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Improved photosynthesis in *Arabidopsis* roots by activation of GATA transcription factors

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Abstract

Plant cells plastically change their functions according to the environment. Although *Arabidopsis* roots are heterotrophic organs, they increase photosynthetic capacity after shoot removal. Transcription factors regulating chloroplast development are involved in this response downstream of positive cytokinin and negative auxin regulation. To dissect the crosstalk of these regulators after shoot removal, we analyzed photosynthetic parameters in roots with chloroplast development enhanced by shoot removal, overexpression of transcription factors, or hormonal treatment. Our data suggest that shoot removal improves electron transfer downstream of PSII in roots, with a decrease in nonregulated energy dissipation. Cytokinin, auxin, and transcription factors affect the photosynthetic capacity of roots in a highly complex manner. Overexpression of two different types of transcription factors (GOLDEN 2-LIKE 1 and class-B GATAs) synergistically increased root chlorophyll content while maintaining high photosynthetic efficiency. Our data demonstrate the flexible regulation of the photosynthetic machinery by hormone signaling and downstream transcription factors.

Additional key words: chlorophyll fluorescence; effective quantum yield of photosystem II; root greening.

Introduction

In seed plants, plastids differentiate into various forms with their respective functions to fulfill the diverse roles of host cells (Jarvis and López-Juez 2013). Development of chloroplasts from other plastids, such as proplastids and etioplasts, is one of the most important cellular processes for plants to establish photoautotrophic growth. Photosynthesis allows plants to grow depending on light energy but with simultaneous threat of photooxidative damage to cells. Therefore, plants should strictly regulate development and the functionality of chloroplasts in coordination with the developmental and functional states of cells and tissues and in response to growth environments. However, the coordination mechanisms of cellular and plastid development remain largely elusive.

In general, roots develop underground as heterotrophic organs with dependence on leaves for their energy and carbon source. In *Arabidopsis thaliana*, chloroplast development in roots is strongly suppressed in part *via* the auxin-signaling pathway, even when the roots are fully illuminated on transparent agar plates (Kobayashi *et al.* 2012). Chlorophyll (Chl) only slightly accumulates in illuminated *Arabidopsis* roots, particularly around the root–hypocotyl junction. Illuminated roots can perform photosynthetic electron transport but with lower photochemical efficiency and larger photoprotective nonphotochemical quenching (NPQ) than leaves (Kobayashi *et al*. 2013). GOLDEN 2-LIKE transcription factors in *Arabidopsis* (GLK1 and GLK2) positively regulate the expression of nuclear-encoded genes associated with Chl biosynthesis and light harvesting by binding directly to their promoter regions (Waters *et al.* 2009). We reported that overexpression of GLK1 and GLK2 (*GLK1ox* and *GLK2ox*) induced chloroplast development in roots (Kobayashi *et al*. 2012). However, the overexpression

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Abbreviations: ARR – ARABIDOPSIS RESPONSE REGULATOR; BA – 6-benzyladenine; B-GATA – class B GATA transcription factor; Chl – chlorophyll; F_v/F_m – maximal quantum yield of PSII; F_v'/F_m' – quantum yield of open PSII under actinic light; GLK – GOLDEN 2-LIKE; GNC – GATA, NITRATE-INDUCIBLE, CARBON METABOLISM INVOLVED; GNL/CGA1 – GNC-LIKE/CYTOKININ-RESPONSIVE GATA TRANSCRIPTION FACTOR 1; IAA – indole 3-acetic acid; MS – Murashige and Skoog; NPQ – nonphotochemical quenching; PAM – pulse amplitude modulation; PCIB – *p*-chlorophenoxyisobutyric acid; q_P – coefficient of photochemical quenching; Φ_{PSII} – effective quantum yield of PSII; Φ_{NO} – quantum yield of nonregulated energy dissipation; Φ_{NPQ} – quantum yield of regulated energy dissipation.
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mainly increased light-harvesting complex (LHC) proteins and antenna pigments in roots, with enhanced grana stacking of the thylakoid membrane but no improvement in photosynthetic efficiency (Kobayashi *et al.* 2013).

We recently revealed that shoot removal promotes chloroplast development in *Arabidopsis* roots, with improved photosynthetic efficiency, *via* a wound-signaling pathway (Kobayashi *et al.* 2017). In response to shoot removal, *WOUND INDUCED DEDIFFERENTIATION* (WIND) transcription factors, which are induced at the wound site, activate cytokinin signaling mediated by type-B ARABIDOPSIS RESPONSE REGULATORs (ARRs) in roots. Double knockout mutation of the major type-B ARRs, ARR1 and ARR12 (Mason *et al.* 2005), blocked photosynthetic remodeling, and Chl accumulation in roots after shoot removal (Kobayashi *et al.* 2017), so these factors are indispensable for the root greening response. Downstream of type-B ARRs, class B GATA transcription factors (B-GATAs), including GATA, NITRATE-INDUCIBLE, CARBON METABOLISM INVOLVED (GNC), and GNC-LIKE/CYTOKININ-RESPONSIVE GATA TRANSCRIPTION FACTOR 1 (GNL/CGA1) (Behringer and Schwechheimer 2015), may play an important role in chloroplast development in roots (Chiang *et al.* 2012, Kobayashi *et al.* 2017). Type-B ARRs activated by shoot removal upregulate B-GATAs, particularly *GNL*, in roots, presumably in addition to direct induction of some photosynthesis-associated nuclear genes. *B-GATA* genes are implicated in the regulation of chloroplast development and diverse developmental processes as well (Behringer and Schwechheimer 2015). In particular, overexpression of *GNC* or *GNL* (*GNCox* or *GNLox*) induces ectopic chloroplast development with increased Chl content and improved photosynthetic efficiency in roots (Chiang *et al.* 2012, Kobayashi *et al.* 2017). The data suggest that B-GATAs are potent regulators of chloroplast development and photosynthetic activity, although the molecular mechanism of how these factors affect chloroplast functionality remains unknown.

Our previous studies indicate that plant hormones auxin and cytokinin and transcription factors GLKs and B-GATAs are involved in regulation of chloroplast development in roots, but how these regulators are intertwined each other to regulate chloroplast functionality is unclear. To gain insight into the signaling crosstalk of these regulators on regulation of chloroplast development, we compared the effects of shoot removal, overexpression of chloroplast-related transcription factors, and hormonal treatment on photosynthetic parameters in roots.

Materials and methods

Plant materials and growth conditions: Plants used in this study were the Columbia ecotype of *Arabidopsis thaliana*. *GLK1ox* (Waters *et al.* 2008), *GNCox,* and *GNLox* lines (Chiang *et al.* 2012) were described previously. Seeds were surface-sterilized, then cold-treated in sterilized water at 4°C for 3 d in the dark before seeding. Plants were grown vertically on solidified Murashige and Skoog (MS) medium (pH 5.7 with KOH) containing 1.0% (w/v) sucrose and 0.7% (w/v) Gelrite (*Wako*, Japan) at 23°C under continuous white light [80 μmol(photon) m^{-2} s^{-1}] for 21 or 28 d after seeding. To prepare detached root samples, roots were excised from 14- or 21-d-old seedlings at the root–hypocotyl junction and further incubated for 7 d on MS medium under the same continuous white light condition. Roots excised from 21- or 28-d-old seedlings immediately before experiments were used as the intact root control. For treatment with 1 μM 6-benzyladenine (BA), 1 μM indole 3-acetic acid (IAA), or 10 μM *p*-chlorophenoxyisobutyric acid (PCIB), detached roots or intact seedlings of 21-d-old plants were transferred to MS medium containing each compound and grown for another 7 d.

