Mitochondria provide the main source of cytosolic ATP for activation of outward-rectifying K⁺ channels in mesophyll protoplast of chlorophyll-deficient mutant rice (OsCHLH) seedlings*

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SUMMARY

The role of mitochondria in providing intracellular ATP that controls the activity of plasma membrane outward-rectifying K+ channels was evaluated. The *OsCHLH* rice mutant, which lacks chlorophyll in the thylakoids, was isolated by T-DNA gene-trapping (Jung et al., 2003, *Plant Cell Physiol.*, 44, 463-472). The *OsCHLH* mutant is unable to fix CO2 and exhibits reduced growth. Wild type and mutant plants exhibit similar rates of respiratory O2 uptake in the dark while the rate of photosynthetic O2 evolution by the mutant was negligible during illumination. During dark respiration the wild type and mutant exhibited similar levels of cytoplasmic ATP. In the mutant oligomycin treatment (an inhibitor of mitochondrial F1F0-ATPase) drastically reduced ATP production. The fact that this was reversed by the addition of glucose suggested that the mutant produced ATP exclusively from mitochondria but not from chloroplasts. In whole-cell patch-clamp experiments, the activity of outward-rectifying K+ channels of rice mesophyll cells showed ATP-dependent currents, which were 1.5-fold greater in wild type than in mutant cells. Channels in both wild type and mutant cells were deactivated by the removal of cytosolic ATP, whereas in the presence of ATP the channels remained active. We conclude that mesophyll cells in the *OsCHLH* rice mutant derive ATP from mitochondrial respiration, and that this is critical for the normal function of plasma membrane outward-rectifying K+ channels.

**Keywords:** intracellular ATP, mesophyll cell, mitochondria, outward-rectifying K+ channel, oxygen uptake, photosynthesis
INTRODUCTION

Mitochondrial metabolism not only impacts on other cellular biosynthetic and catabolic processes, but also plays an important role in providing the overall cellular energy supply. Dark respiration and photosynthesis are metabolic pathways that produce redox equivalents and ATP to meet the energy requirements to support cell growth and maintenance (2). The central role of mitochondria in plants is reflected by the array of mitochondrial transporters and shuttles that transfer metabolites and reducing equivalents back and forth between mitochondria and the cytosol. Oxidative phosphorylation provides the cytosol with ATP, which is required for sucrose synthesis and other cytosolic processes. Studies with specific respiratory inhibitors have shown that oxidative phosphorylation occurs both in darkness and in light (2-4). However, very little is known about the regulation of mitochondrial respiration in photosynthetic mesophyll cells, and the role of mitochondrial ATP production as a source of cytosolic ATP in this process is not fully understood.

Many biochemical reactions in plant and mammalian cells depend on a tightly controlled ratio of ATP to ADP. In mammalian mitochondria, this ratio is preserved by regulatory mechanisms that couple the rate of cellular ATP consumption to the rate of ATP production by oxidative phosphorylation (5-8). Hence, efficient communication between cellular energy stores and membrane metabolic sensors is necessary for regulation of membrane excitability and associated functions. In most mammalian cells, there is a class of K⁺ channels whose activity is closely coupled to metabolism by ATP (9, 10). Recent studies have shown that oxidative phosphorylation, through its effect on ATP synthesis, plays an essential role in the regulation of ATP-sensitive K⁺ channels. (11-13). However, it has also been reported that glycolytic ATP preferentially controls these channels (11, 14, 15).
In plant mesophyll cells, outward-rectifying $K^+$ ($K_{\text{out}}$) channels activated by membrane depolarization serve as the primary pathway for $K^+$ efflux. As $K_{\text{out}}$ channel activity is completely abolished (within 15 min) in the absence of cytosolic ATP (16-19), it can be concluded that $K^+$ efflux in plant cells is ATP-dependent. However, the roles of photophosphorylation and oxidative phosphorylation in the regulation of $K_{\text{out}}$ channels remain controversial. Light activates the $H^+$ pump current (20-23), $K^+$ channels (16, 24-26), and regulates membrane potential (27-29) in photosynthetic cells, suggesting that these cells are sensitive to some aspects of metabolic energy within the cytosol. Light-induced activation of the $H^+$ pump in mesophyll cells may require both photosynthetic products (i.e., sugars) and a non-photosynthetic light effect (20, 28). On the other hand, the light-modulated transport system in mesophyll cells induced a rapid depolarization, which was immediately followed by repolarization and a subsequent slower hyperpolarization (16, 27-31). The hyperpolarization seemed to be regulated by changes in activity of the plasma membrane $H^+$-ATPase and voltage-dependent ion channels (31-33). Analysis of metabolites in the presence of oligomycin, which blocked the $F_1F_0$-ATPase, revealed a dramatic decrease in the mitochondrial and cytosolic ATP concentration (34), indicating that mitochondria-driven ATP production was decreased. While some reports suggest that the activation of $K_{\text{out}}$ channels and membrane potential in mesophyll cells is regulated by ATP derived from photosynthesis (16, 29), a more recent study shows that leaf mitochondria modulate whole cell redox homeostasis (35). These conflicting findings prompted us to evaluate the effects of ATP on the $K_{\text{out}}$ channels in mutant, chlorophyll-deficient rice cells.

