higher Y_{II} in *GNCox* and *GNLox* roots was attributed mainly to the increased fraction of open PSII represented by higher qP and the decreased non-photochemical energy dissipation represented by lower Y_{NPQ} (Fig. 6, G and H), as was observed in detached wild-type roots (Fig. 4A).

Auxin Signaling Regulates Root Greening Independently of Type B ARR Signaling

We reported previously that auxin is involved in the negative regulation of chloroplast development in roots (Kobayashi et al., 2012a). To examine the interaction of auxin and cytokinin signaling during the root greening response, we treated cytokinin mutants with an auxin signaling inhibitor, *p*-chlorophenoxyisobutyric acid (PCIB), and measured Chl content in roots. As reported previously (Kobayashi et al., 2012a), PCIB treatment increased Chl content in intact wild-type roots (Fig. 7A). Similarly, PCIB increased root Chl content in *ahk2 ahk3* and *arr1 arr12* mutants. Moreover, PCIB treatment did not increase type B ARR-mediated GFP fluorescence in intact roots of the *TCSn::GFP* line (Fig. 7B). These data indicate that the inhibition of auxin signaling by PCIB induces root greening independently of type B ARR-mediated cytokinin signaling. As well, the enhanced *TCSn::GFP* fluorescence in detached roots was not suppressed by treatment with an auxin, 1 μM IAA (Fig. 7B), which suggests no inhibitory effect of auxin on type B ARR activation during root greening. Thus, auxin signaling regulates Chl accumulation in roots independently of type B ARR activities.

Next, we examined whether exogenous auxin affects the expression of photosynthesis-associated genes during the greening of detached roots. IAA treatment suppressed the expression of *psbA* and *GOX1*, with a decreasing trend for *LHCB6*, *CHLH*, and *rbcL* expression in detached roots (Fig. 7C). Of note, IAA treatment inhibited the up-regulated *GNL* expression in detached wild-type roots. It also inhibited the suppressed *CuAOx* expression in roots with shoot removal. These data suggest that auxin signaling strongly affects the expression of some genes in detached roots.

We also evaluated photosynthetic activity in intact roots treated with PCIB and detached roots treated with IAA (Fig. 7D). PCIB treatment slightly increased Y_{II} in intact roots, but to a lesser extent than BA (Fig. 6C). Meanwhile, IAA treatment partially suppressed the increased Y_{II} in roots with shoot removal (Fig. 7D). These data suggest that auxin signaling functions to repress photosynthetic activation in roots in contrast to cytokinin signaling.

DISCUSSION

Root Greening as a Wounding Response via Cytokinin Signaling

We previously reported that the *ahk2 ahk3* double mutant for cytokinin receptors showed reduced Chl accumulation in intact roots (Kobayashi et al., 2012a), which suggests the requirement for cytokinin signaling in chloroplast development in roots. By contrast, the type B *arr* mutants showed no reduction in Chl content in intact roots (Fig. 1B). Because the roles of the type B ARR family genes are highly redundant (Mason et al., 2005; Argyros et al., 2008; Ishida et al., 2008), the remaining ARR members may compensate for the loss of each ARR in mutants in terms of Chl accumulation in intact roots. Alternatively, cytokinin pathways may function in Chl accumulation in intact roots independently of type B ARR. Considering that the intact roots of the *arr1 arr12* double mutant had higher Chl content (Fig. 1B) and higher expression of photosynthesis-associated genes than the wild type (Fig. 2A), complex mechanisms downstream of cytokinin reception would be involved in regulating chloroplast differentiation in intact roots. Cortleven et al. (2016) recently reported that the *arr1 arr10 arr12* triple mutant showed an up-regulation of Chl biosynthesis genes and an increased greening rate of cotyledons during the etioplast-chloroplast transition, which is consistent with our data that loss of both ARR1 and ARR12 even increased Chl content in intact roots. An involvement of an as yet unknown compensatory mechanism in the greening phenotype of multiple *arr* mutants is assumed (Cortleven et al., 2016), and future investigations are required to elucidate the mechanism.