Pigment determination: Plant tissues were crushed in liquid nitrogen and then mixed with 80% (v/v) acetone to extract hydrophobic pigments. Cell debris was removed from the extract by centrifugation at 10,000 × *g* for 5 min. The absorbance of the supernatant at 720, 663.2, 646.8, 645, and 470 nm was measured with a *V-730 BIO* spectrophotometer (*JASCO*; Japan) to determine Chl and carotenoid contents as described in Melis *et al*. (1987) and Lichtenthaler (1987), respectively.

Pulse amplitude modulation (PAM) fluorescence analysis of Chl: Photosynthetic quantum yields were analyzed by using an imaging PAM fluorometer *(IMAGING-PAM MAXI*, *Walz*, Germany) and *ImagingWin* software. Seedlings on MS agar plates were dark-incubated for 15 min in the device at room temperature before measurement. After measuring minimal and maximal Chl fluorescence before and during a saturating flash, stationary fluorescence and maximal fluorescence with quenched PSII were determined under actinic illumination. Minimal fluorescence with quenched PSII after actinic illumination was computed by the approximation of Oxborough and Baker (1997). These fluorescence yields were used to calculate the maximal (F_v/F_m) and effective quantum yield of PSII (Φ_{PSII}), quantum yield of open PSII (F_v'/F_m'), coefficient of photochemical quenching (q_P), quantum yield of light-induced energy dissipation *via* NPQ mechanisms (Φ_{NPQ}), and quantum yield of nonregulated energy dissipation (Φ_{NO}) (Maxwell and Johnson 2000; Kramer *et al.* 2004).

Slow induction kinetics and light-response curves of Chl fluorescence were determined by using automated programs provided by the *ImagingWin* software. Slow induction kinetics was obtained under 110 μmol(photon) m^{-2} s^{-1} actinic light with saturating pulses given every

30 s. Light-response curves were determined under actinic light with the intensity increased after every 3 min. Measurement parameters for *IMAGING-PAM* were measuring light intensity = 1, measuring light frequency = 2, damping = 2, gain = 1, saturation pulse intensity = 10.

Results

Photosynthetic remodeling in roots after shoot removal: To examine how transcription factors regulating chloroplast development act on photosynthetic improvement in roots after shoot removal, we compared the induction kinetics of several photosynthetic parameters in intact and detached roots of wild-type *Arabidopsis* and *GLK1ox*, *GNCox*, and *GNLox* lines. In this experiment, we used roots of 28-d-old plants to obtain sufficient Chl fluorescence signals from detached roots, as in a previous study (Kobayashi *et al.* 2017).

We previously reported that shoot removal induces photosynthetic remodeling in wild-type roots, as represented by increased Φ_{PSII} level (Fig. 1*A*) (Kobayashi *et al.* 2017). Image analysis of Chl fluorescence revealed that, in wild-type roots, shoot removal increased Φ_{PSII} levels mainly around the cut site near the root-hypocotyl junction (Fig. 1S, *supplement available online*). Meanwhile, both *GNCox* and *GNLox* increased Φ_{PSII} in roots more broadly. Then we analyzed induction kinetics of various photosynthetic parameters in roots around 1 cm from the root–hypocotyl junction. In intact wild-type roots, Φ_{PSII} level slowly increased with actinic illumination, followed by a slow and weak fluctuation. By contrast, Φ_{PSII} level in detached roots was rapidly and strongly increased and then slightly decreased within a few minutes after actinic illumination, with the level slowly reversed afterward. This kinetics pattern was very similar to that of q_P in the wild type (Fig. 1*B*), a parameter of the redox state of the plastoquinone pool in the "puddle" model (Kramer *et al.* 2004); however, level of F_v'/F_m', representing quantum yield of open PSII under light, was relatively stable in both intact and detached roots (Fig. 1*C*). Similar results were obtained for another photochemical coefficient, q_L, based on the "lake" model (Kramer *et al.* 2004) (*data not shown*). Thus, the redox state of the plastoquinone pool, namely, the openness of PSII, would mainly affect Φ_{PSII} level fluctuation in these roots.

Excess light energy that cannot be used for photosynthetic electron transport in PSII is dissipated as heat or fluorescence in a regulated or nonregulated manner. Here we found that intact wild-type roots showed a rapid increase in Φ_{NPQ} level, the quantum yield of regulated energy dissipation by light-induced NPQ mechanisms (Kramer *et al.* 2004), followed by a slow but continued increase during actinic illumination (Fig. 1*D*). Also, in detached wild-type roots, Φ_{NPQ} level rapidly increased with actinic illumination to a level similar to that in intact roots, but unlike in intact roots, it quickly reached the steady-state level at the middle induction phase. As a result, in detached wild-type roots, Φ_{NPQ} level was higher at the middle phase but lower at the later phase than in intact roots. Level of Φ_{NO}, the quantum yield of nonregulated energy dissipation (Kramer *et al.* 2004), decreased more quickly in detached than that in intact roots (Fig. 1*E*). Thus, in detached roots, the rapid Φ_{PSII} level increase at the early induction phase is inversely related to the rapid decrease in Φ_{NO} level. After the rapid decrease, Φ_{NO} level was maintained at levels lower in detached than that in intact roots, which contributed to the increased Φ_{PSII} level together with suppressed Φ_{NPQ} level in detached roots at later stages.

In all overexpression lines, the kinetics of Φ_{PSII} in roots was similar to that of q_P, with F_v'/F_m' level maintained constant during illumination (Fig. 1*A–C*). In the *GLK1ox* line, Φ_{PSII} level at a steady state was not improved by shoot removal, with a slow and transient Φ_{PSII} increase in intact roots disappearing in detached roots. Also, Φ_{NPQ} and Φ_{NO} levels were not largely changed in *GLK1ox* roots on shoot removal (Fig. 1*D*,*E*). By contrast, in *GNCox* and *GNLox*, the higher Φ_{PSII} level in intact roots than in the wild type (Kobayashi *et al.* 2017) was further increased by shoot removal. The increased Φ_{PSII} level in detached *GNCox* and *GNLox* roots was accompanied by increased q_P and F_v'/F_m' and decreased Φ_{NPQ} levels.

F_v/F_m level was unchanged by shoot removal in all lines, but *GLK1ox* roots showed decreased F_v/F_m level under both conditions (Fig. 2, Fig. 2S, *supplement available online*), which agrees with previous reports (Kobayashi *et al.* 2013, 2017). Thus, the intrinsic photochemical efficiency of PSII is not associated with the increased Φ_{PSII} level in detached roots.

Because the kinetics of photosynthetic electron transport is strongly affected by light intensity, we examined actinic light intensity dependence of photosynthetic parameters in root samples (Fig. 3). Consistent with the induction kinetics analysis (Fig. 1*A*), detached wild-type roots showed higher Φ_{PSII} levels than the intact roots, particularly under middle [80 μmol(photon) m^{-2} s^{-1}] to high [600 μmol(photon) m^{-2} s^{-1}] actinic light conditions. Moreover, Φ_{PSII} levels in intact roots of *GNCox* and *GLK1ox* lines were higher and lower, respectively, than those in intact wild-type roots under most light intensities. In all root samples, the light response kinetics of q_P was similar to that of Φ_{PSII} (Fig. 3*B*), whereas F_v'/F_m' was relatively stable except in *GLK1ox* roots (Fig. 3*C*), which showed remarkably low F_v'/F_m' levels because of the low intrinsic photochemical efficiency of PSII represented by low F_v/F_m (Fig. 2; Fig. 2S). In intact wild-type roots, strongly decreased Φ_{PSII} levels during increased light intensity was accompanied by a steep increase in Φ_{NPQ} levels (Fig. 3*D*). By contrast, in both detached wild-type roots and intact *GNCox* roots, the development of Φ_{NPQ}