To explore this in mesophyll cells, we exploited a chlorophyll-deficient rice mutant ($OsCHLH$), which lacks the largest subunit of Mg$^{2+}$-chelatase (1). This rice mutant is defective in photoperception, and was therefore useful for investigating the source of
cytosolic ATP that controls $K_{\text{out}}$ channels. Here we show that the activity of $K_{\text{out}}$ channel in chlorophyll-deficient mutant cells is controlled by a cytosolic pool of ATP that is predominantly derived from oxidative phosphorylation. Cytosolic ATP production was further enhanced by the addition of glucose. These results strongly suggest that there is metabolic cross-talk between mitochondria and $K_{\text{out}}$ channel activity in rice chlorophyll-deficient mesophyll cells.
MATERIALS AND METHODS

Plant material—Seedlings were grown and mutants screened on media containing 0.44% Murashige and Skoog basal salt, 3% sucrose, 0.2% phytagel, and 0.55 mM myo-inositol (Sigma) for 8 days unless otherwise stated. The plants (Oryza sativa cv. Japonica) were cultured in a temperature-controlled growth chamber at 28 ± 1°C under a 16-h day cycle, at a minimum photon flux density of 90 μmol m⁻² s⁻¹.

Chlorophyll fluorescence imaging—Leaf tissue was harvested from 8-day-old seedlings. Mesophyll protoplasts were prepared as previously described (36). The fluorescence signal was monitored using a fluorescent microscope (Axioplan 2, Zeiss, Jena, Germany). Images were processed using an automatic imaging system (FISH, Carl Zeiss).

Inverse PCR—Inverse PCR methodology was described previously (1). The PCR product was directly subjected to sequencing reactions using the primer 5′-GCAAGGATACAGTCTGTACCT-3′.

Measurements of net CO₂ uptake by leaf gas exchange—Rates of net CO₂ uptake by attached leaves were measured in an open gas exchange system (LCA2, ADC, Hoddesdon, UK) coupled to a mass spectrometer as described previously (37). Air entering the Parkinson leaf chamber was conditioned at 40% relative humidity, 370-380 μL L⁻¹ CO₂, at a flow rate of 200 mL min⁻¹. Leaf temperature in the chamber was maintained at 25°C with a temperature controller (Prime Bio Chemical Co. Seoul, Korea). Incident photon flux densities were supplied by a fiber optic illuminator (Schott, Mainz, Germany) from a halogen lamp (20W, Phillips, Eindhoven, Netherland) and measured with a silicon photodiode detector (MMS, Zeiss). Neutral density filters were used to vary light intensity.

Determination of net O₂ evolution and net O₂ uptake—Leaf discs were excised and
introduced into a leaf disc O₂ electrode chamber (Hansatech Instruments, King’s Lynn, Norfolk, UK) in a closed gas exchange system of air with 5% CO₂ at 26 ± 2°C as described previously (38). After calibration, the steady-state rate of O₂ consumption was monitored until a stable rate was reached. Leaf discs were then illuminated at an irradiance of 60 and 900 µmol m⁻² s⁻¹. Rates of net O₂ evolution and net O₂ uptake were determined as described by Walker (39). The actinic light was provided by a 150-W quartz-halogen slide-projectors fitted with heat filters through neutral density filters.

**Analysis of total cellular ATP levels**—The amount of ATP was measured by the luciferin-luciferase method (40) in intact seedlings (Fig. 4A) and isolated mesophyll protoplasts (Fig. 4B). The OsCHLH rice mutants were harvested about 2 h into the dark and light (90 µmol m⁻² s⁻¹) periods, respectively and stored in liquid nitrogen until analysis. The leaves were extracted and analyzed on the day they were sampled. The protoplasts were incubated in a temperature-controlled room (28 ± 1°C) for 40 min in the dark. The protoplasts (30 µg/mL) were incubated in incubation medium (0.6 M sorbitol, 1 mM CaCl₂, 10 mM KCl, 10 mM Mes-NaOH (pH 6.2)) with various combinations of substrate and inhibitors. ATP was extracted from 100 µl of cell suspension by adding 2.5% trichloroacetic acid using an ENLITEN ATP assay bioluminescence detection kit (Promega, Madison, WI, USA), according to the manufacturer’s recommended protocol. The extracted ATP solution was neutralized by 0.75 M Tris-acetate buffer (pH 7.75) and centrifuged for 10 min at 1,600 x g. The resulting supernatant was used in ATP determination assays. The reaction was initiated by adding 100 µl of the extract to 100 µl of the luciferin-luciferase luminous reagent (Promega). The luminescence was integrated for 5 s using a luminometer (Luminoskan Ascent 2.1 Int. Germany). The actual ATP levels were calculated from an ATP standard curve constructed using commercially supplied ATP.
Whole-Cell Patch-Clamp Recording—Before use in the patch-clamp experiments, mesophyll cell protoplasts were kept in a solution including 1 mM CaCl₂ adjusted with D-sorbitol to 600 mmol kg⁻¹. The dimensions of the protoplasts were fairly consistent, with diameters of 16.6 ± 0.43 µm for the wild type and 15.1 ± 0.35 µm for the mutant. Whole-cell recording was performed as described by Li and Assmann (27, 42). Briefly, the standard patch pipette solution contained (in mM) 100 K-glutamate, 2 EGTA, 2 MgCl₂, 2 KCl, and 10 Hepes with or without 2 MgATP, titrated to pH 7.2 with Tris. The bath solution consisted of 10 K-glutamate, 1 CaCl₂, 4 MgCl₂, and 10 HEPES titrated to pH 7.2 with Tris. Osmolalities were adjusted with D-sorbitol to 696 mmol kg⁻¹ for the pipette solution and 623 mmol kg⁻¹ for the bath solution. During whole-cell recordings, the membrane potential was held at −40 mV, except during voltage steps. Currents across the membrane were measured upon imposition of a series of voltage pulses from −160 to +100 mV with 20 mV increments, as described previously (42). Liquid junction potential was measured and corrected (43). Seal resistances were in the range of 1.5 to 4 GΩ. For whole-cell experiments, application of voltage programs and handling of the data were performed using a Digidata 1200 interface and patch-clamp software pClamp8.0 with Calmpex and Clampfit (Axon instruments). Whole-cell data were low-pass filtered with an eight-pole Bessel filter at a cut-off frequency of 2 kHz. Capacitance and pipette offset potentials were compensated during the patch-clamp experiments.