Meanwhile, the *arr1 arr12* double mutant showed strongly inhibited conditional Chl accumulation in detached roots (Fig. 1B), so cytokinin signaling through ARR1 and ARR12 is specifically required for root
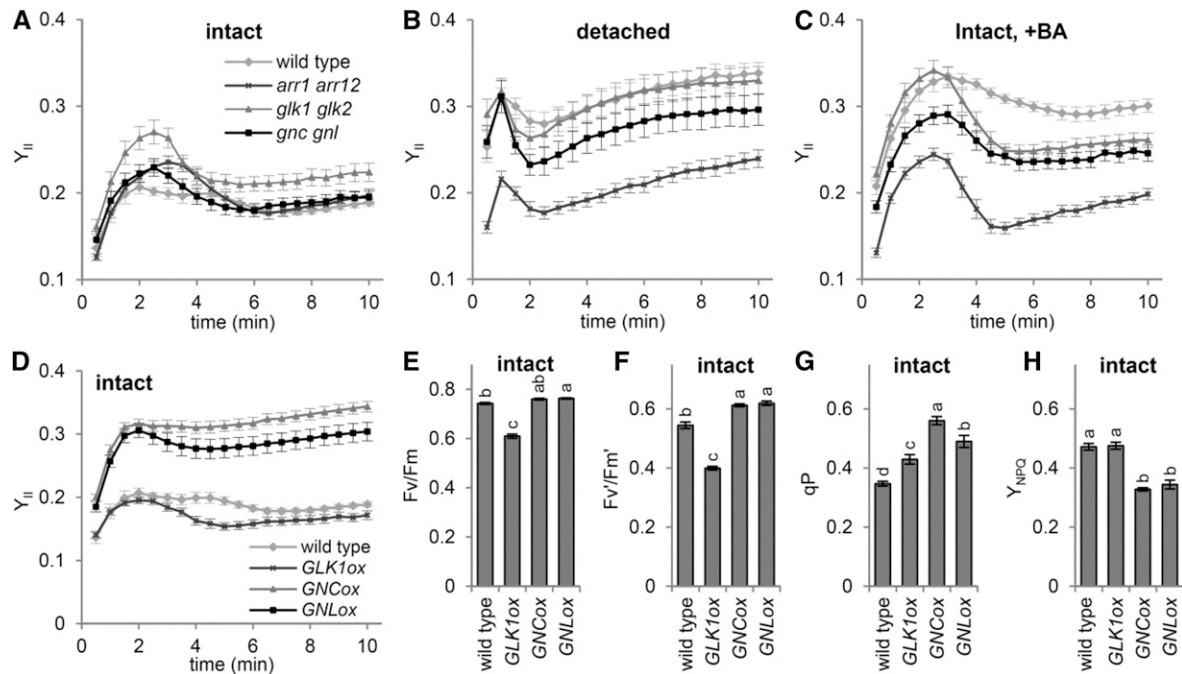
Figure 6. Involvement of transcription factors in root photosynthesis in response to shoot removal. A to D, Y_{II} in intact (A and D), detached (B), and BA-treated intact (C) roots of each line. E to H, Photosynthetic parameters in intact roots of overexpression lines of each transcription factor compared with intact wild-type roots. Data are means ± SE ($n > 12$). Slow induction kinetics under actinic light are shown for Y_{II} (A–D), and other data (E–H) were obtained after actinic light exposure (110 μmol photons m^{-2} s^{-1}) for 10 min. Samples at 21 d old were used for all experiments. Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer multiple comparison test).

greening after shoot removal. Because the dominant repression of WIND1 signaling by the WIND1-SRDX chimeric protein also partially impaired the greening of detached roots (Fig. 1D), WIND1-mediated wounding signaling may be involved in this regulation. WIND1 is an AP2/ERF transcription factor whose expression is strongly up-regulated within a few hours of wounding (Iwase et al., 2011a). WIND1, which plays a central role in regulating cell dedifferentiation after wounding, activates type B ARR-mediated cytokinin signaling at the wounding site, which is necessary for callus induction in response to wounding (Iwase et al., 2011a, 2011b). In fact, the dominant WIND1 repression in *WIND1-SRDX* inhibited type B ARR-mediated *TCS::GFP* expression in response to shoot removal (Fig. 1C). A similar result was observed in *WIND1-SRDX* hypocotyl explants, with repressed *TCS::GFP* expression and Chl accumulation (Iwase et al., 2011a). Thus, the WIND1-mediated wounding response would activate type B ARR signaling, thereby inducing Chl accumulation in detached roots (Fig. 8). However, forced overexpression of *WIND1* causing degreening of green tissues reflects the ability of WIND1 to induce cell dedifferentiation and callus formation (Iwase et al., 2011a, 2011b).

Consistently, artificial induction of *WIND1* expression for 24 h even slightly decreased root Chl content (Fig. 1E). Moreover, despite strong type B ARR

activities, calluses formed at the cutting site of some detached roots accumulated less Chl (Supplemental Fig. S1), as was observed in calluses formed in etiolated hypocotyls with strong *WIND1* expression (Iwase et al., 2011a). Considering the fact that induction levels of *WIND1* are strongly linked with dedifferentiation levels of tissues (Iwase et al., 2011a), there may be an optimum or threshold level of WIND1 in determining cell fate. Strict spatiotemporal regulation of *WIND1* expression in response to shoot removal may be important for promoting callus formation and root greening at each specific site separately. We note that enhanced cytokinin signaling would not solely cause the degreening of dedifferentiating cells in callus or callus-like cell masses, because exogenous cytokinin treatment does not induce degreening but enhances Chl accumulation and photosynthesis in roots (Fig. 4; Kobayashi et al., 2012a). Some other factors induced by WIND1 at the callus-forming site may promote the degreening of dedifferentiating cells together with or independently of cytokinin signals.

