Plant Biotechnol Rep (2010) 4:281–291
DOI 10.1007/s11816-010-0146-z

ORIGINAL ARTICLE

Mitochondrial activity in illuminated leaves of chlorophyll-deficient mutant rice (*OsCHLH*) seedlings

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Received: 18 July 2010 / Accepted: 29 July 2010 / Published online: 25 August 2010

Abstract The rice *CHLH* gene encodes the Mg^{2+}-chelatase H subunit, which is involved in chlorophyll biosynthesis. Growth of the chlorophyll-deficient *oschlh* mutant is supported by mitochondrial activity. In this study, we investigated the activity of mitochondrial respiration in the illuminated leaves during *oschlh* seedling development. Growth of mutant plants was enhanced in the presence of 3% sucrose, which may be used by mitochondria to meet cellular energy requirements. ATP content in these mutants was, however, significantly lowered in light conditions. Low cytosolic levels of NADH in illuminated *oschlh* mutant leaves further indicated the inhibition of mitochondrial metabolism. This down-regulation was particularly evident for oxidative stress-responsive genes in the mutant under light conditions. Hydrogen peroxide levels were higher in *oschlh* mutant leaves than in wild-type leaves; this increase was largely caused by the impairment of the expression of the antioxidant genes, such as *OsAPX1*, *OsRAC1*, and *OsAOXc* in knockout plants. Moreover, treatment of mesophyll protoplasts with ascorbic acid or catalase recovered ATP content in the mutants. Taken together, these results suggest that the light-mediated inhibition of mitochondrial activity leads to stunted growth of *CHLH* rice seedlings.

Keywords Mitochondria · Light · H_2O_2 · *OsCHLH* · Rice (*Oryza sativa* L.)

Abbreviations

AOXc	Alternative oxidase c
APX	Ascorbate peroxidase
AsA	Ascorbic acid

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CAT	Catalase
CHLH	Mg^{2+}-chelatase H subunit
GPT	Glutamate pyruvate transaminase
GRX	Glutaredoxin
LDH	Lactate dehydrogenase
MT	Metallothionein
NADH	Nicotinamide adenine dinucleotide
PS II	Photosystem II
Rac1	Small GTP binding protein
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TRX	Thioredoxin

Introduction

The respiratory activity of plants in light conditions has been debated for a long time, particularly with regard the level of respiration that occurs in photosynthetic cells in the presence of light, with several early studies suggesting the inhibition of respiration during the daytime (Budde and Randall 1990; Krömer 1995; Villar et al. 1995; Tcherkez et al. 2005). During the in vivo respiratory metabolism of illuminated leaves, the down-regulation of several metabolic pathways [e.g., glycolysis and tricarboxylic acid (TCA) cycle] accompanies the light/dark transition and emphasizes the decrease of the decarboxylations in the TCA cycle as a metabolic basis of the light-dependent inhibition of mitochondrial respiration (Krömer 1995; Tcherkez et al. 2005). In contrast, it has been reported that mitochondrial respiration is not only active in the light but that certain respiratory pathways (especially the electron transport chain) are also required for maximal rates of photosynthetic carbon assimilation (Krömer 1995; Dutilleul et al. 2003; Raghavendra and Padmasree 2003). Mitochondrial electron transport and ATP synthesis play an important role in the optimization of photosynthesis by reoxidizing the excess redox equivalents generated by chloroplasts, thus protecting against photoinhibition of photosynthesis (Sasadadevi and Raghavendra 1992; Noctor et al. 2007) and providing the ATP required for sucrose synthesis in the cytosol (Hoefnagel et al. 1998). This indicates that mitochondrial electron transport and ATP synthesis processes function in light conditions. However, the nature of day respiration in illuminated leaves has yet to be fully clarified and, in particular, the regulation mechanisms that operate during mitochondrial respiration remain poorly understood.

Previous studies have shown that the respiratory activity of plants in the light is between 25 and 100% of the levels observed in the dark, which suggests that respiration is inhibited during photosynthesis (Krömer 1995). Other enzymes may also be inhibited, including pyruvate kinase, the pyruvate dehydrogenase complex, TCA cycle enzymes, and mitochondrial isocitrate dehydrogenase (Lin et al. 1989; Budde and Randall 1990; Krömer 1995; Igamberdiev and Gardeström 2003; Tovar-Mendez et al. 2004; Tcherkez et al. 2005), and inhibition of malic acid decarboxylase (Hill and Bryce 1992). Photoinhibition is probably also involved in the inhibition of pyruvate dehydrogenase, as this enzyme is down-regulated by NH_3, which is a byproduct of photorespiratory glycine decarboxylation (Krömer 1995). Light can directly influence the respiratory electron transport chain via photoreceptor-mediated transcriptional control, which may be necessary to support the photosynthetic metabolism (Escobar et al. 2004; Islam et al. 2009). Respiration in the light may also affect cellular carbon and nitrogen assimilation (Foyer and Noctor 2000; Yoshida and Noguchi 2009). The physiological changes would result from severe alterations in mitochondrial dynamics including mitochondrial swelling, transmembrane loss, and the cessation of mitochondrial movement (Gao et al. 2008; Zhang and Xing 2008), probably by production of reactive oxygen species (ROS) (Gao et al. 2008; Zhang et al. 2009).

The chlorophyll-deficient rice mutant *OsCHLH*, which lacks the largest subunit of Mg^{2+}-chelatase, was isolated by T-DNA gene trapping (Jung et al. 2003). This mutant exhibits dysfunctional chloroplast activity (Jung et al. 2003), yet similar growth rates and somewhat greater respiratory O_2 uptake were observed in continuously dark-grown seedlings of the *oschlh* mutant when compared with wild-type seedlings (Goh et al. 2007). However, the *oschlh* mutant showed a light-dependent decrease in CO_2 uptake and O_2 evolution in intact leaves in response to light intensity (Goh et al. 2004). Therefore, this chlorina-type plant is of great interest to investigate the respiratory activity of mitochondria, particularly with respect to the state of illumination, as the photosynthetic electron transport rates do not occur in light conditions.