Data Analysis—Group data are expressed as mean ± SE. Comparisons among groups were made by ANOVA (F-test). Bonferroni-adjusted t-tests were used for multiple group comparisons and paired or unpaired t-tests were used, as appropriate, for single comparisons. Nonlinear least-square curve fitting was performed with CLAMPFIT in pCLAMP 8.0.
RESULTS

The OsCHLH mutation affects chlorophyll pigmentation of the rice seedlings—To study the mitochondrial contribution to the cytosolic ATP pool in photosynthetic cells, we used the chlorophyll-deficient OsCHLH rice mutant (Fig. 1A). We previously identified this mutant as a knockout of OsCHLH, the gene encoding Mg$^{2+}$-chelatase which is involved in chlorophyll biosynthesis (1). The OsCHLH mutant contains only 0.27% or less of the normal chlorophyll content (on a per leaf area basis) (1) and did not exhibit chlorophyll fluorescence in its intact leaves (Fig. 1A, panel c) or in mesophyll cells (Fig. 1C, panel d). This chlorina phenotype co-segregated with a T-DNA insertion in which the OsCHLH mutation was a single recessive mutation (1). The vector used for mutagenesis carried the promotorless β-gluçuroidase (GUS) reporter gene immediately adjacent to the 3‘ border of T-DNA. The T-DNA-tagged lines could be therefore verified by GUS assay (data not shown). This mutant carries a stable DNA mutation that affects chlorophyll synthesis in the thylakoid light-harvesting complexes (1, 44).

The growth of OsCHLH mutant seedlings was comparable to the wild type (Fig. 1A, panel a). The fresh weight of 8-day-old seedlings was 0.061 ± 0.011 and 0.031 ± 0.008 g for wild type and OsCHLH mutant plants, respectively. The addition of sucrose, which stimulates ATP generation by respiration (36), enhanced the growth in both wild type and mutant plants, showing 33.3% and 52.9% increases for the wild type and OsCHLH mutant plants, respectively (data not shown). Although the mutants did not live longer than 4-5 weeks, these data indicate that mitochondria can contribute to the OsCHLH mutants' cellular energy requirements.

Photosynthetic CO$_2$ uptake—The photosynthetic capacity of the OsCHLH mutant was
studied in intact plants by CO₂ gas exchange (Fig. 2A). Whereas in the wild type there was significant CO₂ uptake in the light, there was no CO₂ uptake in the OsCHLH mutant plants. The OsCHLH mutant plants also released more CO₂ than the wild type during dark respiration (Fig. 2B). Although it showed a substantial decrease, CO₂ release was observed in the OsCHLH mutant even under saturating light conditions. On the basis of chlorophyll concentration of this mutant, the decrease in CO₂ release appears to be due to the inhibition of mitochondrial respiration by light. This result implies that the OsCHLH mutant plants have no CO₂ fixation capacity.

Respiratory O₂ uptake and photosynthetic O₂ evolution—In light, transients in chlorophyll fluorescence and oxygen evolution reflect mechanisms regulating CO₂ assimilation (36, 45, 46). The transients might be related to regulatory mechanisms involving, particularly, the cytosolic [ATP]/[ADP] ratio which favors electron transport (26). We need therefore to investigate the activity of O₂-evolving photosystem II (PS II) in the mutant chloroplasts. In preliminary experiments, CP43 immunoreactivity was readily detected in isolated thylakoid membranes from wild type, but not in OsCHLH mutant seedlings (data not shown), suggesting that PS II activity is impaired in the OsCHLH mutant. We measured then oxygen evolution as a function of light intensity in leaf discs (Fig. 3). The voltage output from the oxygen sensor from a typical trial is shown in Fig. 3A. Each leaf was placed in darkness for 9 min, and then exposed to actinic light (L1 and L2) for 3 min. This was followed by another 6 min period of darkness. In wild type, the photosynthetic O₂ evolution increased from 0.71 to 3.98 µmol min⁻¹ g⁻¹ fresh weight, depending on the light intensity (Fig. 3B). There was however no photosynthetic O₂ evolution in the mutant. In addition, mitochondrial respiration in the mutant was regulated by light, in that the rates of oxygen uptake were decreased from 1.5 to 0.3 µmol min⁻¹ g⁻¹ fresh weight with light
treatment, suggesting that light inhibited mitochondrial respiration in the mutant (for review see ref. 2, 34). In the dark, there were no statistically significant differences in O₂ evolution between wild type and mutant leaves (t-test, α=0.042). Taken together, these data indicate that the OsCHLH mutant seedlings exhibited high levels of mitochondrial respiration similar to wild type, but completely lacked the capacity to perform photosynthesis.