Transcription Factors Functioning in Root Greening Downstream of Cytokinin Signaling

We showed that shoot removal substantially up-regulated *GNL* expression in wild-type roots (Fig. 2B).
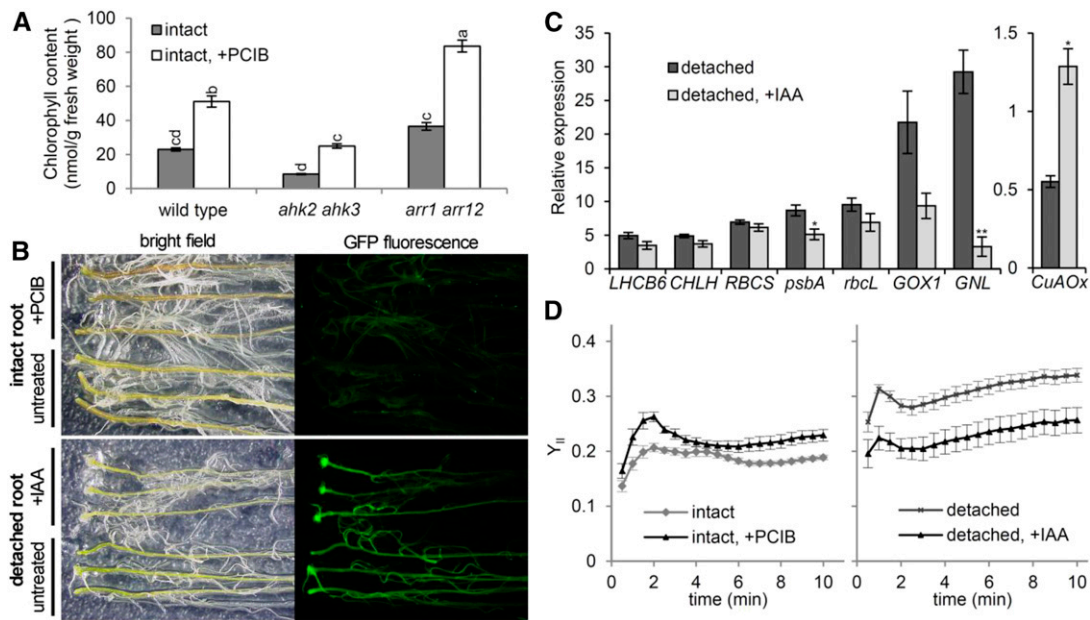
Figure 7. Auxin regulates root greening independent of cytokinin signaling. A, Chl content in intact roots of the wild type and cytokinin signaling mutants in the absence (intact) or presence of 10 μM PCIB (intact, +PCIB). Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer multiple comparison test). B, GFP fluorescence in roots of the *TCSn::GFP* line. For the top images, roots of 21-d-old intact seedlings were grown in the absence (untreated) or presence of 10 μM PCIB for 7 d. Aerial parts were detached just before observation. For the bottom images, 21-d-old root samples were detached from 14-d-old seedlings and grown on agar medium containing no or 1 μM indole-3-acetic acid (IAA). C, Gene expression analysis in 21-d-old detached root samples treated with or without 1 μM IAA for 7 d. Data are means $\pm$ SE fold difference from the 21-d-old intact wild-type root samples after normalizing to *ACTIN8* ($n = 3$). Asterisks indicate significant differences from the untreated control (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test). D, Slow induction kinetics of Y_{II} in intact roots of 28-d-old seedlings grown in the absence or presence of 10 μM PCIB for 7 d (left graph) and root samples detached from 21-d-old seedlings and grown on agar medium containing no or 1 μM IAA for 7 d (right graph). Data are means $\pm$ SE ($n > 12$). Actinic light intensity was 110 μmol photons m^{-2} s^{-1}.