In this study, we examined mitochondrial activity in chlorophyll-deficient rice mutant seedlings in order to elucidate the regulatory mechanisms that underlie the inhibition of mitochondrial electron transport in the light. We found that high H_2O_2 levels may be involved in the light-mediated inhibition of mitochondrial activity in this mutant.

Materials and methods

Plant materials and growth conditions

Rice (*Oryza sativa* var. *japonica* cv. Dongjin) seedlings were grown and mutants were screened on media

containing 0.5× Murashige and Skoog basal salts with 0.2% (w/v) phytagel for 8–10 days, unless otherwise stated. Plants were cultured in a temperature-controlled growth chamber (28 ± 1°C) using a 16-h light/8-h dark regime with an intensity of 100 μmol m^{-2} s^{-1}, unless otherwise specified.

Measurement of mesophyll cell membrane potential (V_m)

Changes in membrane potential of mesophyll cells were measured using the method of Kari et al. (2003). Briefly, the secondary leaf was cut and floated on a solution (pH 6.0) of 10 mM KCl and 1 mM $CaCl_2$ overnight in the dark. Leaf strips were then secured to a Plexiglas strip using Terostat (Teroson Werke, Heidelberg, Germany), placed into a perfusion chamber holding a reference electrode and mounted onto a microscope stage. The leaf strip was continuously bathed with solution throughout the experiment with a gravity-fed perfusion system. A microelectrode was inserted under microscopic control using perpendicular green light of less than 1 μmol m^{-2} s^{-1}. Microelectrodes were pulled from borosilicate glass capillaries (Kwik-Fil; World Precision Instrument, Sarasota, FL, USA), backfilled with 0.3 M KCl and used if tip potentials were less than ±10 mV. The V_m of the second or third cell encountered upon insertion of the microelectrode was recorded continuously through light-on/light-off transitions. White light (150 μmol m^{-2} s^{-1}) was supplied directly to a leaf tissue with a projector lamp through a fiberglass light guide.

Total ATP assays

The levels of ATP were measured by the luciferin–luciferase method using the ENLITEN ATP bioluminescence detection kit (Promega, USA), according to the manufacturer's instructions (Goh et al. 2004). The luminescence was integrated for 5 s using a luminometer (Luminoskan Ascent 2.1 Int., Germany).

Determination of pyruvate and L-lactate concentrations and of the NADH/NAD^+ ratio

To determine the NADH/NAD^+ ratio, pyruvate and lactate concentrations were determined using a dual wavelength spectrophotometer (Sigma ZFP 22; Eppendorf, Germany), as described by Planchet et al. (2005). Frozen samples (1 g of fresh weight) were extracted using 2 ml of 7.5% perchloric acid. The extract was centrifuged (12,000*g* for 10 min) and the supernatant was transferred to a new tube. Twenty microliters of 2 M Tris was added to each 1 ml of supernatant and the solution was neutralized to pH 7.0 with 5 M K_2CO_3. After centrifugation (12,000*g* for 10 min), the supernatant was collected and stored at −80°C until further use. Enzymatic determination of the L-lactate concentration was performed using the glutamate pyruvate transaminase (GPT)–lactate dehydrogenase (LDH) system. Fifty microliters of extract was added to 1 ml of buffer solution (100 mM CHES, pH 10) containing glutamate (10 μM), GPT (5 U), and NAD^+ (2 mM). The reaction was initiated by the addition of LDH (3 U). To determine the concentration of pyruvate, 50 μl of extract was added to 1 ml of buffer solution (100 mM Hepes, pH 7.6) containing NADH (0.5 mM) and LDH (0.15 U). After each reaction, an appropriate internal standard was added to the mixture for the determination of the concentration of these two metabolites.

Determination of internal NADH oxidation

Mitochondria were isolated from the leaves of 10-day-old plants, using a self-generating Percoll gradient in combination with a linear gradient of 0–10% (w/v) polyvinylpyrrolidone (PVP) K-30 in a single step, essentially as described by Day et al. (1985). Glycine (2 mM) was added to the gradient and wash media to maintain maximal glycine decarboxylase activity. The isolated mitochondria had a high degree of membrane intactness, as judged by the latency of cytochrome c in the presence or absence of 0.025% (w/v) Tritone X-100 (Møller et al. 1987). The percentage latency of both enzymes was calculated by the previous methods (Møller et al. 1987; Rasmusson and Møller 1991). For determination of internal NADH oxidation, mitochondria were osmotically burst in 1 mM MOPS pH 7.2, 0.1 mM EGTA for 8 min at room temperature in order to permeabilize the inner membrane. The suspension was then supplemented to make up assay medium [10 mM MOPS pH 7.2, 2.5 mM $MgCl_2$, 0.1 mM EGTA, 0.4 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone], and the reaction was started by the addition of NADH. The oxidation of NADH (0.1 mM) was measured on intact mitochondria at 340 nm in assay medium. The internal rotenone-insensitive NADH oxidation was assayed in the presence of 15 μM rotenone. Rotenone-sensitive activity was determined as the difference with and without rotenone. This osmotic treatment disrupted approximately 80% of the inner mitochondrial membranes, as determined by mitochondrial matrix enzyme malate dehydrohenase latency (Møller et al. 1987).

Determination of H_2O_2 content

Whole leaf H_2O_2 was extracted according to the method of Park et al. (2004). The levels of H_2O_2 were determined using a colorimetric assay (Sigma, Product Number CS0270, USA). Absorbance was recorded at 550 nm.

Isolation of mesophyll protoplasts

Mesophyll protoplasts were enzymatically isolated from the leaves of 10-day-old plants, according to the procedure as described previously (Goh et al. 2004). The isolated protoplasts were kept in a solution of 0.6 M sorbitol and 1 mM $CaCl_2$ on ice until use.

Catalase activity assay

Catalase activity was assayed by monitoring the decrease in absorbance at 240 nm caused by the decomposition of H_2O_2 (Park et al. 2004). Protein concentration was determined using the Bradford assay (Bradford 1976).

