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Up-Regulation of Mitochondrial Alternative Oxidase Concomitant with Chloroplast Over-Reduction by Excess Light

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Alternative oxidase (AOX), the unique terminal oxidase in plant mitochondria, catalyzes the energy-wasteful cyanide (CN)-resistant respiration. Although it has been suggested that AOX might prevent chloroplast over-reduction through the efficient dissipation of excess reducing equivalents, direct evidence for this in the physiological context has been lacking. In this study, we examined the mitochondrial respiratory properties, especially AOX, connected to the accumulation of reducing equivalents in the chloroplasts and the activities of enzymes needed to transport the reducing equivalents. We used *Arabidopsis thaliana* mutants defective in cyclic electron flow around PSI, in which the reducing equivalents accumulate in the chloroplast stroma due to an unbalanced ATP/NADPH production ratio. These mutants showed higher activities of the enzymes needed to transport the reducing equivalents even in low-light growth conditions. The amounts of AOX protein and CN-resistant respiration in the mutants were also higher than those in the wild type. After high-light treatment, AOX, even in the wild type, was preferentially up-regulated concomitant with the accumulation of reducing equivalents in the chloroplasts and an increase in the activities of enzymes needed to transport reducing equivalents. These results indicate that AOX can dissipate the excess reducing equivalents, which are transported from the chloroplasts, and serve in efficient photosynthesis.

Keywords: Alternative oxidase — *Arabidopsis thaliana* — Cyanide-resistant respiration — Cyclic electron flow around PSI — Malate/oxaloacetate shuttle.

Abbreviations: AL, actinic light; AOX, alternative oxidase; CEF-PSI, cyclic electron flow around PSI; COX, cytochrome *c* oxidase; CP, cytochrome pathway; DTT, dithiothreitol; ETR, electron transport rate; Fd, ferredoxin; HL, high light; LL, low light; MDH, malate dehydrogenase; NDH, NAD(P)H dehydrogenase; NPQ, non-photochemical quenching; OAA, oxaloacetate.

Introduction

Excess light energy is harmful for plants and leads to disruption of the photosynthetic apparatus (photoinhibition). Since most plants cannot escape exposure to excess light, they have evolved defense systems that dissipate excess light energy (Niyogi 2000). These systems include the thermal dissipation of light energy in pigment–protein complexes in the light-harvesting antennae (Horton et al. 1996, Demmig-Adams and Adams 1996) and the chloroplast electron cycling systems, such as the cyclic electron flow around PSI (CEF-PSI; Kramer et al. 2004, Johnson 2005) and the water–water cycle (Asada 1999, Ort and Baker 2002). While such intra-chloroplastic defense systems have been studied extensively, little is known about the extra-chloroplastic defense systems.

Depending on the developmental stage and/or environment, reducing equivalents generated in the form of NADPH by the photochemical reaction are often in excess in the chloroplast stroma (Allen 2002). Accumulation of reducing equivalents in the stroma causes over-reduction of the photosynthetic electron transport chain and accelerates generation of reactive oxygen species (ROS), leading to photoinhibition. For the last two decades, it has been assumed that excess reducing equivalents generated in the chloroplasts could be transported to the cytosol, peroxisomes and mitochondria via shuttle machineries such as the malate/oxaloacetate (OAA) shuttle (Heineke et al. 1991, Scheibe 2004), and oxidized in metabolic pathways. In mitochondria, the reducing equivalents can be oxidized by the respiratory electron transport chain. In the respiratory electron transport common in all aerobic organisms, reduced ubiquinone is oxidized by the cytochrome *bc1* complex, which transfers electrons to cytochrome *c* and ultimately to O_2 via cytochrome *c* oxidase (COX). While this cytochrome pathway (CP) generates the proton gradient, the

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cyanide (CN)-resistant alternative pathway catalyzed by the alternative oxidase (AOX), the unique terminal oxidase in plant mitochondria, is uncoupled from proton translocation (Day and Wiskich 1995, Siedow and Umbach 2000, Vanlerberghe and Ordog 2002, Finnegan et al. 2004). Although AOX apparently catalyzes the energy-wasteful respiration, some physiological functions have been proposed and its possible role in optimizing photosynthesis has recently attracted much attention (Krömer 1995, Atkin et al. 2000, Gardeström et al. 2002, Raghavendra and Padmasree 2003). AOX is non-phosphorylating and can oxidize the reducing equivalents efficiently without being restricted by the proton gradient across the mitochondrial inner membrane or the cellular ATP/ADP ratio. Therefore, it has been speculated that AOX might have a particular role in relieving the over-reduction of chloroplasts. In fact, we and others have shown that AOX inhibition leads to a decrease in the photosynthetic rate and the over-reduction of the photosynthetic electron transport chain in leaves (Bartoli et al. 2005, Yoshida et al. 2006) and in protoplasts (Padmasree and Raghavendra 1999a, Padmasree and Raghavendra 1999b). Also, it was reported that the amount and activity of AOX dramatically increased in high-light (HL) conditions (Ribas-Carbo et al. 2000, Noguchi et al. 2001a, Noguchi et al. 2005). These results may reflect that AOX would function as a sink for excess reducing equivalents generated in the chloroplasts, but evidence for the role of AOX as a system for dissipation of excess reducing equivalents is still weak.