However, its up-regulation in detached roots was strongly inhibited in the *arr1 arr12* double mutant, which suggests that shoot removal up-regulates *GNL* via type B ARR signaling. This result agrees with the finding that up-regulation of *GNL* by exogenous cytokinin treatment was impaired in the *arr1 arr12* double mutant (Chiang et al., 2012). Moreover, overexpression of *GNL* or its paralog, *GNC*, caused ectopic chloroplast differentiation in the hypocotyl and roots (Chiang et al., 2012). In fact, we confirmed strong Chl accumulation, remarkable up-regulation of photosynthesis-associated genes, and increased number and size of green plastids in roots with *GNL* and *GNC* overexpression as in detached wild-type roots (Fig. 5). Therefore, activated type B ARR signaling by shoot removal likely up-regulates *GNL* expression, thereby inducing chloroplast development in detached roots (Fig. 8). However, the *gnc gnl* double mutant still showed increased Chl accumulation and photosynthetic gene expression in detached roots, despite lower levels than in the wild type (Fig. 5, A and E), so these GATA factors are important but not crucial for the greening of detached roots. In addition to GNC and GNL, four other similar B-GATA paralogs exist in Arabidopsis and function redundantly in part with GNC and GNL in the leaf greening process (Ranftl et al., 2016). Thus, the remaining B-GATAs in the *gnc gnl* mutant may compensate in part for the function of *GNC* and *GNL* in the root greening response.

In contrast to the conditional requirement of B-GATAs after shoot removal, GLK factors are required for Chl accumulation in both intact and detached roots (Fig. 5A). Moreover, detached roots of the *glk1 glk2* double mutant showed no remarkable differences from the wild type in photosynthetic activity (Fig. 6B). These data suggest that GLKs are not specific for the root greening response after shoot removal. However, *GLK2*, which has a more important role in root Chl accumulation than *GLK1* (Kobayashi et al., 2012a), was up-regulated in roots by cytokinin treatment (Kobayashi et al., 2012a). In addition, the *glk1 glk2* mutant showed lower response to BA treatment in terms of root photosynthesis activity than the wild type (Fig. 6C). Thus, in addition to the GATA factors, *GLK2* may contribute in part to the conditional root greening downstream of cytokinin signaling. In addition, ARR10 and ARR12 were recently shown to bind directly to the promoter regions of *HEMA1* and *LHCB6* (Cortleven et al., 2016). Of note, the *HEMA1* expression in roots was induced by shoot removal via ARR1 and ARR12 but not by the overexpression of
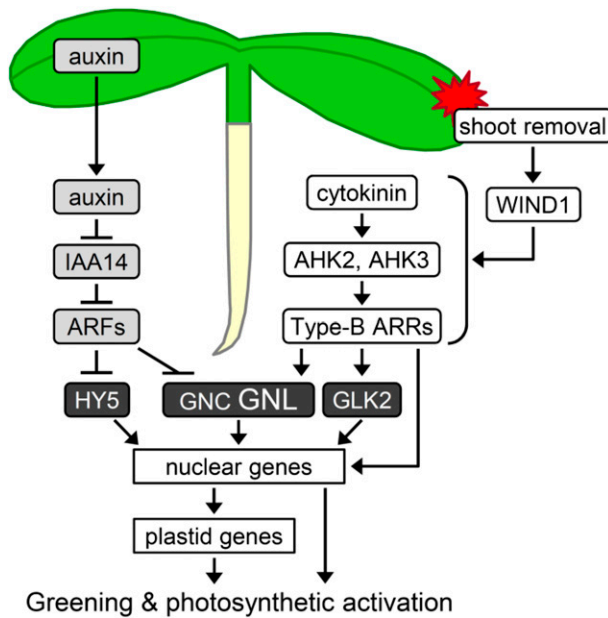
Figure 8. Model of the regulatory pathways of root greening after shoot removal. Chloroplast development in roots is positively regulated by cytokinin signaling via the His kinases AHK2 and AHK3 and negatively regulated by auxin signaling via INDOLE-3-ACETIC ACID INDUC-IBLE14 (IAA14) and AUXIN RESPONSE FACTORs (ARFs). In intact roots, auxin transported from the shoot represses chloroplast development, but in detached roots, the negative auxin regulation is attenuated, and the wound-responsive transcription factor WIND1 is induced in response to shoot removal and activates the type B ARR-mediated cytokinin signaling pathway. Several transcription factors associated with chloroplast development (HY5, GNC, GNL, and GLK2) function downstream of auxin/cytokinin signaling to directly and indirectly up-regulate nucleus- and plastid-encoded genes associated with chloroplast development. Type B ARRs also directly regulate some photosynthesis-associated nuclear genes and induce Chl accumulation and photosynthetic activation in roots. Arrows and bars represent positive and negative regulation, respectively.

GNC and GNL (Supplemental Fig. S2). Thus, type B ARRs may directly up-regulate some photosynthesis-associated nuclear genes in roots in response to shoot removal.