**ATP content in wild type and OsCHLH mutant mesophyll cells**—Since total cellular ATP levels are directly associated with respiratory O₂ uptake and ATP synthesis in mitochondria in darkness (47), we next measured total cellular ATP levels. In dark treated mutant seedlings ATP level was 1.88 whereas in the light it was 0.97 µmol ATP g⁻¹ fresh weight (Fig. 4A). ATP production was significantly (51.4%) inhibited in the light. This result supported the close association between ATP synthesis and respiratory O₂ uptake as shown in Fig. 3B. Fig. 4B shows the ATP content in wild type and mutant mitochondria of mesophyll cell protoplasts in the dark. Cells were incubated in darkness for 40 min in the presence or absence of 5 mM glucose, which stimulates the generation of ATP by respiration, with various combinations of metabolic inhibitors. As expected, cytosolic ATP levels were quite similar in wild type and mutant cells under control conditions (approximately 10 nmol min⁻¹ mg⁻¹ protein). When the F₁F₀-ATPase was blocked by the respiratory inhibitor, oligomycin (2 µg ml⁻¹), ATP production was decreased by a similar level (74% and 77%), in wild type and mutant cells, respectively. The respiration activity indicates that the OsCHLH mutant cells have levels of mitochondrial oxidative phosphorylation similar to that of the wild type. ATP content was decreased by treatment with 10 µM dichlorophenyl-dimethylurea (DCMU) an inhibitor of photosynthetic electron transport. This is probably due to a secondary effect of DCMU on mitochondrial function (48). The combination of oligomycin and DCMU significantly inhibited ATP production in both mutant and wild type cells.
showing that oligomycin is effective in both types of cell. When glycolysis was blocked by the addition of 1 mM iodoacetic acid (I-Ac) to a substrate-free bath solution, cytosolic ATP content was decreased by approximately 20% in both mutant and wild type cells, indicating that glycolysis produced a relatively small amount of ATP. In the presence of glucose in the bath solution, ATP levels were increased by approximately 50% in both wild type and mutant cells. This effect of glucose was largely decreased when oligomycin was included with glucose in the bath solution (oligomycin decreased ATP production by approximately 66% in wild type and 71% in the mutant cells). I-Ac inhibited the production of ATP by approximately 25% in both cell types, relative to the effects of glucose alone. This implies that glycolysis in the mutant cells proceeds at similar rates to that found in the wild type. Taken together, these findings suggest that the cytosolic ATP pool was predominantly produced by mitochondrial oxidative phosphorylation in the mutant.

Voltage- and time-dependent ATP-sensitive outward-rectifying $K^+$ channels ($K_{out}$ channels)—To test the voltage- and time-dependence of membrane currents, a series of voltage pulses was applied to the plasma membrane in the whole-cell configuration. Typical whole-cell current time courses in the presence or absence of ATP are shown in Fig. 5. The whole-cell patch-clamp recordings showed that rice mesophyll cells lacked the inward-rectifying $K^+$ channel activity described in tobacco (49), oat (50), and Vicia mesophyll cells (51). These mesophyll cells display a similar pattern of $K^+$ currents as a yeast mutant (Trk1ΔTrk2Δ) (52). The following experiments demonstrated that ATP was required for the activation of $K_{out}$ channels of both wild type and mutant mesophyll cells, similar to what has been reported previously (17-19, 49-51). The addition of 2 mM ATP to the cytoplasmic side of protoplasts stimulated voltage-dependent $K_{out}$ channels in both wild type and mutant cells (Figs. 5B and 5C, left panels). In whole-cell measurements in the absence of ATP, 8-min run-
down effects of outward current were observed in both cell types (Figs. 5B and 5C, right panels). The effect of ATP was statistically significant at positive membrane potentials in both wild type and mutant cells (Fig. 6). In the absence of ATP, whole cell currents were run down in similar rates in both cells; they showed about 78-80% inhibition at +100 mV as compared in those of +ATP conditions in both cells, respectively. The run-down effect of K$_{\text{out}}$ channel activity could be prevented in both cell types by the addition of 2 mM ATP, although the magnitude of channel current was smaller in mutant cells. It is possible that the number of K$^+$ channels in the plasma membrane is greater in wild type cells than in the mutant. The modulation of channel density is regarded as an important mechanism for controlling the transport rate across the plasma membrane in an increasing number of systems (53). This might bring about reduced growth in the mutant seedlings compared to the wild type (Fig. 1A) and therefore result in the reduced size of protoplasts (compare wild type, 16.6 ± 0.43 µm with mutant 15.1 ± 0.35 µm) (Fig. 1C). This ATP dependency of the K$_{\text{out}}$ channel is similar to that observed in a variety of cell types and plant species (17-19, 54-58).