RNA isolation, PCR conditions, and quantification of PCR products

Total RNA was extracted from 8-day-old plants using the TRIzol kit, according to the manufacturer's instructions (Invitrogen, Valencia, USA). The optimum number of cycles for RT-PCR was established for each primer set and serial dilutions were used to ensure linear amplification. The intensity of each band was quantified using the NIH Image J software, version 1.37 (NIH, Bethesda, Maryland, USA: http://rsb.info.nih.gov/nih-image/) and was normalized to that of *OsACTIN1*. PCR with *Taq* DNA polymerase (Life Technologies) was carried out basically for 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min, and finally 72°C for 15 min. For the specific primers, the optimal cycling parameters were 24 cycles for *OsACTIN1* (LOC_Os03g50890; forward primer (F), 5′-ACGGCGAT AACAGCTCCTCTT-3′, reverse primer (R), 5′-CCTCT TCCAGCCTTCCTTCA T-3′); 28 cycles for *OsMT2b* (accession no. NE7013; F, 5′-CAATTCTTGAGCTCAAT CACC-3′; R, 5′-ACACACGCACACACTGACAAC-3′), *OsTRX-h* (accession no. D21836; F, 5′-CTGCCACAAC AAGGACGAGTTCGAC-3′; R, 5′-CGATTTCGCATGAT ATTCGAGGACA-3′), *OsGRX* (accession no. X77150; F, 5′-CGTCTACAGCAAGTCTTACTGTCCT-3′; R, 5′-CAC ACGATACTAGACGGCAACAAGT-3′), *OsAPX1* (accession no. D45423; F, 5′-ATCAAGGAGGAGATACCCAC CATCT-3′; R, 5′-CTCACAGTGGTAGTCTGCTGGTTC A-3′), and *OsFeSOD* (F, 5′-GTGGATAGCTTGAATAAG CAGCTTG-3′; R, 5′-AAGCAGATTTCCACTATTTCAC CGT-3′); and 32 cycles for *OsAPX2* (accession no. AB53297; F, 5′-GTGGCACTCTGCTGGCACCTTCGAT-3′; R, 5′-CACCAGTGGACGGAAGGCTGG GTC A-3′), *OsRAC1* (accession no. NE7013; F, 5′-AGATAGGGCCTA TCTTGCTGATCA TC-3′; R, 5′-CTAGAGTTTCCTAGC TGCAAGC-3′), and *OsAOXc* (F, 5′-CTGAAGAAATC TTACGGCGG-3′; R, 5′-CCAAACAGATAACAGGAC GC-3′) (Saika et al. 2002). For DNA gel blot analysis, 20 μL of the reaction mixture was separated on a 1.0% agarose gel. The PCR products were quantified using EDAS 120 system (Eastman Kodak, Rochester, NY, USA). OsACTIN1 was used as an internal control to normalize all data.

Statistical analysis

All data obtained were subjected to a one-way analysis of variance (ANOVA), and the mean differences were compared by lowest standard deviations test. Comparisons with $P < 0.05$ were considered significantly different.

Results

Characterization of knock-out mutant seedlings of rice *OsCHLH* gene

The growth of wild-type seedlings was significantly greater than that of *oschlh* mutant seedlings (Fig. 1a). The fresh weight of 8-day-old seedlings was 0.193 ± 0.006 and 0.075 ± 0.003 g for wild-type and *oschlh* mutant plants, respectively, in the absence of sucrose (Fig. 1b). The addition of 3% sucrose, which stimulates lateral root formation and the maintenance of root-to-shoot ratios in plants (Macgregor et al. 2008), led to a greater enhancement of the growth rates of the *oschlh* mutant (77.7%) when compared with the wild-type (42.3%) seedlings. On the other hand, white light delivered to an intact wild-type rice leaf induced immediate depolarization of the membrane potential (V_m), followed by rapid recovery (Fig. 2a). Specifically, initial depolarization/repolarization was followed by slow hyperpolarization of V_m. Upon switching the light off, we observed depolarization after a transient hyperpolarization. A leaf of the albino *oschlh* mutant

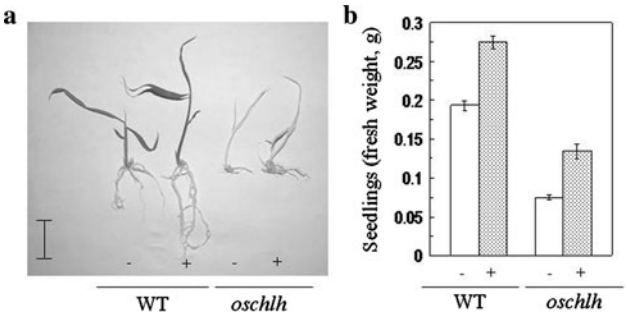

Fig. 1 Growth phenotype of the *oschlh* mutant seedlings. **a** Whole 8-day-old seedlings of wild-type and *oschlh* mutant plants in the presence or absence of 3% sucrose in 100 μmol m^{-2} s^{-1} light. *Bar* 1.4 cm. **b** Growth of *oschlh* mutant seedlings. The weight of fresh seedlings was measured from (**a**). Values are expressed as mean ± SE ($n = 11$ for wild-type and $n = 8$ for *oschlh* plants, $P < 0.05$)

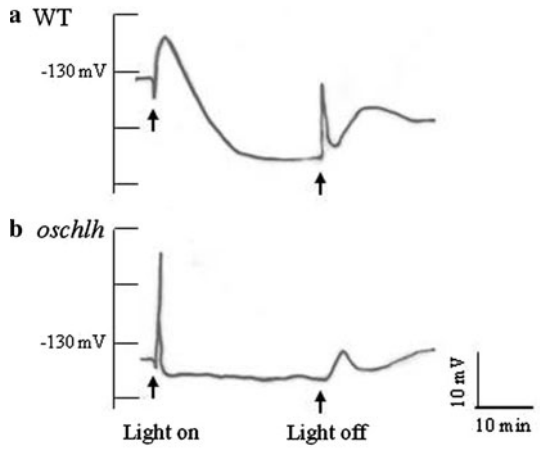

Fig. 2 Light-induced changes of membrane potential (E_m) in the *oschlh* mutant mesophyll cell. **a** WT and **b** *oschlh* mutant seedlings. The leaf strips were submerged and perfused in a flow-through chamber, and a conventional microelectrode was inserted into the first or second layer of mesophyll. After reading a steady value in a mesophyll cell for more than 15 min in the dark, the leaf strip was illuminated. The experimental conditions are described in "Materials and methods"