To assess this possibility directly, we have now performed integrated analyses of photosynthetic electron transport, the accumulation of reducing equivalents in the chloroplasts, the activities of enzymes needed to transport the reducing equivalents and the mitochondrial respiratory properties with an emphasis on AOX capacity. For the detailed analyses of how the accumulation of reducing equivalents in the chloroplasts would affect the respiratory properties, we used *Arabidopsis thaliana* mutants defective in the CEF-PSI as plant material. In higher plants, CEF-PSI consists of two partially redundant pathways. Arabidopsis *pgr5* (*proton gradient reduction*) is deficient in the ferredoxin (Fd)-dependent CEF-PSI pathway (Munekage et al. 2002), while *crr2-2* (*chlororespiratory reduction*) is deficient in the NAD(P)H dehydrogenase (NDH)-dependent pathway (Hashimoto et al. 2003). As reported previously, CEF-PSI is essential for the prevention of stroma over-reduction (Munekage et al. 2004). We had expected that if AOX could dissipate the reducing equivalents generated in the chloroplasts, AOX in the CEF-PSI mutants would be up-regulated to compensate for the defect. Here we demonstrate that a long-distance electron cycling system based on the mitochondrial AOX provides plants with more flexible photo-protection.

Results and Discussion

Redox states of the photosynthetic electron transport chain and stroma in chloroplasts

When the plants were grown under low-light (LL) conditions (40 μmol photon $m^{-2} s^{-1}$), there were no visible phenotypic differences between the wild type and the CEF-PSI mutants (Fig. 1A), although chlorophyll contents in *pgr5* were 21–26% lower than those in the wild type (data not shown). In spite of such similar phenotypes, when the plants were transferred from LL to HL conditions (320 μmol photon $m^{-2} s^{-1}$), photoinhibition was evident in *pgr5* (Fig. 1B). In *pgr5* under HL, induction of non-photochemical quenching (NPQ), the thermal dissipation of excess light energy from PSII antennae (Demmig-Adams and Adams 1996, Horton et al. 1996), was inhibited (Fig. 1C), and the electron transport rate (ETR) was saturated at a low level (Fig. 1D). These results suggest that the Fd-dependent CEF-PSI is essential for NPQ induction and efficient electron transport, which are important for protection from the excess light.

To examine the stromal redox state, we monitored the redox state of P700, the reaction center of PSI. Under HL, most of the P700 in *pgr5* was reduced (Fig. 1E), indicating that downstream of PSI (Fd and $NADP^+$) was over-reduced in *pgr5*. P700 was more reduced in *crr2-2* than in the wild type (Fig. 1E), although the parameters of chlorophyll fluorescence in *crr2-2* were nearly identical to those in the wild type (Fig. 1B–D). This result indicates that the accumulation of reducing equivalents in the stroma was slightly higher in *crr2-2* than in the wild type. The results shown in Fig. 1E confirm that CEF-PSI, especially the Fd-dependent pathway, is essential for the prevention of stroma over-reduction.

The transport activities of reducing equivalents by the malate/OAA shuttle

To examine the accumulation of reducing equivalents in the stroma from another viewpoint, we analyzed the NADP-malate dehydrogenase (NADP-MDH) activities in the chloroplast stroma. This is the key enzyme in the malate/OAA shuttle, which is the major machinery for the transport of reducing equivalents from chloroplasts to mitochondria (Heineke et al. 1991, Scheibe 2004), and the activity is modulated by light energy via the Fd–thioredoxin system (Buchanan and Balmer 2005). Initial activities of NADP-MDH in *pgr5* and *crr2-2* were 40–50% higher than in the wild type before the HL treatment (Fig. 2A). Similarly, the activation states (initial/total activity) of this enzyme were higher in these mutants, whereas the total activities were almost the same level as the wild type (Fig. 2B, C). These results suggest that, because the electron acceptance by PSI downstream was limited in the CEF-PSI