**Analysis of K$_{\text{out}}$ channels activated by membrane depolarization in OsCHLH rice mutant cells**—The activity of ATP-dependent K$^+$ efflux in plasma membranes was maintained in the presence of ATP in the cytosolic solution for 10 min in both wild type (Fig. 7A) and mutant cells (Fig. 7B). When cytosolic ATP was depleted using an ATP-free pipette solution, K$_{\text{out}}$ channel activities declined in a time-dependent manner. K$_{\text{out}}$ channel activity at 10 min was only 25% of initial levels and showed the potential of half-activation (V$_{1/2}$) at approximately 5.5 min in both wild type and mutant cells. In addition, oligomycin significantly reduced the activity of the K$_{\text{out}}$ channel in mutant cells (13.2% less than wild type) (data not shown). These results suggest that the cytosolic ATP originated from
mitochondria-activated ATP-dependent $K_{\text{out}}$ channels.
DISCUSSION

In animal cells there is good evidence that mitochondria are the predominant source of ATP (5-13). In plants, biomass production is ultimately determined by the ratio between photosynthesis and mitochondrial respiration (59). These metabolic pathways produce redox equivalents and ATP to meet the cellular energy requirements for growth and maintenance (2). However, in plants it remains unclear to what extent mitochondria contribute to cytosolic ATP levels.

In order to address this question, we investigated the activities of ATP-dependent outward-rectifying K⁺ channels (K<sub>out</sub> channels) in mesophyll cells from seedlings of the OsCHLH rice mutant. The chloroplasts of the OsCHLH rice mutant lack the largest subunit of Mg<sup>2+</sup>-chelatase, which is a key enzyme in the chlorophyll branch of the tetrapyrrole biosynthetic pathway (1). The mutant plants exhibited no chlorophyll fluorescence in intact leaves or in mesophyll cells, indicating that OsCHLH rice mutant plants lacked photosynthetic capacity (Fig. 1). This was further supported by measurements of photosynthetic activity (CO₂ gas exchange (Fig. 2) and O₂ evolution (Fig. 3)). Light-induced growth of the mutant was approximately 50% of that observed in wild type plants (data not shown). Despite impaired photosynthesis and slower growth, the light-grown OsCHLH rice mutant seedlings were well developed with fully expanded leaves, indicating that growth can occur in the absence of photosynthesis (20, 30). It is likely that the cytochrome and its branched alternative respiratory pathways in mitochondria coordinately balance ubiquinone pool oxidation/reduction and carbon skeleton turnover in response to cytosolic ATP levels (60). Sucrose enhanced the growth of the wild type and mutant seedlings, although the growth of the mutant in the presence of sucrose was still only 58.2% of that of wild type
Sucrose-induced increases in growth were most likely due to enhanced metabolic pathways that produced redox equivalents and ATP via the activation of mitochondria functions.

Mitochondrial functions of the OsCHLH mutant were similar to those of the wild type, as determined by measuring O₂ concentrations in intact leaves (Fig. 3B). No statistically significant differences in O₂ consumption were observed in dark respiration. On the other hand, the OsCHLH mutant plants released higher levels of CO₂ during dark respiration, indicating that mitochondrial activity of the OsCHLH mutant were 62.5% higher than that of wild type (Fig. 2B). This quantitative difference between O₂ uptake and CO₂ release of the OsCHLH mutant plants in the dark seems to depend on the plant age and/or the experimental conditions. Photosynthetic O₂ evolution was modest (10-15% when it was normalized at 900 µmol m⁻² s⁻¹) in wild type seedlings at a low irradiance of 60 µmol m⁻² s⁻¹ and robust (~4 µmol min⁻¹ g⁻¹ fresh weight) at a saturating light pulse of 900 µmol m⁻² s⁻¹. In contrast, no photosynthetic O₂ evolution was observed in the OsCHLH mutant seedlings, even under saturating light conditions. We believe that the observation of O₂ consumption in the dark with the same blank (control) is artifactual and is caused by the 3 minute light pulse. Even though we used heat filtration this is because the rate of oxygen diffusion to the detector is temperature limited in a closed system. The absence of O₂ evolution in the OsCHLH mutant plants is attributable to the lack of chlorophyll (Fig. 1) and CP43 immunoreactivity (data not shown) in the thylakoid membranes. The CP43 subunit of photosystem II (PS II) functions in light harvesting, and the absence of CP43 expression is consistent with the lack of photosynthetic activity in these plants.