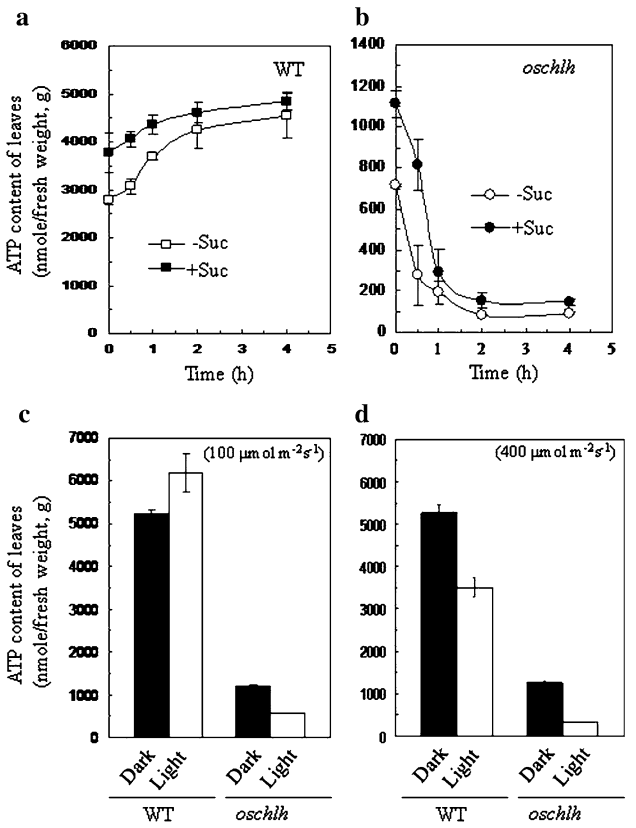

Fig. 3 ATP content of *oschlh* mutant seedlings. **a** WT and **b** *oschlh* Time course of ATP production in the absence or presence of 3% sucrose in the growth medium. **c**,**d** Inhibition of ATP production by light in intact seedlings of wild-type and *oschlh* mutant. Leaves were harvested 2 h after the application of 100 μmol m^{-2} s^{-1} (**a**–**c**) and 400 μmol m^{-2} s^{-1} (**d**) light following an 8-h dark cycle. Other experimental conditions are as described in Fig. 1. Data are expressed as the mean ± SE ($n = 3$)

displayed no electrical responses to light (Fig. 2b). However, changes of V_m in both wild-type and *oschlh* mutant were observed, although the magnitude of the response was different when the light was off. We contend therefore that dark responsive electric currents in theses mutant plants are due to mitochondrial respiration, indicating that the maintenance of growth rate is associated with a high efficiency of respiratory ATP production in dark condition.

ATP content of *oschlh* mutant leaves in dark and light conditions

We examined the levels of ATP in *oschlh* mutant leaves after an 8-h dark adaptation. Interestingly, a large drop in the ATP concentration was observed in the *oschlh* mutant 1 h after initiating light treatment (Fig. 3b), whereas a gradual increase in ATP levels was observed in wild-type plants over time in 100 μmol m^{-2} s^{-1} light (Fig. 3a). Moreover, the addition of sucrose induced a significant increase in ATP levels in wild-type and chlorina mutants in the dark, indicative of higher mitochondrial respiration. In light with an intensity of 100 μmol m^{-2} s^{-1}, the ATP content increased by approximately 18% in the wild-type plants, but decreased by about 52% in the *oschlh* mutant (Fig. 3c). Under a higher light intensity of 400 μmol m^{-2} s^{-1}, the ATP content decreased in wild-type and *oschlh* mutants, 2 h after the initiation of light treatment, resulting in 33.5 and 74.3% decreases, respectively (Fig. 3d), and suggesting an inhibition of mitochondrial respiration. In wild-type plants, this decrease could result from photoinhibition of photosynthetic ATP synthesis due to the actinic light treatment for 2 h at 400 μmol m^{-2} s^{-1} or it could be partially due to the photorespiratory metabolic consumption. In fact, the photosynthetic electron transport rates decreased in wild-type during the shift to higher light intensity (400 μmol m^{-2} s^{-1}) from 55 μmol m^{-2} s^{-1} of actinic light (data not shown). These data therefore indicate that light influences the ATP production in *oschlh* mutant plants, suggesting that mitochondrial electron transport is inhibited in illuminated mutant leaves.

Determination of the cytosolic NADH/NAD^+ ratio in *oschlh* mutant leaves in dark and light conditions

To determine whether cytosolic nicotinamide adenine dinucleotide (NADH) is also regulated by light in the *oschlh* mutant leaves, we attempted to estimate the cytosolic NADH/NAD^+ ratio by measuring the lactate/pyruvate ratio in the leaves of wild-type and *oschlh* mutants; this ratio is coupled to the cytosolic NADH/NAD^+ ratio via LDH

Table 1 Pyruvate and L-lactate production and cytosolic NADH/NAD^+ ratio in leaves of WT and *oschlh* mutant seedlings

	Pyruvate (nmol g^{-1} FW[a])	L-lactate (nmol g^{-1} FW[a])	NADH/NAD^+ ratio ($\times 10^{-4}$)
WT			
Dark	236.1 ± 28.9	528.9 ± 57.4	0.68 ± 0.16
Light	221.8 ± 31.4	969.4 ± 202.4	1.33 ± 0.24
OsCHLH mutants			
oschlh-1			
Dark	145.3 ± 19.3	339.4 ± 53.3	0.71 ± 0.11
Light	33.2 ± 13.5	33.9 ± 10.7	0.31 ± 0.06
oschlh-2			
Dark	150.6 ± 27.1	341.9 ± 41.8	0.69 ± 0.13
Light	48.5 ± 16.9	41.5 ± 14.8	0.26 ± 0.09