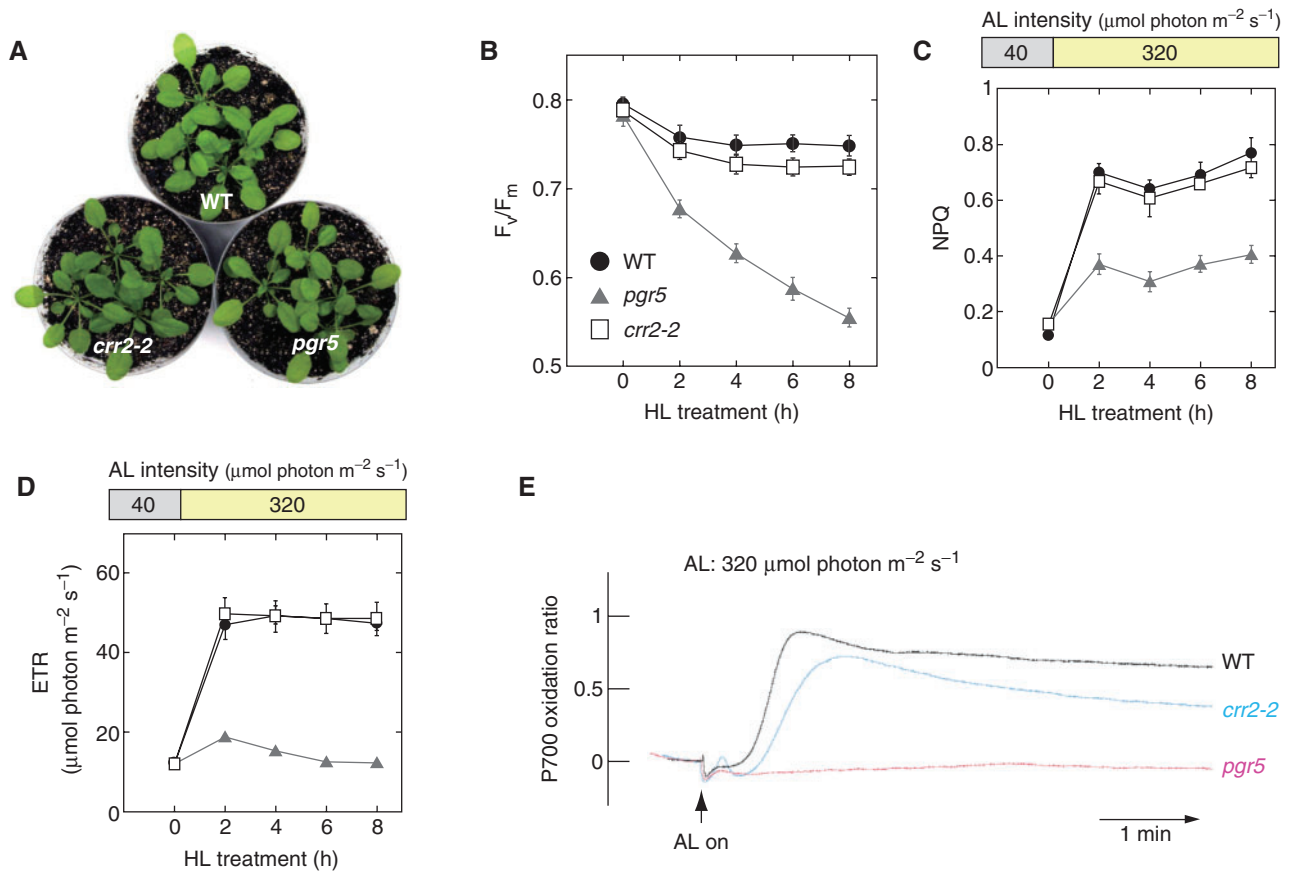

Fig. 1 Characteristics of the photosynthetic electron transport in the wild type (WT) and mutants. (A) Visible phenotype of plants. The plants were grown for 5 weeks in long-day conditions. (B) Effect of high-light (HL) treatment on the PSII maximum quantum yield (F_v/F_m). (C) Effect of HL treatment on the non-photochemical quenching (NPQ). (D) Effect of HL treatment on the electron transport rate (ETR). ETR was calculated as $\Phi_{PSII} \times$ actinic light (AL) intensity $\times 0.84 \times 0.5$. NPQ and ETR were measured at 40 or 320 μmol photon m^{-2} s^{-1} AL intensity. (E) Traces of P700 oxidation ratio ($\Delta A/\Delta A_{max}$) under the HL. ΔA and ΔA_{max} are in vivo absorbance at 830 nm during AL and far-red light illumination, respectively. The arrow indicates the point at which 320 μmol photon m^{-2} s^{-1} AL was switched on. Each value represents the mean ± standard error ($n = 5$).