Interestingly, the OsCHLH mutant still exhibited mitochondrial-mediated CO₂ release (Fig. 2B) as well as O₂ consumption (Fig. 3B) in the light which progressively decreased.
These results are consistent with light-induced inhibition of mitochondrial respiration, which has been reviewed previously (2). Light regulation of mitochondrial ATP production was also observed in the mutant seedlings as evidenced by a dramatic reduction of ATP levels (Fig. 4A) as O2 consumption is closely coupled to mitochondrial ATP synthesis (2, 48). However, the decrease of ATP levels in the light is not due to CO2 assimilation in the OsCHLH mutant as in the mutant light did not induce CO2 uptake as it did in the wild type (compare traces in Fig. 2A). It is important to note that the decrease in O2 consumption that we measured in the mutant resulted from light-induced inhibition of mitochondrial respiration. The rates of net O2 uptake, which are nearly identical to net CO2 release, are comprised of O2 evolution in the Hill reaction of PS II, O2 consumption by the Mehler reaction and the glycolytic pathway, and O2 consumption in mitochondrial respiration in the light, whereas photorespiratory CO2 evolution is accompanied by CO2 uptake in the Calvin cycle and CO2 release from mitochondrial respiration (61). Based on these facts and the photosynthetic properties of the OsCHLH mutant it is possible that respiration in the light may depend on the amount of photosynthetic primary products (61, 62), which the mutants are expected to lack because there is no photosynthetic CO2 fixation. This could explain, as it does in drought stressed plants (61) the substantial decrease of mitochondrial O2 uptake and CO2 release in the OsCHLH mutant in the light. This has been well established in both animal and plant cells, emphasizing that inhibition of ATP synthesis by light occurs in mitochondria (63-65). However, light markedly stimulates ATP synthesis via oxidative phosphorylation and O2 uptake in photosynthetic cells (4, 66, 67), tobacco (68), wheat (64, 69), and barley mitochondria (70). A large body of evidence suggests that mitochondria are involved in photosynthetic metabolism, particularly with respect to ATP production (2). The level of ATP increased by 11-26% after exposure to light (71). In this case, the cytochrome
and alternative pathways of mitochondrial electron transport might be involved in such reactions. How are the contradictory findings in the literature concerning mitochondrial respiration in light to be explained? Further studies in the regulation of mitochondrial activity during photosynthesis are needed. Our data revealed that the OsCHLH rice mutant plants have similar levels of mitochondrial activity as the wild type but lack photosynthetic capacity. The mutant plants provide ATP to meet the cellular energy requirements for growth and maintenance solely from mitochondrial functions.

During oxidation of the redox equivalents in the mitochondrial respiratory electron transport chain, O₂ is consumed and a proton gradient is formed across the inner mitochondrial membrane. This proton gradient provides energy for ATP synthesis in the mitochondria, a process termed oxidative phosphorylation. ATP production in darkness was similar in wild type and mutant cells (Fig. 4B), which is consistent with the similar rates of O₂ uptake (Fig. 3B). Oligomycin (2 µg ml⁻¹), which is known to inhibit mitochondrial H⁺-ATP synthase (72), significantly inhibited the level of cytosolic ATP in both wild type and mutant cells, effectively inhibiting mitochondrial respiration. DCMU also inhibited ATP production in both wild type and mutant cells, although OsCHLH plants lack photosynthetic capacity. This appears to be due to the inhibition of cytochrome b oxidation-reduction by DCMU at one ubiquinone site, presumably ubiquinone (Q1) redox near the inner side of the mitochondrial membrane (48). The combined inhibitory effects of oligomycin and DCMU on ATP production were synergistic although the binding sites for the two compounds were specific.

In the presence of glucose as a substrate, oxidative phosphorylation was 154% and 152% of control levels, for wild type and mutant cells, respectively. Oligomycin reduced ATP levels by 66% and 70% in wild type and mutant cells, whereas it showed a much
greater effect in the absence of glucose, decreasing ATP levels by ~75% in both cell types. I-Ac inhibited ATP production by 36% in both wild type and mutant cells. Taken together, these findings suggest that glycolysis is functional in both wild type and mutant cells. However, glycolysis, which is the initial stage of glucose metabolism, produces a relatively small amount of ATP in plant cells (Fig. 4B). Therefore, oxidative phosphorylation represents the primary source of ATP in the chlorophyll-deficient OsCHLH rice mutant. These cells are capable of maintaining a steady-state flux of energy from mitochondrial oxidative phosphorylation, which provides ATP for the cytosolic ATPases to perform essential cellular functions.

K⁺ is the most abundant cation in the cytoplasm of living cells, and it regulates ionic strength, osmotic potential, and membrane polarization. Permeability to K⁺ is mediated by voltage-dependent K⁺ channels, which are by far the best-characterized plasma membrane ion channels in plant cells (73). It has been established in a variety of cell types that activation of K_out channels under steady-state conditions is dependent on cytoplasmic ATP, and a lack of ATP causes channel “run-down” (16, 17, 19, 24, 54-58). The activation of various protein kinases, PP1, PP2A, and PP2C are required for the up-regulation of K_out channel currents in Samanea, Tobacco and Vicia mesophyll cells (56, 74, 75). Our data show that rice mesophyll cells also exhibit voltage-dependent K⁺ current and that ATP is required for its activation (Figs. 5-7). The magnitude of current in the wild type cells was greater in the presence of ATP than that of mutant (Fig. 6). The smaller whole cell current in the mutant cells might be due to a combination of environmental conditions and epistatic interactions, which are the two main factors controlling the effects of deleterious mutations (76). One could therefore expect that the number of K⁺ channels in the plasma membrane is different in the wild type and mutant cells because the mutant seedlings were poorly
developed (Fig. 1A), although the mitochondrial respiratory activity is quite similar to that of wild type (Figs. 3B and 4B). Interestingly, rice mesophyll cells lacked the inward K⁺ current that is found in guard cells (16, 19, 26, 49-52, 77, 78). The ATP-activated Kₐₜ channel current was maintained over periods of 10 min from the beginning of patch recording in both wild type and mutant cells.