Ten-day-old seedlings were placed for 2 h in light conditions (100 μmol m^{-2} s^{-1}), 8 h after dark incubation. The leaf materials were harvested from plants incubated in water for an additional 20 min, in dark or light (100 μmol m^{-2} s^{-1}) conditions. Data represent the mean ($n = 6$ for WT and $n = 3$ for *oschlh* mutants) ±SE. The *oschlh-1* and *oschlh-2* indicates homozygous rice plants with either a T-DNA or *Tos17*-inserted allele of *OsCHLH,* respectively. *Tos17* mutant (line name ND9060) means a chlorina-type plant that carry the *Tos17* insertion in the *OsCHLH* gene (Jung et al. 2003)

[a] *FW* Fresh weight

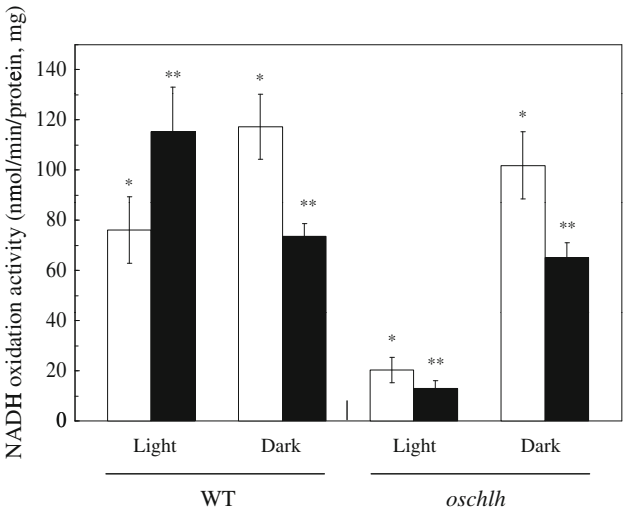

Fig. 4 NADH oxidation activities of mitochondria purified from leaves of *oschlh* mutant seedlings. Internal rotenone-sensitive NADH oxidation (*white boxes*). Internal rotenone-insensitive NADH oxidation (*black boxes*). *Asterisks above* the *columns* indicate values that are significantly different from control values ($P < 0.05$). *Data* represent the mean ± SE ($n = 8$ for wild-type and $n = 6$ for *oschlh* plants). The experimental conditions are described in "Materials and methods"

(Table 1). In illuminated leaves, the cytosolic NADH/NAD^+ ratio was 1.33×10^{-4}; this ratio was higher than that observed in leaves kept in the dark (0.68×10^{-4}). As expected, the ratio was significantly decreased in *oschlh* mutants, which further indicates that light inhibits the mitochondrial metabolism in *oschlh* mutant leaves.

On the other hand, induction of internal type II NADH dehydrogenase genes suggests that alternative respiratory chain may be specifically activated in the light (Amirsadeghi et al. 2007). It was proposed that this alternative respiratory pathway might be required to accommodate the increased levels of matrix NADH generated by the glycine oxidation step of photorespiration (Svensson and rasmusson 2001). We compared the content of matrix NADH in the *oschlh* mutant leaves in dark and 100 μmol m^{-2} s^{-1} light conditions (Fig. 4). The internal rotenone-sensitive NADH oxidation (via complex I) in isolated mitochondria (wild-type) was approximately 33% lower after plants were exposed to light for 2 h. In the *oschlh* mutant leaves, NADH oxidation via complex I decreased by approximately 80%. The internal rotenone-insensitive NADH oxidation activity was 61% higher in wild-type after plants were exposed to light for 2 h, whereas it was 21% lower in the *oschlh* mutant leaves. Our results indicate that NADH oxidation by the main respiratory chain (complex I) is significantly inhibited in illuminated *oschlh* mutant leaves.

H_2O_2 content in leaves of *oschlh* mutant seedlings

We hypothesized that the inhibition of mitochondrial activity by light results in elevated levels of reactive oxygen species (ROS), which include H_2O_2, in *oschlh* mutant leaves in the light. To examine the production of ROS in *oschlh* and wild-type plants in vivo, we examined the leaf H_2O_2 content over the course of a diurnal cycle, as the contribution of leaf mitochondria to whole cell H_2O_2 production varies as a function of light (Fig. 5a). In both genotypes, global leaf H_2O_2 increased significantly during the 16-h light period, but decreased over the 8-h dark period. At all times, however, the H_2O_2 content was higher in *oschlh* mutant plants than in wild-type plants, with this difference being most significant in the light. Our findings therefore imply that internal H_2O_2 levels may play a significant role in mitochondrial activity in *oschlh* mutant leaves.

Catalases (CATs) are the major H_2O_2 scavengers and remove the bulk of cellular H_2O_2. The alteration of the levels of expression of these enzymes allows the in planta modulation of H_2O_2 concentration (Vandenabeele et al. 2004). We examined catalase abundance in *oschlh* and wild-type plant leaves during the diurnal cycle (Fig. 5b). The catalase activity patterns were similar in both genotypes and were enhanced by light in both cases. These results indicate that catalase operates normally but is not able to effectively reduce H_2O_2 levels in the mutant cells, which suggests that alternative H_2O_2-scavenging enzymes are necessary to regulate H_2O_2 levels in the *oschlh* mutant.

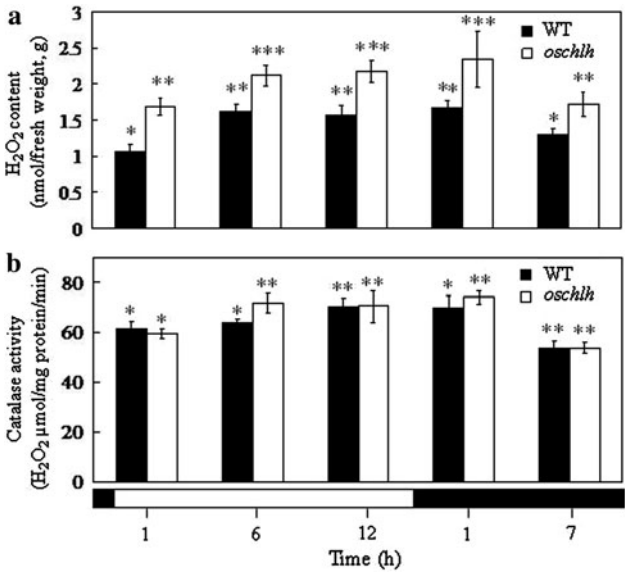

Fig. 5 Diurnal changes in H_2O_2 content (**a**) and associated catalase activity (**b**) in *oschlh* mutant leaves. Plants were grown in the presence of 3% sucrose for 10 days using a 16-h light/8-h dark cycle with 100 μmol m^{-2} s^{-1} light and were then exposed to a light intensity of 100 μmol m^{-2} s^{-1}. Leaf samples were harvested at the indicated times during a diurnal cycle. The *white* and *black horizontal bars* indicate the light and dark periods, respectively. The *open* and *closed columns* represent *oschlh* mutant and wild-type plants, respectively. *Values* represent the mean ± SE from three independent experiments. *Asterisks above* the *columns* indicate values that are significantly different from the wild-type values after 2-h treatment in the dark in each box ($P < 0.05$)