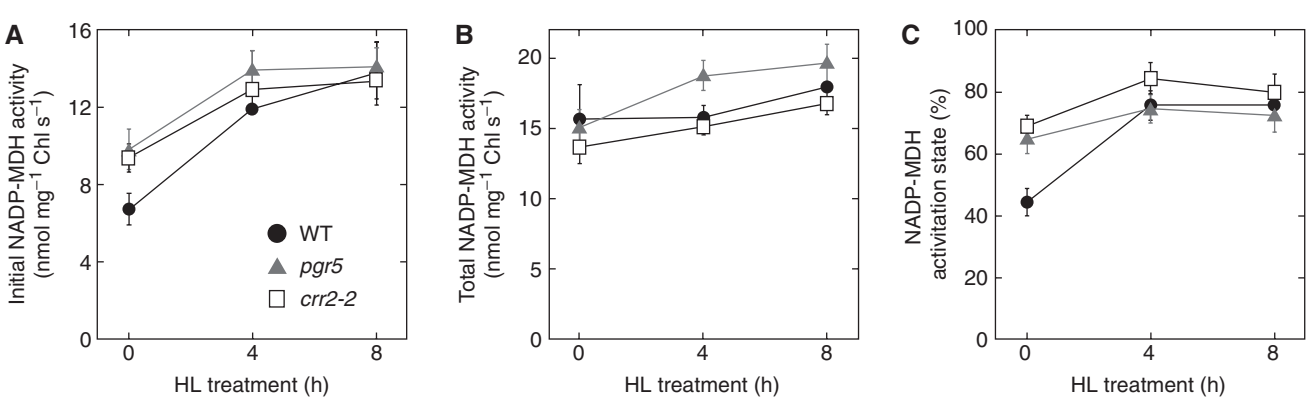

Fig. 2 Characteristics of NADP-malate dehydrogenase (NADP-MDH) in the chloroplast stroma in the wild type (WT) and mutants. NADP-MDH is needed to export the reducing equivalents from the chloroplasts. (A) Effect of high-light (HL) treatment on the initial NADP-MDH activity. The initial activity was measured directly in the sample preparation. (B) Effect of HL treatment on the total NADP-MDH activity. The total activity was measured after the full activation by DTT treatment. (C) Effect of HL treatment on the NADP-MDH activation state. The activation state was expressed as initial/total activity × 100 (%). Each value represents the mean ± standard error ($n = 4$–6).

mutants, NADP-MDH in these mutants was more activated by the Fd–thioredoxin system. These results also suggest that these CEF-PSI mutants exported the reducing equivalents from the chloroplasts at a potentially higher rate and could prevent the accumulation of reducing equivalents in the stroma even under LL, where the mutants did not show a drastic phenotype (Fig. 1A). Even in the wild type, the initial activities and the activation state of NADP-MDH increased after the HL treatment (Fig. 2A, C). This result indicates that, even in the presence of CEF-PSI, higher export of reducing equivalents is essential under HL.

In the malate/OAA shuttle, the reducing equivalents exported from the chloroplasts in the form of malate are metabolized by NAD-MDH in the cytosol, peroxisomes and mitochondria (Scheibe 2004). Similar to NADP-MDH, the activity of NAD-MDH was higher in the CEF-PSI mutants even before the HL treatment (Fig. 3A). The NAD-MDH activities were increased by the HL treatment especially in *pgr5* (Fig. 3A). These results are also consistent with the notion that, when the reducing equivalents accumulate in the stroma, the capacities for their transport and metabolism in the cytosol and mitochondria increase. Although activities of other enzymes in the cytosol and mitochondria [citrate synthase (CS), NADP-isocitrate dehydrogenase (NADP-ICDH) and NAD-malic enzyme (NAD-ME)] were the same before the HL treatment irrespective of the mutants, only the *pgr5* mutant showed an increase in their activities slightly after the HL treatment (Fig. 3B–D). These increases would be attributed to the elevation of organic acids derived from malate, and support the idea that more reducing equivalents were transported from chloroplasts in *pgr5* under HL.

Properties of the mitochondrial respiratory chain

The reducing equivalents in mitochondria could be oxidized by the respiratory chain. The reducing equivalents transported from the chloroplasts to mitochondria could also be oxidized in transit by the external NAD(P)H dehydrogenase (NDex) in the respiratory chain (Rasmusson et al. 2004). In this situation, which electron transport pathway in plant mitochondria (CP or AOX) plays the major role in the dissipation of chloroplast-derived reducing equivalents? Immunoblot analysis revealed that the amounts of AOX protein in the CEF-PSI mutants were higher than in the wild type before the HL treatment (Fig. 4A, B). The amounts of AOX protein especially in the wild type and *pgr5* increased after the HL treatment (Fig. 4A, B). To examine the contributions of AOX to the electron transport, the AOX-dependent respiration rate was measured in the presence of 2 mM KCN. Although the total respiration rates were higher in the CEF-PSI mutants before the HL treatment, these differences became small after the