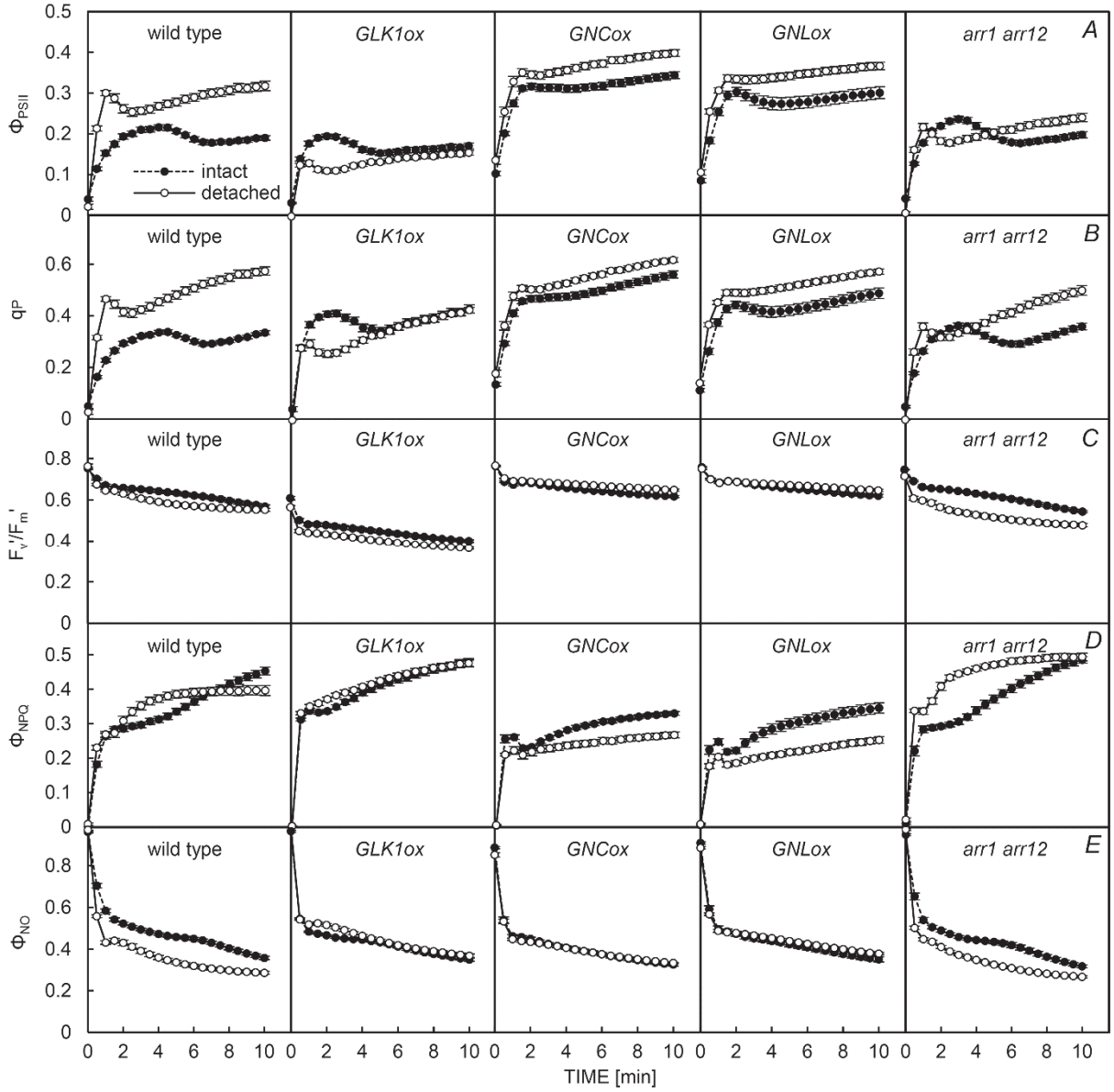

Fig. 1. Induction kinetics of photosynthetic parameters in roots. Chlorophyll fluorescence under actinic light [110 μmol(photon) m^{-2} s^{-1}] was monitored for 10 min with an imaging PAM fluorometer to determine: *A* – effective quantum yield of PSII (Φ_{PSII}), *B* – coefficient of photochemical quenching (q_P), *C* – quantum yield of the open PSII under actinic illumination (F_v'/F_m'), *D* – quantum yield of regulated energy dissipation (Φ_{NPQ}), and *E* – quantum yield of nonregulated energy dissipation (Φ_{NO}). For the detached root sample, roots were excised from 21-d-old seedlings and grown for 7 d, whereas for the intact root control, shoots of 28-d-old seedlings were removed immediately before experiments. Data are mean ± SE from biologically independent samples ($n > 8$). The data for Φ_{PSII}, except for those in detached *GLK1ox*, *GNCox*, and *GNLox* roots, are adapted from Kobayashi *et al.* (2017).

was less prominent than that in intact wild-type roots, and thus Φ_{PSII} levels were higher in these samples. Meanwhile, the Φ_{NO} levels were not greatly altered under increased actinic light in all root samples (Fig. 3*E*).

Content and composition of photosynthetic pigments are changed by shoot removal: In addition to the improved photosynthetic efficiency (Fig. 1), Chl and carotenoid content greatly increased in 28-d-old wild-type roots on shoot removal (Fig. 4*A,B*), which is consistent with previous reports (Kobayashi *et al.* 2012, 2017). The increased pigment content in wild-type roots was accompanied by increased ratio of Chl *a* to Chl *b* and Chl *a* to carotenoid content (Fig. 4*C,D*). *GLK1ox*, *GNCox*, and *GNLox* lines showed substantially increased Chl content in intact roots (Fig. 4*A*) as previously described (Kobayashi *et al.* 2012, 2013, 2017). Carotenoid content was also greatly increased (Fig. 4*B*). Moreover, as in wild-type roots, *GLK1ox*, *GNCox*, and *GNLox* roots showed increased total Chl and carotenoid contents after shoot

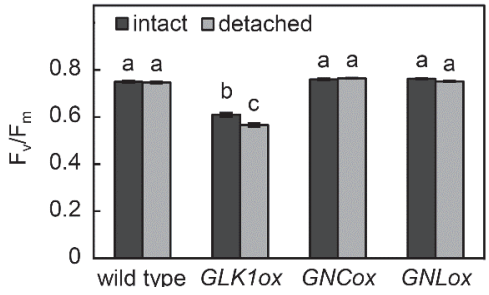

Fig. 2. Maximum quantum yield of PSII (F_v/F_m) in intact and detached roots. For the detached root sample, roots were excised from 21-d-old seedlings and grown for 7 d, whereas for the intact root control, shoots of 28-d-old seedlings were removed immediately before experiments. Data are mean ± SE from biologically independent samples ($n > 8$). *Different letters* indicate significant differences by *Tukey–Kramer* multiple comparison test ($P<0.05$).

removal. In *GNCox* and *GNLox* roots, the Chl *a*/carotenoid ratio increased even without shoot removal, whereas *GLK1ox* roots showed no change in pigment composition in response to shoot removal (Fig. 4*C*,*D*).