We also found that the ATP-dependent Kₐₜ channel current was largely inhibited by oligomycin, which blocks mitochondrial FₐFₒ-ATPase, in both wild type and mutant cells (data not shown). These data further supported the idea that a cytosolic ATP pool derived from mitochondria maintained the activation of Kₐₜ channel current in the mutant cells. However, a previous study suggested that activation of K⁺ channels, including Kₐₜ and non-selective cation channels, was mediated by ATP produced photosynthetically from albino mutant (alb-1) plant cells (16). Thus, the role of mitochondria in the stimulation of the Kₐₜ channel current remains controversial. More recent studies showed that light-induced membrane hyperpolarization is modulated by photosynthesis-dependent plasma membrane H⁺-ATPases in mesophyll cells (27, 29). However, this hyperpolarization clearly occurred in response to light absorption by pigments other than chlorophyll, demonstrating that chlorophyll-deficient cells also exhibit light-induced membrane hyperpolarization (30), which differs from the findings of the earlier study (16). Taken together, the data suggest that light-driven photosynthesis is not directly involved in the activation of Kₐₜ channel current in the plasma membrane, although the possibility of direct K⁺ transport by other ATPases (i.e. K⁺-ATPases) cannot be ruled out (17). Mitochondrial respiration therefore provides the source of cytosolic ATP. From these results, we strongly suggest that mitochondria in the OsCHLH mutant play a major role in meeting the cytosolic energy requirements during plant growth and development.
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FIGURE LEGENDS
Fig. 1. Phenotype and genotype of OsCHLH rice plants.

(A) Whole 8-day-old seedlings of wild type (a, left) and OsCHLH mutant plants (a, right). The OsCHLH mutant lacks chlorophyll in the leaves of the seedlings. Panel b shows chlorophyll fluorescence in a representative wild type leaf and panel c shows chlorophyll fluorescence in an OsCHLH mutant leaf of 8-day seedlings. The bar indicates 1.6 cm. (B) (a) Genomic DNA from T2 plants was subjected to PCR analysis with the primers shown in panel C. Only the 1.1 kb fragment, the 0.9 kb fragment, or both fragments were amplified by PCR in homozygous (Ho), WT (wild type), and OsCHLH heterozygous (He) T3 plants, respectively. M indicates the lambda DNA size marker cut with PstI. (b) Schematic representation of the gene structures in the region that complemented the OsCHLH mutant phenotype. p1, forward primer in OsCHLH; p2, reverse primer in T-DNA; p3, reverse primer in OsCHLH. The p1 and p2 primers amplified the 1.1 kb PCR product from the T-DNA inserted DNA template. The p1 and p3 primers produced the 0.9 kb PCR product from the DNA of wild type plants. In contrast, no PCR band was obtained from the T-DNA inserted template, because the predicted fragment size (7.7 kb) was too large to amplify (1). (C) Chlorophyll fluorescence of homozygous OsCHLH rice mesophyll cells isolated from the leaves of 8-day-old seedlings. (a) and (b) Light micrograph of wild type and OsCHLH mutant protoplasts. (c) and (d) show the red chlorophyll fluorescence from the same field of the cells of (a) and (b), respectively. Mesophyll cells were suspended in a solution including 1 mM CaCl₂ adjusted with D-sorbitol to 600 mmol kg⁻¹. The bars indicate 5 µm.

Fig. 2. Changes of CO₂ exchange rates in intact leaves of wild type and homozygous OsCHLH mutant plants.
(A) Typical recordings of CO₂ exchange during dark-light transitions measured by leaf gas exchange. Attached leaves from 2-week-old seedlings of two wild type or OsCHLH mutant plants were first adapted to dark (D) for 5 min. Light was turned on when indicated by the vertical arrows (L1; 70 µmol m⁻² s⁻¹ for 10 min and L2; 900 µmol m⁻² s⁻¹ for 30 min). Bold and fine lines indicate CO₂ exchange in wild type (WT) and mutant (OsCHLH) leaves, respectively. (B) Histograms show net CO₂ uptake in the dark (0) and under 70 and 900 µmol m⁻² s⁻¹ actinic light, as indicated. Data are expressed as mean ± SE from three separate experiments. The differences between the means were statistically significant (t-test, P=0.05), except for dark experiments (t-test, P=0.144). Other experimental conditions were described in Materials and Methods.

Fig. 3. Oxygen evolution and consumption in detached leaves of wild type and homozygous OsCHLH mutant plants.

(A) The time course of a typical experiment showing the output voltage from an oxygen sensor (logged twice every second). The scaling is linear. Four leaf discs were provided with bicarbonate as a source of carbon dioxide and kept in the dark for 9 min prior to the start of logging and then illuminated. On the vertical (oxygen concentration) axis, + and - indicate net oxygen production and net oxygen consumption, respectively. L1 and L2 indicate applications of actinic light at 60 and 900 µmol m⁻² s⁻¹, respectively, while D represents dark treatment. A blank (without leaf material) was included as control (bottom) and its signal (L2) was increased artifically by increasing light intensity (900 µmol m⁻² s⁻¹), inducing an increase in temperature. (B) Histograms show net oxygen consumption in the dark (0) and net oxygen evolution under 60 and 900 µmol m⁻² s⁻¹ actinic light, as indicated. Data are expressed as mean ± SE from five separate
experiments. The differences between the means were statistically significant ($t$-test, $P=0.05$), except for dark experiments. Net oxygen concentrations were measured over the final 2 min period of the dark and actinic light intervals. Data subtracted from control values and log transformed, such that the gradient of a fitted line, using regression analysis, would constitute the exponential decay constant ($k$) of the form $R_t = R_0 \times e^{-kt}$ (39), where $R_t$ is the respiration rate at the start of the dark period and $R_0$ is the respiration rate at some time, $t$. Other experimental conditions were described in the Materials and Methods.