We previously reported that complete loss of Chl in the hy5 mutant was not rescued by increased cytokinin signaling or inhibited auxin signaling (Kobayashi et al., 2012a). Moreover, overexpression of GLKs only slightly increased root Chl content in the hy5 background. Consistent with these results, shoot removal as well as overexpression of GNC and GNL did not increase root Chl content in the hy5 mutant (Fig. 5B). These data indicate that HY5 acts as a prerequisite for Chl accumulation in roots and that B-GATAs function thoroughly depending on HY5. Although Chl accumulation in the root is strictly regulated in a development- and region-dependent manner, HY5 protein globally accumulates in all root regions independently of developmental stage (Kobayashi et al., 2012a; Kobayashi and Masuda, 2013). Thus, HY5 widely accumulated in roots may

support other factors, such as GLKs and B-GATAs, to regulate chloroplast development in specific areas and under particular conditions.

Up-Regulation of Photosynthesis-Associated Genes in Roots by Shoot Removal

In detached roots, all photosynthesis-associated nuclear genes investigated were strongly up-regulated in the wild type but not in the arr1 arr12 mutant (Fig. 2A), so ARR1 and ARR12 mediate the up-regulation of these genes after shoot removal. The expression of GOX1 and GLDP1, which are associated with the photorespiratory metabolism in mitochondria and peroxisomes, respectively, also was increased in detached roots in an ARR1- and ARR12-dependent manner (Fig. 2D), whereas the expression patterns of root-specific genes after shoot removal varied largely among genes (Fig. 2E). Thus, genes associated with photosynthesis, including the processes outside chloroplasts, may be the main targets of ARR1- and ARR12-mediated transcriptional remodeling during the root greening response.

In addition to the nuclear genes, plastid-encoded photosynthetic genes also were up-regulated via the ARR1- and ARR12-mediated signaling pathway (Fig. 2A). Our data are consistent with the finding that BA treatment of detached barley (Hordeum vulgare) leaves activates chloroplast transcription in the light (Zubo et al., 2008). Because ARR proteins are localized to the nucleus (Sakai et al., 2000; Hwang and Sheen, 2001), other factors acting downstream of cytokinin signaling would induce the expression of plastid-encoded genes. SIGs, which are encoded in the nucleus but function in plastids as transcription initiation factors of the PEP complex (Schweer et al., 2010), may be involved in the up-regulation of plastid-encoded photosynthetic genes. Indeed, many SIG genes, particularly SIG4, were up-regulated in roots on shoot removal via ARR1 and ARR12 (Fig. 2C). Consistent with these data, the expression of SIG4 was increased substantially in GNLox roots along with plastid-encoded psbA and rbcL (Fig. 5F), but induction of these genes in detached roots in the gnc gnl double mutant was weakened (Fig. 5E). These data suggest that GNL activates plastid gene expression downstream of ARR signaling during root greening. Of note, shoot removal increased the levels of plastid-encoded genes in roots without increasing plastid DNA content (Figs. 2A and 5G), which suggests that photosynthetic gene loci in plastid nucleoids are transcriptionally activated in detached root cells. In addition, the induction of plastid-encoded genes was stronger with GNLox than GNCox in roots (Fig. 5F), although these lines showed similar plastid DNA contents (Fig. 5G). Thus, unlike GNC, GNL can enhance the transcriptional activity of the photosynthetic gene loci in plastid nucleoids besides increasing plastid number and plastid genome copies in roots. B-GATAs may act on chloroplast-related genes in an indirect manner (Hudson et al., 2011), and the mode of function of B-GATAs in photosynthesis gene expression awaits future investigations.

Shoot Removal Improves Photosynthetic Electron Transport in Roots via Cytokinin Signaling

In addition to the increased Chl content and number and size of chloroplasts, photosynthetic efficiency was increased in wild-type roots on shoot removal (Fig. 4A), which indicates the quantitative and qualitative development of chloroplasts in detached roots. The mean F_{PSI}/F_{PSII} ratio was greater in detached roots than in intact roots (Fig. 3B), so light energy input to PSI is increased in root chloroplasts on shoot removal. This result is consistent with the data that electron transport around PSI was modified in root chloroplasts on shoot removal (Fig. 3A). Indeed, the PSII acceptor side limitation, represented by low qP, in root chloroplasts was improved to the leaf level in detached roots (Fig. 4A). In parallel to or as a result of the sufficient electron transport to PSI, thermal dissipation of light energy (Y_{NPQ}) decreased to the leaf level in detached roots. Thus, as shown by the change in F_{PSI}/F_{PSII} ratio, rearrangement of light energy input between PSII and PSI may improve the efficiency of electron transport downstream of PSII and decrease thermal energy dissipation within PSII in detached root chloroplasts. Because psaA, psbA, and rbcL were markedly up-regulated along with rpoB and SIGs in roots with shoot removal (Fig. 2), the global induction of plastid-encoded photosynthetic genes may increase photosynthetic efficiency in root chloroplasts. By contrast, the slow electron transport within PSII (Fig. 3A) as well as the low F_v/F_m (Fig. 4A) in root chloroplasts was not improved by shoot removal. Therefore, certain components in PSII may remain less efficient in roots even after shoot removal.