Enhanced Chl accumulation by simultaneous overexpression of GLK1 and B-GATAs: Differences in expression profiles of photosynthesis-associated genes and photosynthetic characteristics in roots between *GLK* overexpression lines (*GLK1ox* and *GLK2ox*) and B-GATA overexpression lines (*GNCox* and *GNLox*) suggest that these two transcription-factor families are differentially involved in regulation of chloroplast development (Kobayashi *et al.* 2013, 2017). To examine the crosstalk between GLKs and B-GATAs, we obtained an F1 generation overexpressing both transcription-factor families by crossing homozygous *GLK1ox* with homozygous *GNCox* or *GNLox* lines. For comparison, heterozygous lines were generated for each overexpression line by crossing each homozygous overexpression line with the wild type. The F1 seedlings of *GLK1ox GNCox* and *GLK1ox GNLox* lines, which carried each transgene in the heterozygous state, developed green roots without severe arrest of root growth (Fig. 5*A*). Pigment analysis revealed higher Chl and carotenoid content in double overexpression roots than that in roots of each single homozygous overexpression line (Fig. 5*B,C*). By contrast, Chl and carotenoid contents were lower in roots of heterozygous F1 seedlings of single overexpression lines than their parental homozygous lines, presumably due to halved copy number of transgenes by crossing with the wild type. The Chl *a*/carotenoid ratio increased in both homozygous and heterozygous *GNCox* and *GNLox* roots but not in *GLK1ox* roots (Fig. 5*E*), which was generally consistent with the data in 28-d-old plants. *GLK1ox GNCox* and *GLK1ox GNLox* roots also showed increased Chl *a*/carotenoid ratio, but the changes in the Chl *a*/*b* ratio were less distinct (Fig. 5*D*).

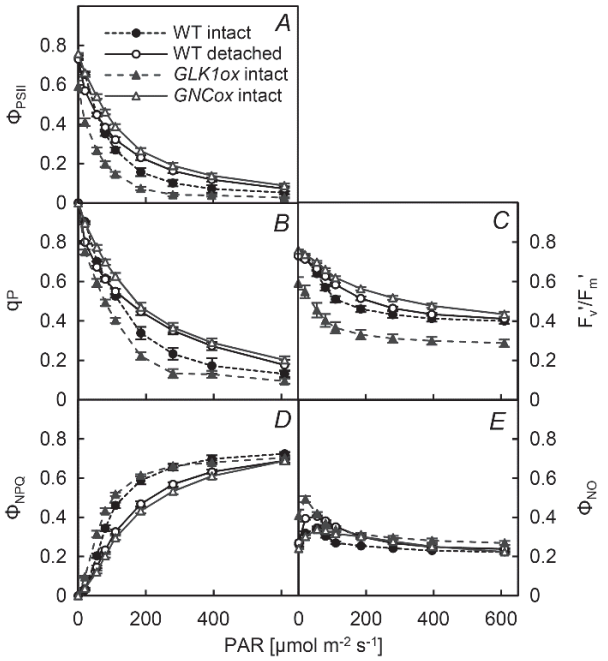

Fig. 3. Light-response curves of photosynthetic parameters in roots. Intact and detached roots of wild type (WT) and intact roots of *GLK1ox* and *GNCox* were dark-adapted for 15 min and exposed for 3 min to each photosynthetically active radiation (PAR). *A* – effective quantum yield of PSII (Φ_{PSII}), *B* – coefficient of photochemical quenching (q_P), *C* – quantum yield of the open PSII under actinic illumination (F_v'/F_m'), *D* – quantum yield of regulated energy dissipation (Φ_{NPQ}), and *E* – quantum yield of nonregulated energy dissipation (Φ_{NO}) were determined with an imaging PAM fluorometer.

***GNCox* and *GNLox* improve photosynthetic efficiency in *GLK1ox* roots**: To assess whether *GNCox* and *GNLox* affect root photosynthesis in the *GLK1ox* background, we analyzed the slow induction kinetics of photosynthetic parameters in roots of double overexpression lines. For this analysis, we used roots from 21-d-old intact seedlings, which showed photosynthetic kinetics similar to that for 28-d-old roots in the wild type and all homozygous overexpression lines (Figs. 1, 6). Thus, photosynthetic characteristics in mature roots were unchanged during development. As in 28-d-old roots (Fig. 1*A*), in 21-d-old roots, *GLK1ox* did not improve and even decreased Φ_{PSII} level, whereas *GNCox* and *GNLox* strongly increased Φ_{PSII} level (Fig. 6*A*). Both *GLK1ox GNCox* and *GLK1ox GNLox* roots showed induction kinetics of Φ_{PSII} similar to that in the *GNCox* and *GNLox* single lines. Thus, even in the *GLK1ox* background, *GNCox* and *GNLox* improved photosynthetic efficiency in roots. Both q_P and F_v'/F_m' level increased in roots of double overexpression lines as compared with single *GLK1ox* roots (Fig. 6*B*,*C*). Moreover, decreased F_v/F_m in roots with *GLK1ox* was recovered in the double overexpression lines (Fig. 6*D*). Thus, electron transport efficiency both within and downstream of PSII would be improved in *GLK1ox* roots by simulta neous overexpression of *GNC* or *GNL*.

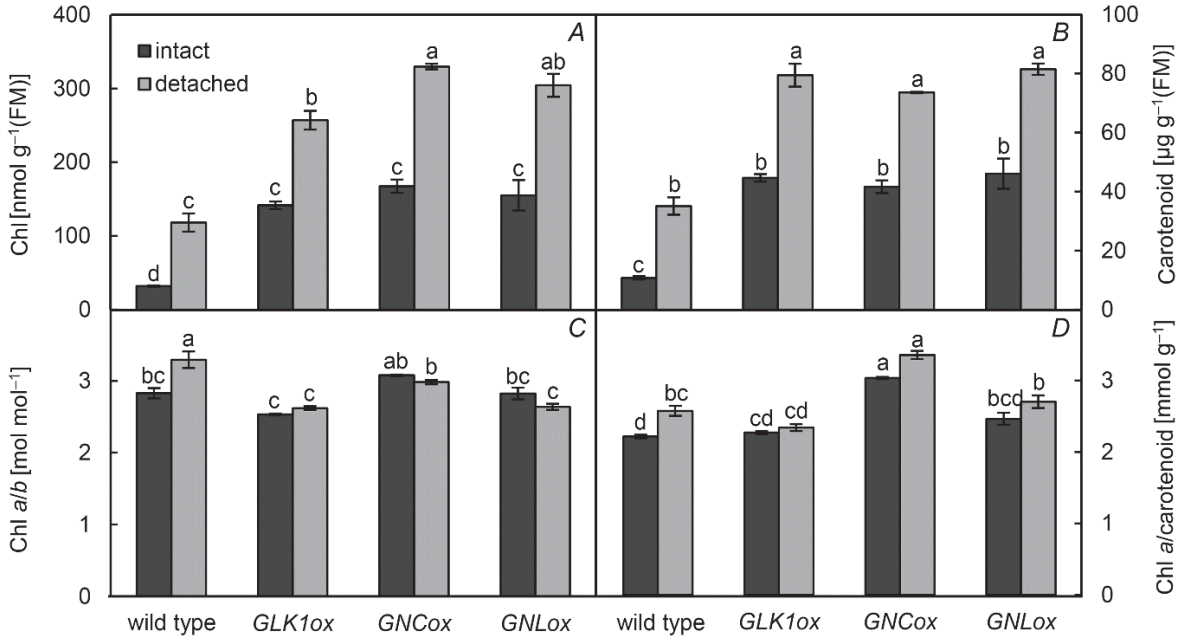

Fig. 4. Content and composition of photosynthetic pigments in intact and detached roots. For the detached root sample, roots were excised from 21-d-old seedlings and grown for 7 d, whereas for the intact root control, shoots of 28-d-old seedlings were removed immediately before experiments. Data are mean ± SE from biologically independent samples ($n > 5$). *Different letters* indicate significant differences by *Tukey–Kramer* multiple comparison test ($P<0.05$). Chl – chlorophyll; FM – fresh mass.