**Fig. 4. ATP content in intact seedlings and mesophyll protoplasts of homozygous OsCHLH mutant plants.**

(A) The mutant seedlings were grown for 8 days in 16-h day cycle, at a minimum photon flux density of 90 $\mu$mol m$^{-2}$s$^{-1}$ and then harvested 2 h after dark and light (90 $\mu$mol m$^{-2}$s$^{-1}$) treatments, respectively. ATP production was determined as described in Materials and Methods. Data are expressed as mean $\pm$ SE (n=6). The differences between the means were statistically significant ($t$-test, $P=<0.001$). (B) Cells were incubated with various combinations of substrate (glucose, 5 mM), oligomycin (Oligo, 2 $\mu$g ml$^{-1}$), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, 10 $\mu$M), and iodoacetic acid (I-Ac, 1 mM) for 40 min under darkness. Glucose was included as a mitochondrial substrate via glycolysis. Oligomycin and DCMU were used to block mitochondrial $F_1F_0$-ATPase and photosynthetic electron transport, respectively. I-Ac was used to block glycolysis. Data are expressed as mean $\pm$ SE (n=3). The differences between the means were statistically significant ($t$-test, $P=0.05$). The amount of protein was determined by the method of Bradford (41) using BSA as a standard.
Fig. 5. Whole-cell currents in mesophyll protoplasts of wild type and homozygous OsCHLH mutant plants.

(A) Voltage protocol for all whole-cell measurements. Representative current measurements in (B) wild type cells and (C) mutant OsCHLH cells are shown. +ATP and –ATP indicate the presence and absence of ATP in the pipette solution, respectively. Currents were measured after 8 min of perfusion in either the presence (left panels of B and C) or absence (right panels of B and C) of 2 mM ATP in the pipette solution. The holding potential was set at –40 mV, and voltage steps were increased by 20-mV increments from –160 to +100 mV. The right panels of B and C show the current traces at 4.5 scales for easier visualization of kinetics. The stimulation of K_out channel current by intracellularly administered ATP as induced by the voltage steps shown in A. Left panels show a K_out channel current when 2 mM ATP (Tris salt) was perfused into the cell interior with the patch pipette solution. Seal resistance, R_s = 2.83 GΩ; whole-cell capacitance, C = 10.12 pF (upper trace), R = 2.70 GΩ; C = 10.18 pF (lower trace). Right panels show a K_out channel current in the absence of ATP (control). R = 2.07 GΩ; C = 9.78 pF (upper trace), R = 2.18 GΩ; C = 10.06 pF (lower trace). The average cell diameters were 15.7 and 15.2 µm for wild type and mutant cells, respectively.

Fig. 6. Current-voltage (I-V) relationship of whole-cell currents in mesophyll protoplasts of wild type and homozygous OsCHLH mutant seedlings.

Recordings were made after perfusing the cells for 8 min in the presence (+ATP, open symbols) or absence (–ATP, closed symbols) of 2 mM ATP in the pipette solution, as indicated. Data are derived from currents shown in Fig. 5 and are presented as the
average ± SE (n=9). The voltage steps ranged between –160 and +100 mV in 20-mV increments from a holding potential of –40 mV. W, wild type (circles); M, mutant (triangles). Other experimental conditions are as in Fig. 5.

**Fig. 7. Time course of whole-cell currents at +80 mV in rice mesophyll protoplasts of wild type and mutant plants.**

The channel currents were measured immediately after gaining access to whole cells (within 20 seconds) in the presence or absence of 2 mM ATP in the pipette solution. Whole-cell current magnitude at +80 mV, (relative to control current at that voltage) is shown. Data are presented as the average ± SE of mesophyll cells (n=6 for wild type, n=8 for mutant). The voltage steps ranged between –160 and +100 mV in 20-mV increments from a holding potential of –40 mV. A, wild type; B, mutant. +ATP and –ATP indicate the presence and absence of ATP in the pipette solution, respectively. Other experimental conditions are as in Fig. 5.
**Fig. 5**

A. Voltage protocol:

-160 mV
-40 mV
+100 mV

B. + ATP

C. - ATP

90 pA  640 ms

20 pA  640 ms
Fig. 7

A. WT

B. OsCHLH

Whole-cell current at +80 mV (% of control)

Time (min)

-10 1 2 3 4 5 6 7 8 9 10

+ ATP

- ATP

+ ATP

- ATP
Mitochondria provide the main source of cytosolic ATP for activation of outward-rectifying K⁺ channels in mesophyll protoplast of chlorophyll-deficient mutant rice (OsCHLH) seedlings

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