In wild-type roots, cytokinin treatment improved photosynthetic efficiency similar to that with shoot removal (Fig. 4A). By contrast, the photosynthetic modifications with shoot removal and cytokinin treatment were inhibited in arr1 arr12 roots (Fig. 4B). Moreover, the shoot removal-induced up-regulation of photosynthetic genes was strongly impaired in detached arr1 arr12 roots (Fig. 2). Therefore, cytokinin signaling via ARR1 and ARR12 plays a crucial role in the quality control of root chloroplasts and photosynthetic activities. In addition, GNC and GNL overexpression greatly improved photosynthetic efficiency in roots (Fig. 6). Both GNCox and GNLox increased the expression of plastid-encoded genes to similar or higher levels compared with nucleus-encoded photosynthetic genes (Fig. 5E), which may help to balance the amount of photosynthetic electron transport components. However, the effect of GNLox on photosynthetic gene expression was much stronger than that of GNCox, although Chl accumulation and photosynthetic activities in roots were similar between these two lines. Thus, the up-regulation of photosynthetic genes in GNCox may be sufficient to induce the remodeling of photosynthetic activity in roots. In contrast to GNCox and GNLox roots, GLK1ox roots showed lower PSII efficiency than wild-type roots, which may be attributed to the unbalanced

photosynthetic machinery caused by the excessive expression of GLK1-targeted genes associated specifically with peripheral light-harvesting antennae (Waters et al., 2009; Kobayashi et al., 2013).

Involvement of Auxin Signaling in Root Greening after Shoot Removal

Our previous study suggested that auxin transported from the shoot, absent in detached roots, represses root greening (Kobayashi et al., 2012a). In fact, exogenous IAA treatment partially inhibited the up-regulation of photosynthesis-associated genes and photosynthetic remodeling in detached roots (Fig. 7, C and D). By contrast, inhibition of auxin signaling by PCIB slightly increased photosynthetic efficiency in intact roots (Fig. 7D). These data suggest that auxin signaling negatively regulates photosynthetic activity in roots. This regulation by auxin is independent of type B ARR activities (Fig. 7, A and B). However, IAA treatment strongly repressed the GNL expression in detached roots (Fig. 7C). GNC and GNL are negatively regulated by auxin signaling, and the expression of GNC and GNL is increased in some mutants defective in auxin signaling (Richter et al., 2013), including solitary root1, which has greenish roots (Kobayashi et al., 2012a). Thus, B-GATAs may function at the point of convergence of cytokinin and auxin signaling on chloroplast development in roots.

CONCLUSION

In combination with previous studies (Kobayashi et al., 2012a), we propose a model of regulatory pathways of root greening after shoot removal (Fig. 8). In intact seedlings, auxin transported from the shoot represses HY5 protein accumulation and GNL gene expression via IAA14 and its regulatory targets the ARFs in roots, which leads to the suppression of chloroplast-associated genes. After shoot removal, chloroplast-associated genes are released from the suppression by shoot-derived auxin. At the same time, shoot removal activates type B ARR-mediated cytokinin signaling in roots via the wound-responsive transcription factor WIND1. The cytokinin receptors AHK2 and AHK3 are essential for the root greening response, although the mechanism intertwining the wounding signaling by WIND1 and cytokinin signaling via AHKs remains elusive (Iwase et al., 2011a). Activated type B ARRs, particularly ARR1 and ARR12, strongly induce GNL and moderately induce GNC and GLK2 and also may directly increase the expression of chloroplast-associated nuclear genes (Cortleven et al., 2016). The up-regulation of nuclear genes associated with photosynthesis and plastid transcription is accompanied by an increased expression of plastid-encoded genes, which together result in Chl accumulation and photosynthetic activation in detached roots.