Different regulation of root photosynthesis by cytokinin and auxin: We recently revealed that the double knockout mutation of ARR1 and ARR12 (*arr1 arr12*) strongly impaired root greening response, namely, Chl accumulation, photosynthetic gene expression, and photosynthetic improvement, on shoot removal (Kobayashi *et al.* 2017). Hence, type-B ARR-mediated cytokinin signaling may play a central role in this response. In fact, shoot removal did not notably increase the steady-state Φ_{PSII} level in *arr1 arr12* roots (Fig. 1*A*) (Kobayashi *et al.* 2017). However, as in the wild type, in *arr1 arr12*, shoot removal changed the curve pattern of the Φ_{PSII} induction kinetics, with Φ_{PSII} rapidly and transiently increasing after actinic illumination only in detached roots. The data suggest that the transient increase in Φ_{PSII} during the early induction phase is regulated differently from the steady-state Φ_{PSII} level.

To ascertain whether the *arr1 arr12* roots modify the induction kinetics in response to shoot removal similar to wild-type roots, we compared the kinetics of other photosynthetic parameters in mutant roots with or without shoot removal (Fig. 1, right-most panels). As in detached wild-type roots, in detached *arr1 arr12* roots, q_P rapidly and transiently increased on shoot removal, although the level was lower than in the wild type. The kinetics of Φ_{NPQ} level was also changed in *arr1 arr12* roots with shoot removal as in the wild type but with higher levels than in detached wild-type roots. In addition, unlike in the wild type, *arr1 arr12* roots showed decreased F_v'/F_m' on shoot removal. Meanwhile, the kinetics and level of Φ_{NO} were similar between the wild type and *arr1 arr12*. These data suggest that the transient development of Φ_{PSII} and q_P in detached roots is independent of ARR1 and ARR12 signaling, although these ARRs are required for the increased steady-state level of Φ_{PSII} in roots on shoot removal.

We reported that treatment with BA, a synthetic cytokinin, or PCIB, an auxin-signaling inhibitor, increases Chl content and Φ_{PSII} level in intact *Arabidopsis* roots, whereas an auxin, IAA, partially inhibits the Chl accumulation and the increased Φ_{PSII} level in detached roots (Kobayashi *et al.* 2012, 2017). We confirmed that 28-d-old seedlings treated with BA or PCIB for 7 d showed increased Chl content in intact roots, whereas IAA treatment inhibited the enhanced Chl accumulation in detached roots (Fig. 7*A*), which is consistent with data for 21-d-old seedlings (Kobayashi *et al.* 2012). Total carotenoid content in roots was similarly changed by the hormone treatments (Fig. 7*B*). The IAA treatment appeared to slightly decrease Chl *a*/*b* and Chl *a*/carotenoid ratios in detached roots, but the differences were not statistically significant (Fig. 7*C*,*D*). BA treatment increased only the Chl *a*/carotenoid ratio, and PCIB did not change any ratios.

In order to understand how hormonal signaling is involved in photosynthetic remodeling in roots, we examined the induction kinetics of the photosynthetic parameters in 28-d-old roots treated with the growth regulators for 7 d (Fig. 8). Similar to detached roots, in intact roots, increased Φ_{PSII} by BA treatment was accompanied by increased q_P, with almost no change in F_v'/F_m'. However, unlike in detached roots, BA-treated roots showed no steep induction of q_P and Φ_{PSII} on actinic illumination. Meanwhile, PCIB-treated roots showed a

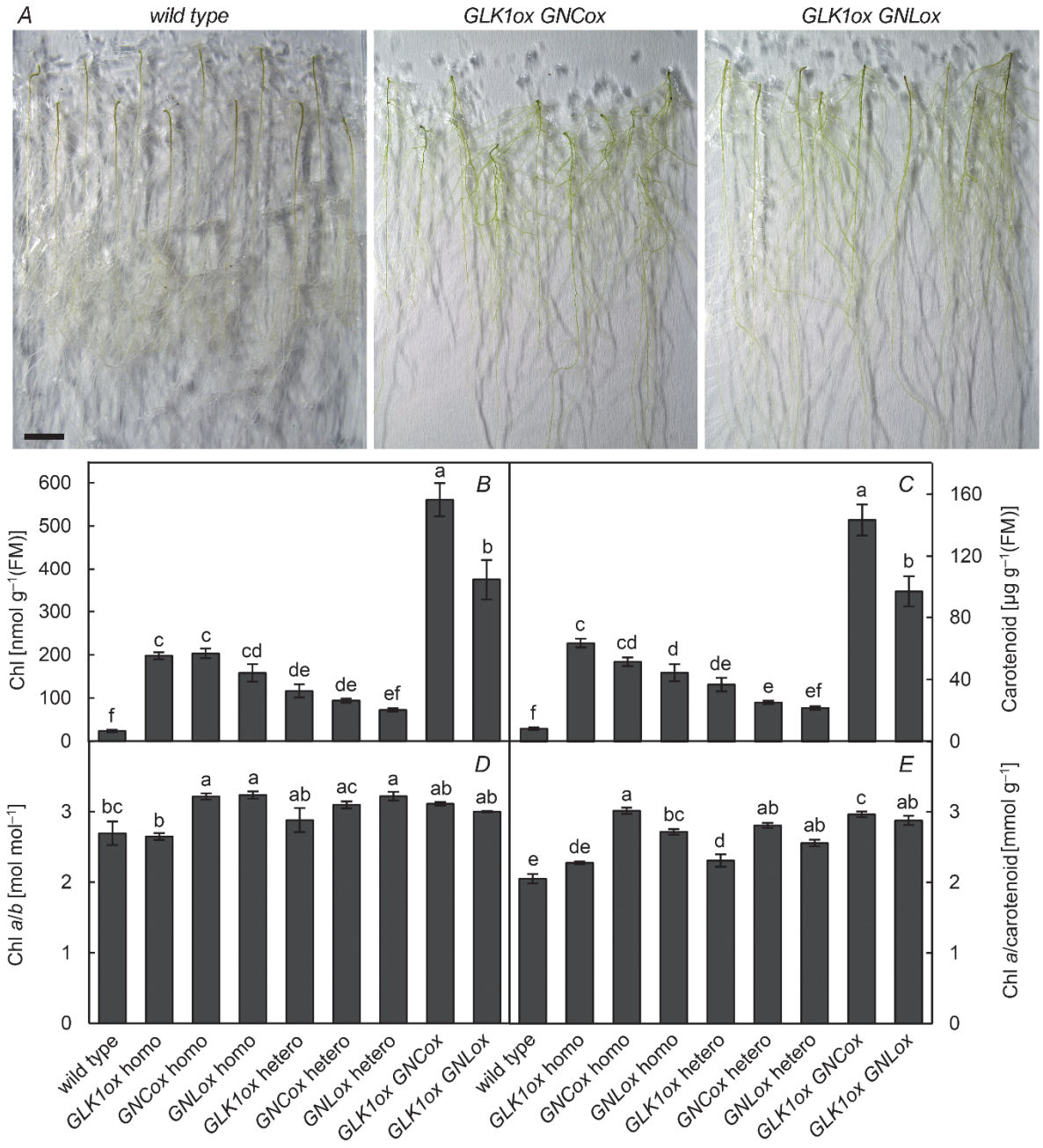

Fig. 5. Enhanced root greening by double overexpression of *GLK1* and B-GATA factors (*GNC* or *GNL*). *A* – Visible phenotype and *B–E* – pigment content and composition in roots of various overexpression lines. *GLK1ox GNCox* and *GLK1ox GNLox* are double overexpression lines carrying each transgene in the heterozygous state. Data are mean ± SE from biologically independent samples ($n > 3$). *Different letters* indicate significant differences by *Tukey–Kramer* multiple comparison test ($P<0.05$). Chl – chlorophyll; FM – fresh mass. A bar in (*A*) represents 1.0 cm.

rapid and transient induction of these parameters. Both BA- and PCIB-treated roots showed a slightly faster decrease in Φ_{NO} level. In addition, BA treatment strongly suppressed Φ_{NPQ} in roots, which mainly contributed to increased Φ_{PSII}. Φ_{NPQ} level was higher in PCIB-treated than that in untreated intact roots in the middle induction phase, as it was observed in detached roots. IAA-treated detached roots showed induction patterns of parameters similar to that in untreated detached roots, but the transient induction of q_P and Φ_{PSII} at the early induction phase was partially suppressed. These data suggest that cytokinin and auxin differentially affect the photosynthetic machinery developed in roots, and complex regulation by these hormones is likely involved in the photosynthetic remodeling in detached roots.