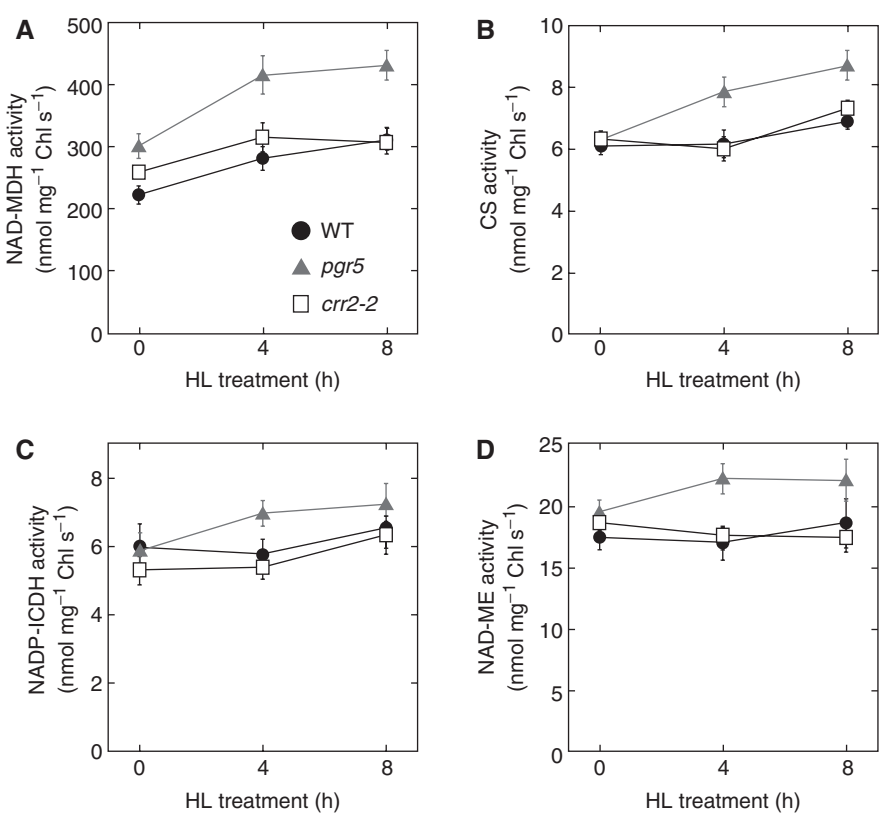

Fig. 3 Characteristics of several enzymes in the cytosol and mitochondria in the wild type (WT) and mutants. (A) Effect of high-light (HL) treatment on NAD-malate dehydrogenase (NAD-MDH) activity. NAD-MDH is needed to transport the reducing equivalents in the malate/oxaloacetate shuttle. (B) Effect of HL treatment on citrate synthase (CS) activity. (C) Effect of HL treatment on NADP-isocitrate dehydrogenase (NADP-ICDH) activity. (D) Effect of HL treatment on NAD-malic enzyme (NAD-ME) activity. Each value represents the mean ± standard error ($n = 4$–6).

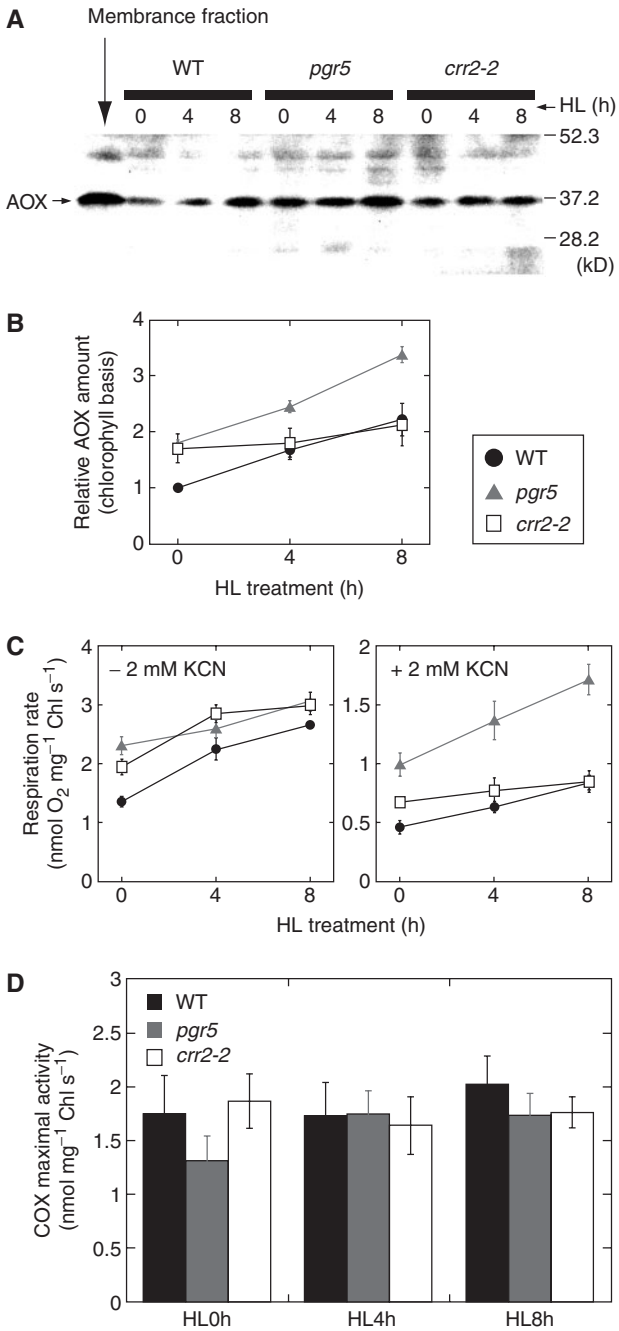

Fig. 4 Characteristics of the mitochondrial respiratory chain in the wild type (WT) and mutants. (A) Immunodetection of alternative oxidase (AOX) in the mitochondria. The lanes were loaded with protein equivalent to 40 μg BSA standards from whole leaf extracts. The protein in the leaf membrane fraction was loaded as a positive control. (B) Effect of high-light (HL) treatment on the amounts of AOX on the basis of the amount of chlorophyll. AOX amounts are shown as relative values when the WT of HL treatment at 0 h is 1. (C) Effect of HL treatment on the total leaf respiration (−2 mM KCN; left panel) and the cyanide-resistant respiration (+2 mM KCN; right panel). (D) Effect of HL treatment on the cytochrome *c* oxidase (COX) maximal activity. Each value represents the mean ± standard error ($n = 3$–6).