Land plants are at constant risk of serious damage or removal of photosynthetic tissues by physical attacks from herbivores and pathogens and by abiotic stresses such as heat and drought. We reported previously that root photosynthesis can contribute to carbon assimilation in Arabidopsis (Kobayashi et al., 2013). Thus, the root greening system regulated by cytokinin and auxin may function as a survival strategy of land plants upon the loss of main photosynthetic tissues.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All Arabidopsis (*Arabidopsis thaliana*) plants used in this study were in the Columbia ecotype. *ahk2-2 ahk3-3* (Higuchi et al., 2004), *arr1-3, arr12-1, arr1-3 arr10-5, arr1-3 arr11-2*, and *arr1-3 arr12-1* (Mason et al., 2005; Argyros et al., 2008), *TCS::GFP* (Müller and Sheen, 2008), *TCSn::GFP* (Zürcher et al., 2013), *WIND1-SRDX* and *XVE-WIND1* (Iwase et al., 2011a), *glk1 glk2* (Fitter et al., 2002), *GLK1ox* (Waters et al., 2009), *gnc gnl, GNCox*, and *GNLox* (Chiang et al., 2012), and *hy5-215* (Oyama et al., 1997) were described previously. The *hy5 GNCox* and *hy5 GNLox* lines were generated by crossing *GNCox* and *GNLox* with the *hy5-215* mutant and segregating double homozygous lines. Plants were grown on Murashige and Skoog (MS) medium (adjusted to pH 5.7 with KOH) containing 1% (w/v) Suc solidified with 0.7% (w/v) Gelrite (Wako) on vertical plates at 23°C under continuous white light (40 μmol photons m^{-2} s^{-1}). For analyses of detached roots, roots were excised from 14-d-old seedlings at the root-hypocotyl junction and further incubated for 7 d on MS medium under continuous white light. Intact roots of 21-d-old seedlings were used as the untreated control. For the imaging PAM analysis in Figures 6 and 7D, roots excised from 21-d-old seedlings were incubated for 7 d and used for analysis along with 28-d-old seedling controls. For the experiment in Figure 1E, 21-d-old *XVE-WIND1* seedlings were transferred to MS medium containing 10 μM 17β-estradiol. For treatment with 1 μM BA, 1 μM IAA, or 10 μM PCIB, detached roots or intact seedlings of 14- or 21-d-old plants were transferred to MS medium containing each compound and grown for another 7 d. For experiments in Figures 3 and 4, leaves of 21-d-old Arabidopsis wild type were considered as photosynthetically competent tissues.

GFP Fluorescence Detection

The GFP fluorescence of *TCS::GFP* and *TCSn::GFP* roots was observed with a stereomicroscope (MZ 16FA; Leica) with a band-pass filter set (GFP Plants; Leica) and a color CCD camera (VB-7010; Keyence) in darkness. All images were obtained with the same microscope settings and CCD camera to compare fluorescence intensity fairly among samples. Background signals in blue and red channels were eliminated from images with the use of ZEN 2011 (Carl Zeiss), and brightness was linearly adjusted to the same extent for all images. We analyzed 21-d-old *TCS::GFP* and *TCSn::GFP* seedlings as the untreated control along with detached roots. In the untreated control, shoots were removed just before observation to clarify the fluorescence around the root-hypocotyl junction.

Real-Time PCR Analysis of Gene Expression

Total RNA was extracted by use of the RNeasy Plant Mini kit (Qiagen). Genomic DNA digestion and reverse transcription to obtain cDNA involved the use of the PrimeScript RT Reagent kit with gDNA Eraser (TaKaRa Bio). cDNA amplification involved the use of the Thunderbird PreMix kit (Toyobo) and 200 nM gene-specific primers listed in Supplemental Table S1. Signal detection and quantification involved the use of a MiniOpticon real-time PCR machine (Bio-Rad) as described (Kobayashi et al., 2013).

Real-Time PCR Analysis of Plastid DNA Content

Total DNA was extracted from root samples by use of the DNeasy Plant Mini kit (Qiagen). Plastid and nuclear DNA were amplified using *rpoB*-specific primers and *ACTIN8*-specific primers, respectively (Supplemental Table S1), by

use of the Thunderbird PreMix kit (Toyobo) and the CFX Connect Real-Time System (Bio-Rad). Cycle threshold values of the amplified plastid DNA signals were normalized to those of nuclear DNA signals, and plastid DNA content in each root sample relative to that of intact wild-type roots was calculated by a PCR-based mathematical model (Pfaffl, 2001). The same result was obtained with the use of *psaA*-specific primers for plastid DNA and the use of *CHLH*-specific primers for nuclear DNA (Supplemental Fig. S3B). Three independent biological experiments were carried out for each sample.

Chl Determination and Photosynthetic Measurement

Spectroscopic Chl determination and kinetic analysis of transient Chl fluorescence induction were performed by measuring Chl fluorescence directly from root segments as described (Kobayashi et al., 2013). Fluorescence transients were normalized between minimal and maximal Chl fluorescence to range from 0 to 1.