Discussion

Rapid and transient Φ_{PSII} development in detached roots is independent of cytokinin signaling: We recently reported that shoot removal not only increases Chl content but also improves photosynthetic efficiency in *Arabidopsis* roots (Kobayashi *et al.* 2017). Image analysis of Chl fluorescence in roots revealed that shoot removal locally increased Φ_{PSII} around the cut site near the root–hypocotyl junction, although intact wild-type roots showed more uniform Φ_{PSII} levels from the basal to the middle areas (Fig. 1S). The data indicate that photosynthetic remodeling by shoot removal is a local response around the cut site. The data is consistent with the finding that chloroplast development is triggered by a local wounding response mediated by WINDs and type-B ARRs (Kobayashi *et al*. 2017). By contrast, ectopic overexpression of *GNC* and *GNL* increased Φ_{PSII} over a wide area of the root, which suggests that *GNC* and *GNL* function to improve root photosynthesis at downstream of wounding and cytokinin-signaling pathways. In detached roots, the cytokinin signaling around the wounding site may locally upregulate B-GATAs, particularly *GNL*, which subsequently induce chloroplast development and photosynthetic improvement around the cut site.

Shoot removal greatly changes the induction kinetics of Φ_{PSII} in wild-type roots, inducing transient Φ_{PSII} development while rapidly suppressing Φ_{NO} within a few minutes after actinic illumination. Type-B ARRs functioning downstream of cytokinin signaling, particularly ARR1 and ARR12, play a central role in the root greening response after shoot removal, upregulating transcription factors involved in chloroplast development, particularly *GNL* (Kobayashi *et al.* 2017), presumably in addition to directly inducing the expression of some photosynthesis-associated genes (Cortleven *et al.* 2016). However, although *arr1 arr12* roots failed to accumulate Chl and increase steady-state Φ_{PSII} level in response to shoot removal, they still showed a rapid and transient increase in Φ_{PSII} on actinic illumination (Fig. 1*A*). Moreover, cytokinin treatment increased Φ_{PSII} in wild-type roots *via* ARR1 and ARR12 as with shoot removal, but the induction kinetics greatly differed from that in detached roots, particularly lacking the steep transient increase in Φ_{PSII} level and q_P (Fig. 8*A*,*B*). Therefore, changes in photosynthetic kinetics at the early induction phase in detached roots may be independent of cytokinin signaling.

In addition to positive cytokinin signaling, negative auxin signaling is involved in the root greening response after shoot removal (Kobayashi *et al.* 2017). In fact, inhibition of auxin signaling by PCIB slightly increased Φ_{PSII} level along with Chl and carotenoid content in intact roots, whereas IAA treatment suppressed the enhanced Φ_{PSII} level and pigment accumulation in roots on shoot removal (Figs. 7*A*,*B*; 8*A*). Of note, IAA treatment slightly suppressed the transient increase in Φ_{PSII} and q_P specific to detached roots, whereas PCIB treatment partially mimicked the effect of shoot removal on these parameters (Fig. 8). These data may reflect an involvement of auxin signaling in the transient Φ_{PSII} increase on actinic illumination in roots. However, the effects of PCIB and IAA on Φ_{PSII} and other parameters were limited, so the contribution of auxin signaling to the regulation of root photosynthesis would be only partial. Consistent with this result, auxin treatment to detached roots only slightly affected the expression of photosynthesis-associated genes (Kobayashi *et al*. 2017). Auxin signaling appears to regulate chloroplast development in roots independently of type-B ARR-mediated cytokinin signaling (Kobayashi *et al*. 2017). Thus, cytokinin, auxin, and presumably other factors are likely to affect photosynthetic processes in roots in a highly complex manner.

B-GATA factors may play a role in regulating chloroplast development in roots downstream of hormonal signaling. Φ_{PSII} and q_P were rapidly induced in intact roots of *GNCox* and *GNLox* lines after actinic illumination as in detached wild-type roots (Fig. 1*A*,*B*), so enhanced activity of these factors in response to shoot removal may be associated with photosynthetic remodeling in detached roots. This suggestion is supported by the fact that shoot removal did not largely change the kinetics of Φ_{PSII} and q_P in *GNCox* and *GNLox* roots, particularly at the early induction phase, which implies that GNC and GNL are in the same pathway as that activated in response to shoot removal. However, loss of function of both GNC and GNL by the *gnc gnl* double mutations did not impair the steep transient increase in Φ_{PSII} level in roots (Kobayashi *et al.* 2017), so these factors are not essential for this process in roots. Considering that *Arabidopsis* has 4 other B-GATA paralogs closely related to *GNC* and *GNL* (Behringer and Schwechheimer 2015, Ranftl *et al.* 2016), the remaining B-GATAs or other factors functioning in the same pathway may compensate for the function of *GNC* and *GNL* in transient Φ_{PSII} development in the *gnc gnl* double mutant.

Enhanced oxidation of the plastoquinone pool increases Φ_{PSII} in detached roots: Φ_{PSII} can be considered a product of q_P, the openness of PSII, and F_v'/F_m', the quantum efficiency of the open PSII (Maxwell and Johnson 2000). The very similar fluctuation patterns between q_P and Φ_{PSII} in all root samples, with F_v'/F_m' level being more stable, suggest that the fluctuating PSII redox state mainly determines Φ_{PSII} kinetics during actinic illumination. In the wild type, reoxidation of PSII, represented by increased q_P on actinic illumination, was faster in detached than intact roots (Fig. 1*B*), which suggests that electron transfer from the plastoquinone pool to the downstream components is more efficient in detached roots. The efficient electron transport would decrease nonregulated energy dissipation, as reflected by the rapidly decreased Φ_{NO} in detached roots (Fig. 1*E*). Moreover, the enhanced q_P in detached roots persisted to the later stages