Global leaf H_2O_2 concentrations were higher in the *oschlh* mutant in light condition, showing a 2- to 3-fold increase over time (Fig. 6a). The content of H_2O_2 in the leaves of both genotypes fluctuated over time, suggesting the activity of enzymes responsible for antioxidant defense. Furthermore, H_2O_2 was produced in high amounts in *oschlh* mutants in the dark. To establish the further role of H_2O_2 in mesophyll protoplasts of the chlorina type mutants, we examined the effects of ascorbic acid (AsA), a natural antioxidant, and CAT on the ATP content in light condition over time. As shown in Fig. 3b, ATP content of chlorina-type mutants was decreased in light condition over time (Fig. 6b). However, pre-incubating the protoplasts with AsA (Fig. 6c) or CAT (Fig. 6d) maintained the ATP content over time. Our findings therefore suggest that internal H_2O_2 levels inhibit the mitochondrial activity in *oschlh* mutant leaves.

Analysis of H_2O_2-scavenging enzymes in leaves of *oschlh* mutant seedlings

From a typical microarray experiment, we identified significant changes in the expression levels of genes involved in oxygen and ROS metabolism (3.4 and 18.5% for genes up- and down-regulated, respectively) and in the response to oxidative stress (3.7 and 20.4% for genes up- and down-regulated, respectively) (data not shown). We therefore performed a PCR experiment to confirm the expression of H_2O_2-scavenging enzymes (Fig. 7a). Ascorbate peroxidase (APX) is a class I peroxidase that is localized in the cytosol (Teixeira et al. 2004). *OsAPX1* was highly expressed in plants in the dark and its expression was further induced by light in wild-type plants, but not in chlorina-type plants (*oschlh-1* and *oschlh-2*) (Fig. 7b-a). The expression of *OsAPX2* (which is a class II peroxidase) was induced by high levels of light in the two chlorina-type plants (Fig. 7b-b). Light induced an increase in the cytosolic expression of thioredoxin (*OsTRX-h*) (Santos and Rey 2006); this gene was also up-regulated in the dark in the chlorina mutants when compared with wild-type plants (Fig. 7b-c). Interestingly, the promoters of *OsTRX-h*, glutaredoxin (*OsGRX*) (Minakuchi et al. 1994) (Fig. 7b-d), and cytosolic superoxide dismutase (*OsFeSOD*) (Feng et al. 2006) (Fig. 7b-f) contain a novel *cis*-element that regulates expression in response to methyl viologen, but does not respond to H_2O_2 (Santos and Rey 2006). The iron-associated superoxide dismutase gene *OsFeSOD* produced two protein isoforms of different sizes (Fig. 7a). In contrast with a previous report that indicated that the accumulation of both isoforms increases in response to light (Feng et al. 2006), our results showed that *OsFeSODb* (708 bp) appears to act antagonistically to *OsFeSODa* (859 bp) in the light. Metallothioneins are small, ubiquitous Cys-rich proteins involved in H_2O_2 scavenging and metal homeostasis (Wong et al. 2004). The expression of the metallothionein gene *OsMT2b* was significantly induced in all genotypes studied, in both dark and light conditions (Fig. 7b-e). In the genotypes examined here, *OsRAC1* functioned in response to light as a suppressor of H_2O_2 scavengers, assuming that its enzyme activity is 27% lower in mutants in the light. Taken together, these results suggest that H_2O_2-scavenging enzymes would function normally except for *OsAPX1* and *OsRAC1* genes in the mutant. The expression of the alternative oxidase c (*OsAOXc*) gene, which encodes a mitochondrial antioxidant, was markedly induced in response to light in wild-type but not in mutant plants (Fig. 7b-i). The expression levels of this gene in the dark are negligible. Hence, low expression of light-inducible *OsAOXc* transcripts may also be responsible for the high levels of H_2O_2 detected in the illuminated leaves of the mutant. For this reason, it is possible that the inhibition of mitochondrial activity by light in *oschlh* mutant plants results in the accumulation of high levels of H_2O_2.

Discussion

The results described here revealed the mechanisms underlying the regulation of mitochondrial respiration by light in