HL treatment (Fig. 4C, left panel). On the other hand, the CN-resistant respiration rate was higher in *pgr5* and further increased after the HL treatment (Fig. 4C, right panel). The maximal activity of COX, the terminal oxidase of CP, did not differ among the plants even after the HL treatments (Fig. 4D). Also, there was little change in the protein amounts of COX subunit II (data not shown). Taken together, in the two ubiquinol-oxidizing pathways (CP and AOX), AOX was specifically up-regulated in the CEF-PSI mutants and under HL conditions.

Coordinated up-regulation of malate/OAA shuttle activities and AOX capacity

The increase in amounts of AOX protein was synchronized with the accumulation of reducing equivalents in the stroma and the increase in the activities of enzymes that drive the metabolite shuttles across the organelle membranes (Fig. 5A). Also, the CN-resistant respiration was very strongly positively correlated with the amounts of AOX protein (Pearson's $r = 0.93$; Fig. 5B). These coordinated up-regulations suggest that AOX functions as a sink for excess light energy and serves in the alleviation of photoinhibition. This is further strengthened by the results of our previous study showing that AOX inhibition caused the decrease in photosynthetic rates and the over-reduction of the photosynthetic electron transport chain (Yoshida et al. 2006). This conclusion raises new questions of how many reducing equivalents are exported from the chloroplasts, and how many of them are oxidized via AOX. Elucidation of these matters is essential for further clarification of the extent to which AOX contributes to dissipation of excess light energy.

Possible machineries for AOX up-regulation in CEF-PSI mutants

Even under LL, the transport activity of reducing equivalents and AOX capacity were increased in both CEF-PSI mutants (Figs. 2, 3A, 4A–C). However, they were hardly increased after the HL treatment in *crr2-2* (Figs. 2, 3A, 4A–C). While the Fd-dependent CEF-PSI is thought to be the predominant pathway in C_3 photosynthesis (Munekage et al. 2004), the significance of the NDH-dependent pathway is still under discussion. It has been demonstrated that NDH-inactivated tobacco showed a delay of NPQ induction, more sensitivity to oxidative damage and a decrease in photosynthesis under some stressful conditions, although its growth was normal in the optimal environments (Burrows et al. 1998, Shikanai et al. 1998, Horváth et al. 2000, Wang et al. 2006). It has also been shown that, in C_4 photosynthesis, the NDH-dependent (not Fd-dependent) pathway plays a central role for extra ATP production to concentrate CO_2 (Takabayashi et al. 2005). These reports indicate that NDH has some

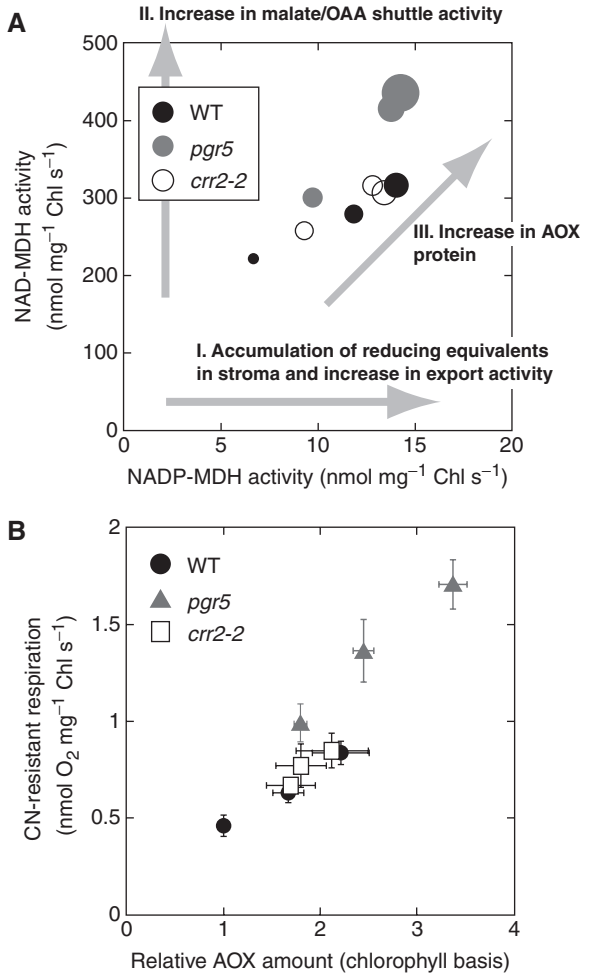

Fig. 5 Up-regulation of mitochondrial alternative oxidase (AOX), accompanied by the accumulation of reducing equivalents in the chloroplast stroma and malate/oxaloacetate (OAA) activity. (A) Relationships among NADP-malate dehydrogenase (NADP-MDH) activity, NAD-MDH activity and relative AOX amount. The diameter of each circle expresses the relative amount of AOX on the basis of the amount of chlorophyll. (B) Relationships between the amount of AOX on the basis of the amount of chlorophyll and cyanide (CN)-resistant respiration.

physiological significance in plant acclimation. Furthermore, the results of our present study indicate that the NDH-dependent pathway, as well as the Fd-dependent pathway, contributes to preventing stroma over-reduction even under non-stressful conditions, although its contribution would be masked under HL (Figs. 2, 3A, 4A–C). As the dysfunction of CEF-PSI causes over-reduction on the acceptor side of PSI, the electrons are liable to reduce oxygen, leading to H_2O_2 production by the Mehler reaction (Asada 1999, Ort and Baker 2002). It is possible that ROS generated in the chloroplasts increase the capacity of AOX as an electron sink, because it has been well established that AOX has a role in preventing ROS generation in the mitochondria and ROS induce AOX expression (Wagner 1995, Vanlerberghe and McIntosh 1996, Maxwell et al. 1999, Sweetlove and Foyer 2004).