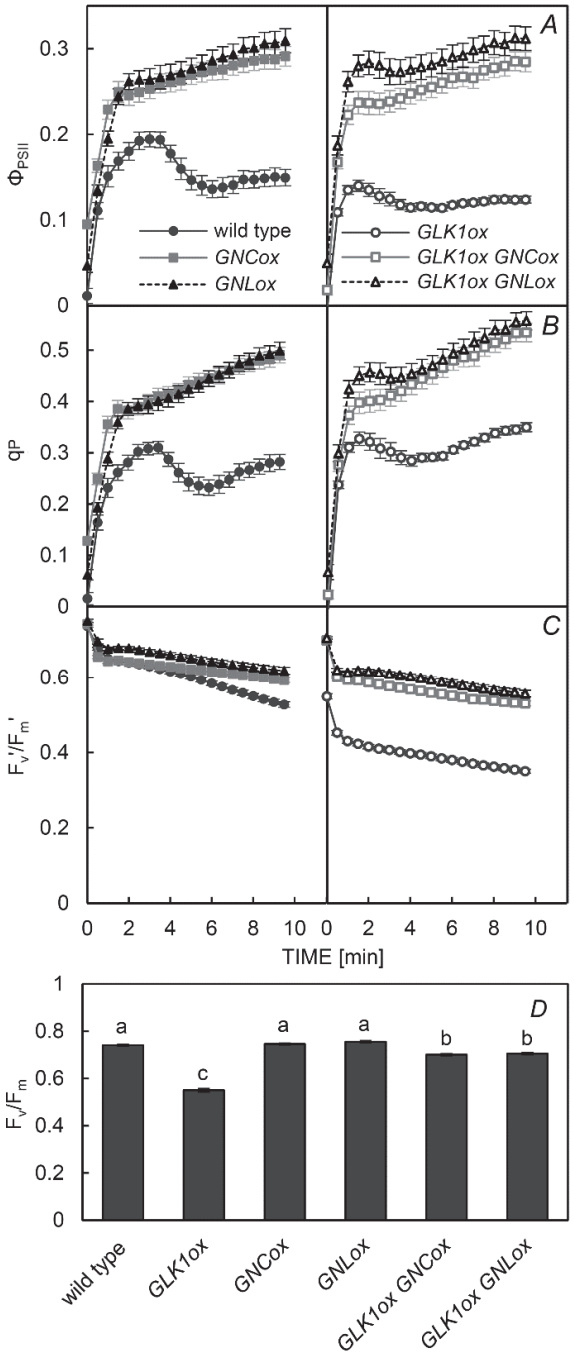

Fig. 6. Photosynthetic parameters in intact roots of various overexpression lines grown for 21 d. Slow induction kinetics of *A* – effective quantum yield of PSII (Φ_{PSII}), *B* – coefficient of photochemical quenching (q_P), *C* – quantum yield of the open PSII under actinic illumination [110 μmol(photon) m^{-2} s^{-1}] for 10 min (F_v'/F_m'), and *D* – maximum quantum yield of PSII (F_v/F_m). *GLK1ox GNCox* and *GLK1ox GNLox* are double overexpression lines carrying each transgene in the heterozygous state. Data are mean ± SE from biologically independent samples ($n > 6$). In (*D*), *different letters* indicate significant differences by *Tukey–Kramer* multiple comparison test ($P<0.05$).

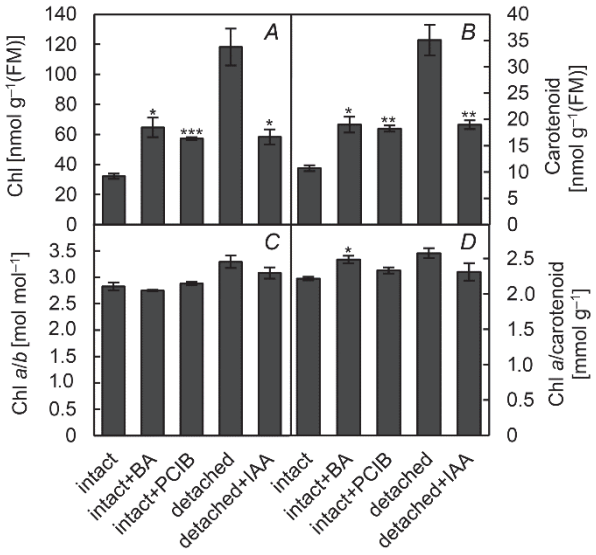

Fig. 7. Effect of hormone signaling on photosynthetic pigments in intact and detached roots. Detached roots or intact seedlings of 21-d-old plants were treated with 1 μM 6-benzyladenine (BA), 1 μM indole 3-acetic acid (IAA), or 10 μM *p*-chlorophenoxy-isobutyric acid (PCIB) for 7 d. Data are mean ± SE from biologically independent samples ($n > 3$). *Asterisks* indicate significant differences from the wild-type (* – $P<0.05$, ** – $P<0.01$, *** – $P<0.001$, *Student*'s *t*-test after a *Bonferroni* correction for multiple comparison). Chl – chlorophyll; FM – fresh mass.

of the induction kinetics. Therefore, detached roots increase Φ_{PSII} level by maintaining an oxidized plastoquinone pool. Because plastoquinone oxidation by the cytochrome b_6/f complex is generally the rate limiting step of the linear electron transport under saturating light conditions (*see* review by Tikhonov 2015), the electron transport capacity of this complex may be somehow improved in detached roots. This assumption is supported by the light-response curve analysis of photosynthetic parameters (Fig. 3). In the intact wild-type roots, q_P strongly decreased as actinic light intensity increased. This was more evident in intact *GLK1ox* roots. We previously reported that PSI in *GLK1ox* roots was in a more oxidized state than in leaf chloroplasts due to donor-side limitations, which implies that intersystem electron transport through cytochrome b_6/f is limited in this root sample (Kobayashi *et al.* 2013). In intact wild-type and *GLK1ox* roots, the electron transport capacity of the cytochrome b_6/f complex may be low, so the plastoquinone pool may be strongly reduced even under lower actinic light conditions. By contrast, as did shoot removal to wild-type roots, *GNCox* and *GNLox* increased q_P in roots, with F_v'/F_m' level only slightly affected (Fig. 1*B*,*C*). Moreover, detached wild-type roots and intact *GNCox* roots showed higher q_P particularly under middle to high light conditions (Fig. 3*B*). Thus, shoot removal, which is mimicked by overexpression of B-GATAs at least partially, may improve the intersystem electron transport and thereby increase the electron transport rate in root chloroplasts.