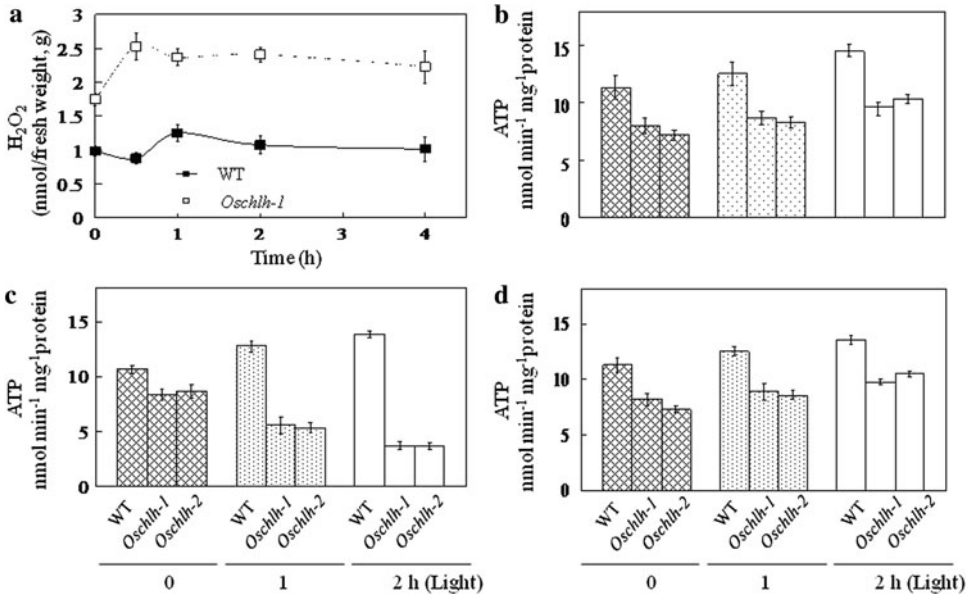

Fig. 6 H_2O_2 content (**a**) and effects of antioxidants on ATP content (**b–d**) in mesophyll protoplasts of wild-type and chlorina mutants. **a** H_2O_2 content in leaves of intact seedlings. Plants were grown with 3% sucrose in the medium for 10 days using a 16-h light/8-h dark cycle with 100 μmol m^{-2} s^{-1} light and were then exposed to light intensities of 100 μmol m^{-2} s^{-1}. Leaf samples were harvested at the indicated times from the seedlings after 8-h dark adaptation. **b–d** ATP content in mesophyll protoplasts. The protoplasts (40 μg protein/ml) incubated in incubation medium (0.6 M sorbitol, 1 mM $CaCl_2$, 10 mM KCl, 10 mM Mes-NaOH, pH 6.2) without (**b**) or with AsA at 1 mM (**c**) or with CAT at 100 Uml^{-1} (**d**) for 30 min in the dark and then exposed to light intensities of 100 μmol $m^{-2}s^{-1}$. Protoplast samples were harvested at the indicated times after light treatment at RT. Values are the mean ± SE from three independent experiments

chlorophyll-deficient rice mutant plants. The growth rate of the *OsCHLH* knockout mutant was considerably enhanced by incubation in sucrose (Fig. 1b). As the addition of sucrose to the growth medium increases the rate of mitochondrial respiration (Journet et al. 1986), the growth of the mutant seems to be closely associated with the respiratory metabolism. These results imply that mitochondrial function supports the cellular energy requirements of the *oschlh* mutant. On the other hand, aleaf of the albino *oschlh* mutant had no electrical responses to light, but did when the light was off (Fig. 2b). This light response indicates that ATP produced photosynthetically affects membrane potential (Spalding and Goldsmith 1993). However, changes of V_m in both wild-type and *oschlh* mutant were observed, although the magnitude of the response was different when the light was off. These dark-induced changes to the membrane potential (V_m) were not inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a photosynthetic electron transport inhibitor (Stahlberg et al. 2000). We contend therefore that dark responsive electric currents in theses mutant plants are due to mitochondrial respiration, indicating that the maintenance of growth rate is associated with a high efficiency of respiratory ATP production. These results imply that they were supported by the mitochondrial functions for *oschlh* mutants' cellular energy requirements.

The main function of the mitochondria is the generation of ATP by oxidative phosphorylation (Fernie et al. 2004). In the current study, the mitochondrial activity displayed comparative levels of ATP in the *oschlh* plant leaves was inhibited by light (Fig. 3b). The ATP concentration in *oschlh* mutants were largely affected by light treatment, which implies that the respiratory metabolism is regulated by light. Some studies on leaf respiration have reported that the rate of mitochondrial respiration in the light is less than that in darkness (Brooks and Farquhar 1985; Krömer 1995; Atkin et al. 2002), with the degree of inhibition ranging from 16 to 77%. Under illumination, mitochondrial respiration may be down-regulated because photosynthesis supplies sufficient amounts of ATP and NADPH or the enzymes of the TCA are potentially inactivated (Loreto et al. 2001; Pinelli and Loreto 2003). Some reports based on O_2 consumption suggest an inhibition of respiration in light conditions (Canvin et al. 1980; Bate et al. 1988), whereas others suggest that the rate of respiration is invariant, regardless of illumination conditions (Gerbaud and Andre 1980). Based on our current analysis, we hypothesized that this phenomenon is due to the inhibition of mitochondrial respiration by light in the mutant, as chlorina-type plant leaves have no PS II activity.

Mitochondria are the main target of oxidative damage in illuminated leaves (Foyer and Noctor 2000; Bartoli et al. 2004). Mitochondrial activity in the dark includes the oxidation of nicotinamide adenine dinucleotide phosphate (NAD(P)H) in the mitochondrial electron transport chain