Conclusion

A scheme for the long-distance electron cycling system involving AOX-dependent respiration is shown in Fig. 6. We propose that the major physiological function of AOX, the unique respiratory pathway widely distributed in photosynthetic organisms, is to serve as an electron sink that prevents over-reduction of the photosynthetic apparatus and thereby mitigates photoinhibition when leaves are exposed to excess light. In this role, AOX in plant mitochondria becomes an essential accessory for stabilizing the autotrophic system of higher plants.

Other mitochondrial energy-dissipating proteins might also support photosynthesis. Sweetlove et al. (2006) recently demonstrated that mitochondrial uncoupling protein (UCP) contributes to photosynthesis through efficient oxidation of glycine produced during photorespiratory metabolism. Consequently, AOX probably works in conjunction with other energy-dissipating respiratory proteins, such as UCP, in the light. This idea provides intriguing research areas to examine the significance of this redundancy and how these multiple respiratory components interact in illuminated leaves.

Materials and Methods

Plant materials and HL treatment

Arabidopsis thaliana wild type (ecotype Columbia *gl1*) and mutants were cultured in soil for 4–6 weeks under LL; 40 µmol photon $m^{-2}s^{-1}$, 16/8 h light/dark cycle and 22–23°C. For HL treatments, plants were transferred to 320 µmol photon $m^{-2}s^{-1}$ light conditions at 2–3 h after the beginning of the light period.

Measurements of chlorophyll fluorescence

Chlorophyll fluorescence was measured using a PAM (pulse-amplitude modulation) fluorometer (PAM-101, Waltz, Effeltrich, Germany). Chlorophyll fluorescence parameters were calculated as previously described (Genty et al. 1989, Bilger and Björkman 1994). After 20 min dark incubation, the plants were given the saturating pulse (SP) at 7,000 µmol photon $m^{-2}s^{-1}$ (KL 1500 LCD, Schott, Cologne, Germany) and the maximum quantum yield of PSII (F_v/F_m) was measured. After that, the actinic light (AL) at 40 or 320 µmol photon $m^{-2}s^{-1}$ was provided (KL 1500 electronic, Schott). SP for the estimate of F'_m was given 5 min after the onset of AL irradiation, when the chlorophyll fluorescence attained steady-state levels. The ETR was calculated as $\Phi_{PSII} \times$ AL intensity $\times 0.84 \times 0.5$.

Measurements of P700 redox state

The redox state of P700 was measured as previously described (Schreiber et al. 1988), using ED-P700DW-E (Waltz) as the emitter-detector unit. The P700 oxidation ratio was expressed as $\Delta A/\Delta A_{max}$, where ΔA and ΔA_{max} are in vivo absorbance at 830 nm during AL and far-red light illumination, respectively.

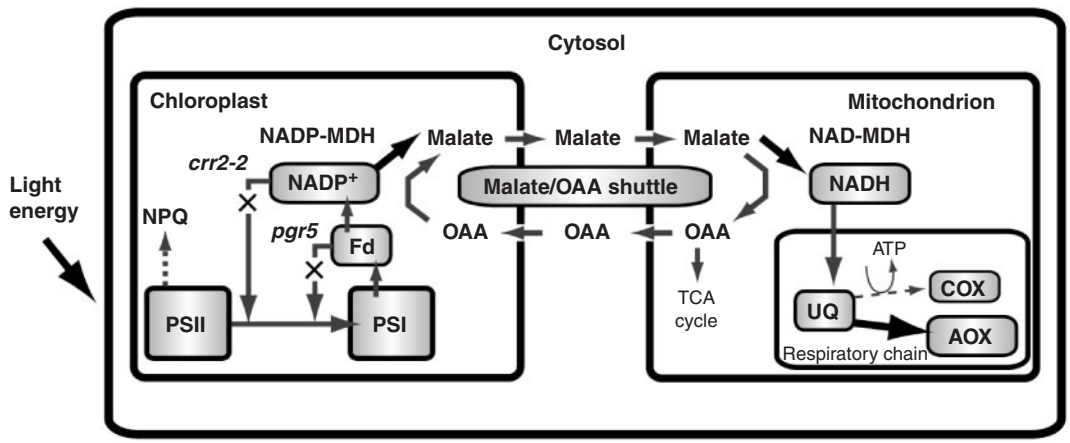

Fig. 6 Schematic model of the transport of reducing equivalents from chloroplast by the malate/oxaloacetate shuttle and the dissipation by alternative oxidase. In the mutants, the reducing equivalents are liable to accumulate in the chloroplasts. Therefore, this scheme is important even under low light. Under high light, this scheme is important even in the wild type. See text for details. AOX, alternative oxidase; COX, cytochrome *c* oxidase; Fd, ferredoxin; NAD(P)-MDH, NAD(P)-malate dehydrogenase; NPQ, non-photochemical quenching; OAA, oxaloacetate; PSI(II), photosystem I(II); UQ, ubiquinone.