To measure fluorescence emission spectra of Chl proteins at 77K (Fig. 4B), root samples were pulverized in liquid nitrogen and homogenized in a cold buffer (0.33 M sorbitol, 5 mM MgCl$_2$, 5 mM EDTA, and 50 mM HEPES-KOH, pH 7.6). The homogenate was filtered through a single layer of Miracloth (Calbiochem) with gentle hand pressure. After centrifugation at 5,000g for 10 min at 4°C, the supernatant was discarded and the pellet was resuspended in a cold buffer to obtain 2 μg mL^{-1} Chl-containing membrane fractions. We should note the possibility that PSI-enriched small membrane fragments may remain in the supernatant under this mild centrifugation condition, which may slightly affect the F_{PSI}/F_{PSII} ratio. Chl fluorescence spectra of the membrane fractions from 620 to 800 nm were measured in liquid nitrogen by the use of an RF-5300PC spectrofluorometer (Shimadzu) under 435-nm excitation.

In Figure 4, photosynthetic parameters were analyzed by the use of a fiber-optic PAM fluorometer (Junior-PAM; Heinz Walz). The automated induction program provided by the WinControl-3 software (Heinz Walz) was used with default settings to measure light and saturation pulse. Single leaves or batches of 1-cm segments of primary roots from the root-hypocotyl junction were dark incubated for 15 min on the leaf clip. After measuring minimum Chl fluorescence (F_o) in the dark, maximal Chl fluorescence (F_m) was determined with a saturating pulse. Then, actinic light (420 μmol photons m^{-2} s^{-1}) was supplied for 10 min with recording of stationary fluorescence (F), and saturating pulses were given every 20 s to determine maximum fluorescence (F_m') under the actinic light. In Figures 6 and 7D, the IMAGING-PAM MAXI fluorometer and ImagingWin software (Heinz Walz) were used to analyze photosynthetic parameters under 110 μmol photons m^{-2} s^{-1} actinic light with saturating pulses given every 30 s. Photosynthetic parameters were calculated as follows (Maxwell and Johnson, 2000): $F_o' = F_o/(F_v/F_m + F_o/F_m')$, $F_v/F_m = (F_m - F_o)/F_m$, $F_v'/F_m' = (F_m' - F_o')/F_m'$, $Y_{II} = (F_m' - F)/F_m'$, and $qP = (F_m' - F)/(F_m' - F_o')$. Y_{II} can be transformed into a product of qP and F_v'/F_m': $Y_{II} = (F_m' - F)/F_m' = qP \times F_v'/F_m'$. Y_{NPQ} and Y_{NO} were determined as described (Kramer et al., 2004).

Root Plastid Observation

Primary roots cut between 1 and 1.5 cm from the root-hypocotyl junction were vacuum infiltrated in an enzyme solution containing 2% (w/v) cellulase, 1% (w/v) macerozyme, 0.2% (w/v) pectolyase, 2 mM CaCl$_2$, 10 mM KCl, 500 mM D-mannitol, and 5 mM MES-KOH (pH 5.8). Then, root samples were incubated in the same enzyme solution on a glass slide at room temperature for 3 h to digest cell walls. After fixing with 3.5% (w/v) glutaraldehyde, cells were loosened from tissues by tweezers and gently covered with a cover glass. The number of green plastids was counted in released or loosened cells with a light microscope (BX50; Olympus). Cells used for the measurement were selected randomly, but those obviously containing no plastids were eliminated from the data. Differential interference contrast images of the cells were taken with a digital camera (MC120 HD; Leica) on the BX50 microscope, and the size of green plastids was measured using ImageJ (https://imagej.nih.gov/ij/).

Accession Numbers

Sequence data from this article can be found in TAIR under the following accession numbers: *CHLH* (AT5G13630), *CuAOx* (AT1G31710), *GLDP1* (AT4G33010), *GLK1* (AT2G20570), *GLK2* (AT5G44190), *GNC* (AT5G56860), *GNL* (AT4G26150), *GOX1* (AT3G14420), *HEMA1* (AT1G58290), *HEMA2* (AT1G09940), *LHCA4* (AT3G47470), *LHCB6* (AT1G15820), *psaA* (ATCG00350), *psbA* (ATCG00020), *rbcL* (ATCG00490), *RBCS* (AT1G67090), *rpoB* (ATCG00190),

RPOTp (AT2G24120), *RPOTmp* (AT5G15700), *SIG1* (AT1G64860), *SIG2* (AT1G08540), *SIG3* (AT3G53920), *SIG4* (AT5G13730), *SIG5* (AT5G24120), and *SIG6* (AT2G36990).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Fluorescence microscopy of roots from *TCS::GFP* and *TCSn::GFP* transgenic lines.

Supplemental Figure S2. Quantitative RT-PCR analysis of mRNA expression of *HEMA1* and *HEMA2* encoding glutamyl-tRNA reductase.

Supplemental Figure S3. Chloroplast development in detached wild-type roots and intact roots of *GNCox* and *GNLox* plants.

Supplemental Table S1. Oligonucleotide primers used in this study.

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