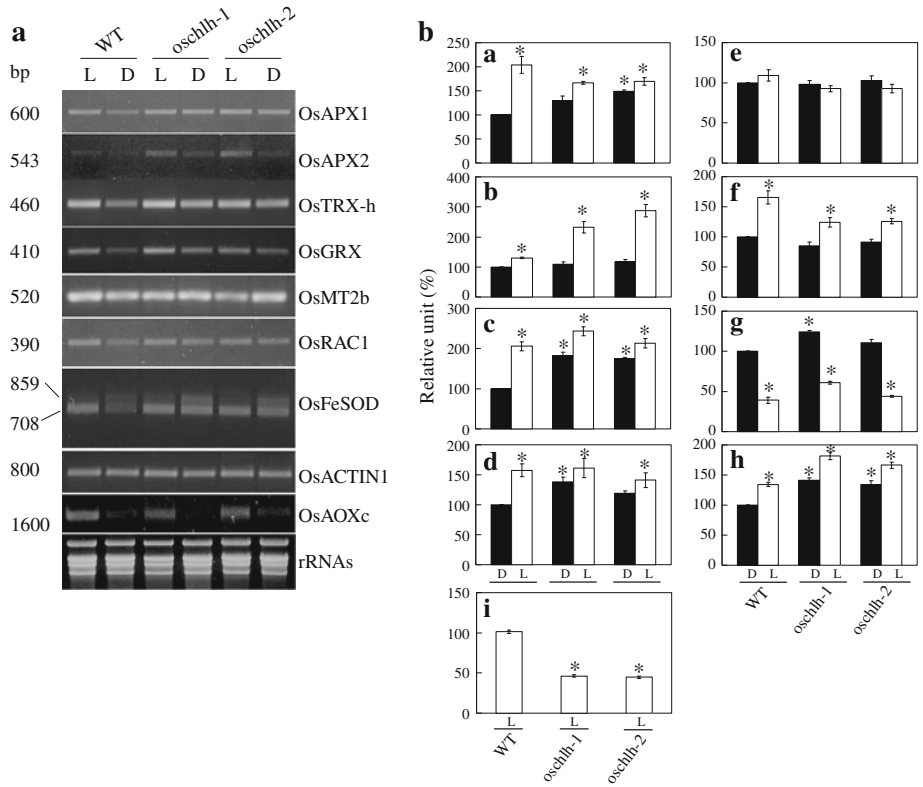

Fig. 7 RT-PCR analysis of the comparative expression levels of antioxidant genes in *oschlh* mutant leaves. After an 8-h dark adaptation period, leaves were exposed to light (100 µmol m^{-2} s^{-1}) for 2 h and were then harvested. Total RNA from each sample (20 µg) was subjected to RNA gel blot analysis on the same membrane; the bands shown are the products of RT-PCR. **a** Gene expression was assessed using probes for the indicated genes and using *OsACTIN1* as a loading control. Representative RNA gel blots are shown. **b** Quantitative analysis of expressed genes. Relative transcript levels were given by normalizing expression to the highest value for each gene. These experiments were repeated at least three times. *a OSAPX1*; *b OsAPX2*; *c OsTRX-h*; *d OsGRX*; *e OsMT2b*; *f OsRAC1*; *g OsFeSOD* (859 bp); *h OsFeSOD* (708 bp); *i OsAOXc*. *L* light, *D* dark. *Black boxes* dark treatment, *white boxes* light treatment. *Asterisks above* the *columns* indicate values that are significantly different from the wild-type values after 2-h treatment in the dark in each box ($P < 0.05$)

(Nunes-Nesi et al. 2007). A biochemical measurement revealed a drop in the cytosolic NADH/NAD^+ ratio in *oschlh* mutant leaves, which was verified at the level of the cellular NAD(H) pool in the cytosol (Table 1). On the other hand, we compared the level of matrix NADH generated by the glycine oxidation step of photorespiration in the *oschlh* mutant leaves in dark and 100 µmol m^{-2} s^{-1} light conditions (Fig. 4). Our results indicate that NADH oxidation by the main respiratory chain (complex I) is significantly inhibited in illuminated *oschlh* mutant leaves.

The bulk of cellular H_2O_2 seems to be formed during substrate oxidation at the level of the mitochondrial complex II (Braidot et al. 1999). Because the addition of mitochondrially-targeted antioxidants prevents the inhibition of respiration (Murphy 2004) and the contribution of leaf mitochondria to whole cell H_2O_2 production varies between light and dark conditions, we further compared leaf H_2O_2 production throughout a diurnal cycle (Fig. 5a). The H_2O_2 content was under light and diurnal regulation in plants of both genotypes. The *oschlh* mutants produced high levels of H_2O_2 in the light and these levels decreased in the dark, which seems to indicate that mitochondria are affected by light. In contrast, abscisic acid (ABA), which mediates H_2O_2 production in cells, is partly involved in this phenomenon because of the impairment of ABA signal transduction mechanisms in chlorina *Arabidopsis* (Shen et al. 2006). As O_2 consumption by mitochondria is significantly decreased after H_2O_2 treatment (Sweetlove et al. 2002), the activity of CAT may be induced to minimize oxidative damage in these experimental conditions (Figs. 5b and 6d). The treatment of protoplasts with ascorbic acid (AsA) also recovered ATP content that might be inhibited by internal H_2O_2 levels (Fig. 6c). Therefore, the generation of H_2O_2 in the cytosol results in the inhibition of mitochondrial activity in the mutant.

Changes in the respiratory metabolism in illuminated leaves may be indicative of changes in oxygen and ROS metabolism. The genes investigated, particularly *OsAPX1* and *OsRAC1*, which are expressed at comparatively low levels in *oschlh* mutant plants (Fig. 7b). *OsAOXc*

mitochondrial antioxidant transcripts were also poorly induced by light in the leaves of mutant plants and thus failed to lower H_2O_2 levels in this organelle. Increased levels of H_2O_2 result in ATP depletion in plant cells (Tiwari et al. 2002). Therefore, our results indicate that illumination leads to the accumulation of high H_2O_2 levels in cells and thereby inhibits mitochondrial electron transport in *oschlh* mutant plant leaves, which ultimately suppresses plant growth during the day. Furthermore, we consider this result to be a strong indication that the activity of mitochondria in the illuminated leaves of wild-type plants is closely related to the chloroplast activity.

On the other hand, complex I and III of the mitochondrial respiratory chain are the major site of intracellular ROS production. Mitochondria are the sensitive target of ROS attack and play a primary role in triggering and/or executing ROS-dependent apoptotic cell death in animals (Dat et al. 2003; Apel and Hirt 2004). In plants, mitochondria may also serve as first relay stations where the initial alteration in ROS homeostasis is triggered (Gao et al. 2008). Our results have shown that the contribution of leaf mitochondria to whole cell H_2O_2 production varied as a function of light. In the light regulation of plant respiratory activity, we have here questioned how mitochondria are governed by the light-associated processes. Many photoreceptors reside in the nucleus, cytosol or plasma membrane, with some photoreceptors communicating between the cellular compartments upon exposure to light (Chen et al. 2004), especially to blue light (Islam et al. 2009). In contrast, cryptochrome 3 is localized to chloroplasts and mitochondria (Kleine et al. 2003), indicating that these organelles may directly perceive light. We need to further investigate the photoreceptor-mediated control of mitochondria elsewhere.

Acknowledgments This work was supported by grants from the Korea Research Foundation (KRF-2004-015-C00532, to C.-H. Goh), the Crop Functional Genomics Center of the 21st Century Frontier Research Program (CG2111-2, to J.S. Jeon) and the World Class University program (R33-2008-000-10168-0, to J.S. Jeon) of the Korean Ministry of Education, Science and Technology. This work was also partly supported by Priority Research Centers Program through the National Research Foundation (NRF) funded by the Ministry of Education, Science and Technology (KRF-2009-0094060).

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