Enzyme assays

Enzymes in the soluble fraction were extracted from the leaves as previously described (Dutilleul et al. 2003). About 60 mg FW of leaves were ground in liquid N_2 and extracted into 25 mM HEPES-KOH (pH 7.5) buffer containing 10 mM $MgSO_4$, 1 mM Na_2EDTA, 5 mM dithiothreitol (DTT), a protease inhibitor tablet (Roche, Mannheim, Germany), 5% (w/v) insoluble polyvinylpyrrolidone and 0.05% (v/v) Triton X-100. After centrifugation for 5 min at 10,000×*g*, the enzyme activity in the supernatant was measured with a U-3310 spectrophotometer (Hitachi, Tokyo, Japan). The NADP-MDH activities in chloroplasts were measured according to Dutilleul et al. (2003). The initial activity was measured directly in the supernatant. The total activity was measured after the enzyme was fully activated by pre-incubation of the supernatant in 40 mM Tricine-KOH (pH 9.0), 0.4 mM Na_2EDTA, 120 mM KCl, 100 mM DTT and 0.0025% Triton X-100. Assays were performed in 25 mM Tricine-KOH (pH 8.3), 150 mM KCl, 1 mM Na_2EDTA, 5 mM DTT, 0.2 mM NADPH and 2 mM OAA, plus the sample. The NAD-MDH activity in the cytosol and mitochondria was assayed in 50 mM TES-HCl (pH 7.2), 10 mM $MgCl_2$, 0.02% (v/v) Triton X-100, 0.2 mM NADH and 2 mM OAA, plus the sample, according to Millar and Leaver (2000). The activities of CS, NADP-ICDH and NAD-ME were measured according to MacDougall and ap Rees (1991), Gray et al. (2004) and Millar et al. (1998), respectively.

Extraction and activity measurement of COX were performed as previously described (Hendry 1993). About 60 mg FW of leaves were ground in liquid N_2 and extracted into 50 mM KPi (pH 7.5) buffer containing 250 mM sorbitol and 0.2 mM Na_2EDTA. After the filtration with 26 μm nylon mesh, the homogenate was centrifuged for 3 min at 15,000×*g*. The pellet was re-suspended in 25 mM KPi (pH 7.5) buffer, and the COX activity was measured in 25 mM KPi (pH 7.5) buffer and reduced cytochrome *c*. The reduced cytochrome *c* was prepared by desalting 0.5 ml of 25 mM KPi (pH 7.5) buffer that contained 5 mg of horse heart cytochrome *c* and 11 mg of ascorbic acid with a Sephadex G 50 column.

Leaf respiration measurement

Leaf respiration rates were measured using a Clark-type O_2 electrode (Rank Brothers, Cambridge, UK) as previously described (Noguchi et al. 2001b, Kurimoto et al. 2004). The detached leaves were kept above the stirrer bar and electrode surface by a piece of nylon mesh. Reaction medium contained 100 mM sucrose, 50 mM HEPES, 10 mM MES (pH 6.6) and 0.2 mM $CaCl_2$. The CN-resistant respiration rates were measured in the presence of 2 mM KCN.

SDS–PAGE and immunoblotting

Proteins of the whole leaf tissue were extracted in the SDS sample buffer [2% (w/v) SDS, 62.5 mM Tris–HCl (pH 6.8), 7.5% (v/v) glycerol and 0.01% (w/v) bromophenol blue] including 50 mM DTT and a protease inhibitor tablet (Roche). After boiling for 5 min, the homogenate was centrifuged at 15,000 *g* for 10 min. Proteins in the supernatant were separated by 12.5% SDS–PAGE according to Laemmli (1970). As a positive control, the proteins in the leaf membrane fraction, which were isolated according to Noguchi et al. (2005), were also loaded. The lanes were loaded with protein equivalent to 40 μg bovine serum albumin (BSA) standards. Protein concentration was determined according to Peterson (1977).

For immunoreaction experiments, proteins were transferred to a polyvinylidene fluoride membrane (Hybond-P, Amersham, Piscataway, NJ, USA). Immunodetection of AOX was performed with the AOA monoclonal culture supernatant reacting with AOX at a dilution of 1 : 50 (Elthon et al. 1989). Chemiluminescence was used for detection of horseradish peroxidase-conjugated secondary antibodies and visualized using LAS 1000 (Fuji, Tokyo, Japan). The AOX blots were quantified using the IMAGE GAUGE v 3.0 software (Fuji).

Chlorophyll measurement

Chlorophyll contents and the *a*/*b* ratio were determined according to Porra et al. (1989).

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