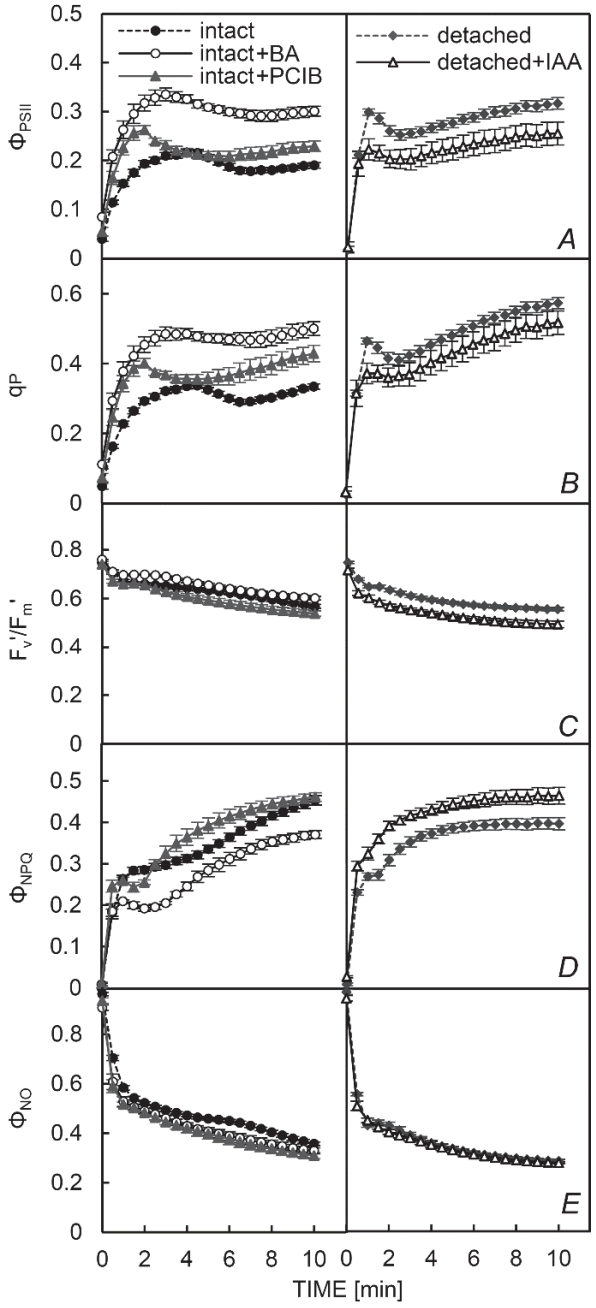

Fig. 8. Effect of hormone signaling on photosynthetic parameters in intact and detached roots. (*A*) effective quantum yield of PSII (Φ_{PSII}), (*B*) coefficient of photochemical quenching (q_P), (*C*) quantum yield of the open PSII under actinic illumination (F_v'/F_m'), (*D*) quantum yield of regulated energy dissipation (Φ_{NPQ}), and (*E*) quantum yield of nonregulated energy dissipation (Φ_{NO}). For the detached root sample, roots were excised from 21-d-old seedlings and grown for 7 d, whereas for the intact root control, shoots of 28-d-old seedlings were removed immediately before experiments. For hormonal treatment, 21-d-old plants were treated with 1 μM 6-benzyladenine (BA), 1 μM indole 3-acetic acid (IAA), or 10 μM *p*-chlorophenoxyisobutyric acid (PCIB) for 7 d. Data are mean ± SE from biologically independent samples ($n > 8$). The data for Φ_{PSII} are adapted from Kobayashi *et al*. (2017).

Considering that *GNCox* and *GNLox* upregulate plastid-encoded photosynthetic genes in addition to nuclear-encoded genes (Kobayashi *et al.* 2017), the balanced induction of photosynthetic components may positively affect photosynthetic electron transport. By contrast, in *GLK1ox* roots, q_P was not increased in response to shoot removal. Although *GLK1ox* did not strongly change Chl *a*/*b* and Chl *a*/carotenoid ratios in roots (Figs. 4, 5), mRNA, protein, and Chl fluorescence analyses indicate that *GLK1ox* preferentially induces the formation of antenna complexes and decreases the photochemical efficiency of PSII in roots (Kobayashi *et al.* 2013). Because *GLK1ox* also decreases the PSI/PSII ratio in roots (Kobayashi *et al.* 2013), various components of photosystem complexes including PSI/PSII and antenna/reaction center ratios, the pigment composition in photosystem complexes, and possibly the subunit composition in photosystem cores and LHCII complexes, would be unbalanced in the *GLK1ox* roots. Therefore, in *GLK1ox*, the forced imbalance in photosystem complexes may cancel the photosynthetic remodeling in roots by shoot removal.

In the early stages of induction kinetics, the rapid induction of Φ_{NO} was inversely associated with the transient induction of Φ_{PSII} in detached roots. In the later stages, in addition to continuing the lower Φ_{NO} level, suppressed Φ_{NPQ} contributed to a gradual increase in Φ_{PSII} in detached wild-type roots (Fig. 1*D*). Φ_{NPQ} was also suppressed in *GNCox* and *GNLox* roots, which mainly contributed to the increased Φ_{PSII} in these overexpression lines after shoot removal. In intact wild-type roots, after a rapid induction, Φ_{NPQ} slowly and continuously increased until later stages, whereas that in detached roots reached near to a steady-state level within several minutes (Fig. 1*D*). The data indicate that the energy quenching mechanism is different between intact and detached roots. Because *GNCox* and *GNLox* also partially suppressed the slow and continuous NPQ development in intact roots (Fig. 1*D*), these factors may have a function in the remodeling of NPQ systems in roots.

We previously reported that roots have larger antennae relative to reaction centers than that in leaves (Kobayashi *et al.* 2013). However, shoot removal increased the ratio of Chl *a*, the main pigment in reaction centers, to the antenna pigments Chl *b* and carotenoids in wild-type roots (Fig. 4*C*,*D*). Thus, shoot removal may increase the reaction-center size relative to antenna complexes in root chloroplasts, which may decrease NPQ operating in the antennae, particularly in the LHCII trimers. This assumption is essentially consistent with the increased ratio of Chl *a* to antenna pigments in *GNCox* and *GNLox* roots. As we previously discussed (Kobayashi *et al.* 2013), it is possible that the high antenna/reaction center ratio in root chloroplasts is of advantage in the low-light environments of roots growing in the soil. Meanwhile, in the field, shoot removal greatly changes light environment in roots particularly at the basal area on the ground surface. Because, in this study, whole *Arabidopsis* seedlings were

evenly illuminated on vertical transparent agar plates, photosynthetic remodeling in detached roots did not simply a result of light responses, but rather might be an intrinsic mechanism to adjust photosynthetic properties and the growth to altered conditions without the shoot.

B-GATAs and GLK1 synergistically affect chloroplast development in roots: We showed previously and in this study that overexpression of *GLK1* increases Chl content in roots, but the intrinsic photochemical efficiency of PSII (F_v/F_m and F_v'/F_m') substantially decreased (Kobayashi *et al.* 2012, 2013, 2017). However, simultaneous overexpression of *GNC* or *GNL* with *GLK1* further increased Chl content and also improved photosynthetic efficiency in roots (Figs. 5,6). The 4- to 5-times higher Chl and carotenoid content in roots of double heterozygous overexpression lines than that of each heterozygous line alone indicates that GLK1 and B-GATAs synergistically act on the accumulation of photosynthetic pigment in roots. Moreover, decreased F_v/F_m, F_v'/F_m', and Φ_{PSII} in *GLK1ox* roots were reversed in the *GLK1ox GNCox* and the *GNL1ox GNLox* lines to a level comparable to that in single *GNCox* and *GNLox* roots. We reported that *GNCox* and *GNLox* increased the expression of plastid-encoded photosynthetic genes in addition to nuclear-encoded photosynthetic genes (Kobayashi *et al.* 2017), whereas *GLK1ox* preferentially upregulates nuclear-encoded genes associated with Chl biosynthesis and light harvesting (Kobayashi *et al.* 2013). Overexpression of *GNC* or *GNL* in *GLK1ox* plants may change the transcriptional balance between plastid-encoded reaction center genes and nuclear-encoded antenna-related genes in roots. In fact, Chl *a*/*b* and Chl *a*/carotenoid ratios in *GLK1ox* roots were increased by simultaneous overexpression of *GNC* and *GNL*, which may reflect improved balance between reaction centers and antennae by *GNCox* and *GNLox* in *GLK1ox* roots. Meanwhile, shoot removal did not improve photosynthetic efficiency in *GLK1ox* roots. The strong effect of *GLK1ox* causing the antenna–reaction center imbalance in roots may override the photosynthetic improvement induced by shoot removal.

Under our growth conditions, total Chl content in mature *Arabidopsis* leaves is ~3,000 nmol g^{-1}(fresh mass) (Kobayashi *et al.* 2013). Thus, the roots of double overexpression lines accumulated Chl to ~20% of the wild-type leaf content while maintaining high photosynthetic efficiency. Both GLKs and B-GATA are involved in a wide range of developmental processes, so overexpression of these factors in the whole plant has negative effects on growth, particularly in the shoot (Waters *et al.* 2008, Richter *et al.* 2010, Hudson *et al.* 2011). Meanwhile, substantial accumulation of photosynthetic pigments in roots of double overexpression lines did not severely impair root growth (Fig. 5*A*). We reported that root photosynthesis can contribute to carbon assimilation (Kobayashi *et al.* 2013). Moreover, overexpression of *GLKs* further increases carbon assimilation in roots despite the lower photochemical efficiency of PSII. Under certain conditions with roots illuminated, enhanced root greening with high photosynthetic efficiency by modulating activities of chloroplast-related transcription factors in roots may increase overall biomass production in plants without severely affecting growth and functions of roots.

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