Journal of Plant Biology, August 2007, 50(4) : 430-439

Activation of Mitochondrial Respiration in Chlorophyll-Deficient Rice Mutant Seedlings

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Previously we described a knock-out mutant of the rice *oschlh* gene, which encodes a Mg-chelatase H subunit and is involved in chlorophyll biosynthesis. This mutant exhibits ATP-dependent activities of plasma membrane outward-rectifying K^+ channel currents that are supported by mitochondrial activation. Here, we have investigated mitochondrial activity in *oschlh* mutants. Growth rates were similar between the wild type and the mutant, and were enhanced by the addition of sucrose under darkness, indicating that the mutants have active mitochondrial respiration. Proteomic analyses led to the identification of 41 proteins ($P < 0.05$) involved in a range of functions that differed between the mutant and the wild type. Of these, 15 were up-regulated and 26 were down-regulated by more than 2-fold in the mutant. We hypothesize that loss of functioning in the chloroplasts, mainly ATP production, can be restored via beneficial interactions with other cellular compartments, especially the mitochondria, through the inter-organellar regulation of metabolites. Oxygen consumption is greater during mitochondrial respiration in chlorina mutants than in the wild type, so that those mutants produce large amounts of ATP in the presence of sucrose. These results imply that gene expression of photosynthetic organisms is strongly connected through energy-driven networks of transcriptional regulators that can control factors in other cellular compartments, thus indicating the re-programming of cellular functions.

Keywords: ATP, chloroplast, mitochondria, oschlh, re-programming, rice

Mitochondria contribute to energy metabolism in photosynthetic cells, especially in relation to ATP production, oxidation of redox equivalents, and the supply of carbon skeletons (Krömer, 1995). Its metabolism, particularly the bioenergetic reactions of oxidative electron transport and phosphorylation, continue to be active in the light and are essential for sustaining photosynthetic activities (Raghavendra and Padmasree, 2003). The concept of the dependence of photosynthesis on mitochondrial metabolism has evoked considerable interest. Impaired chloroplast development affects the level of mitochondrial gene transcripts (Hedtke et al., 1999) and the activity of plasma membrane K^+ channels (Goh et al., 2004). How these organelles communicate with each other is not well understood.

New insights into this issue are being provided through the analysis of transferred DNA (T-DNA) and transposon insertion mutants (Gierl and Saedler, 1992; Bouchez and Hofte, 1998; Jung et al., 2003; Goh et al., 2004) combined with genomics (Holtorf et al., 2002). However, even though the genomes of two key species, *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000) and rice (Eckardt, 2000), are now available, gene functioning cannot be inferred solely through a single approach because gene-deletion sometimes either has little phenotypic influence (May et al., 2003; Yazaki et al., 2004) or produces unexpected phenotypes due to effects on apparently unrelated systems. These responses can result from other genes "compensating" for the loss of the deleted gene (Saga et al., 1992; Normanly and Bartel, 1999; Gu et al., 2003). Thus, the functions of these "compensating" genes might mask those of the deleted ones.

The metabolic requirements and developmental needs of organisms can vary dramatically, depending on environmental conditions and epistatic interactions (Saga et al., 1992; Imhof and Schlötterer, 2001). This is particularly true in plants, under conditions of light and darkness, or in the presence or absence of a carbon supply (Reyes and Chua, 2004; Thum et al., 2004). Environmental signals can regulate gene expression in complex ways, suggesting that cellular functions are linked through intricate networks of transcriptional regulators (Lee et al., 2002; Bergman and Siegal, 2003). Plants must then coordinate the activity of yet another genome, that of their plastids, during growth and development. Information is now being accumulated about the mutual regulation of chloroplast and mitochondrial compartments. However, very little is known about the mechanisms and the extent of regulation of intracellular crosstalk within the framework of the whole plant cell. Here, we investigated whether the loss of functioning of the rice chloroplast gene *oschlh* could be compensated for by regulating the various roles of other organelles in order to maintain plant growth and development.

MATERIALS AND METHODS

Plant Materials

Oryza sativa cv. Japonica seedlings were grown and

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mutants were screened for 8 d on a medium containing 1/2 x MS plus 0.2% (w/v) phytagel. These rice plants were cultured in a temperature-controlled growth chamber (28 ± 1°C) under a 16-h photoperiod (90 µmol m^{-2} s^{-1} light intensity).

Real-Time PCR

Total RNA was extracted from 8-d-old plants, using guanidinium thiocyanate reagent as described previously (Chirgwin et al., 1970). DNA was removed with Dnase I (Sigma, USA), and first-strand cDNA was reverse-transcribed from 0.5 µg of RNA according to the manufacturer's recommendations (QuantiTect® SYBR®, Green RT-PCR; Qiagen, USA). To obtain PCR products of about 200~250-bp at the 3′-UTR (untranslated region) of each cDNA clone (listed in Table 1), we designed the following gene-specific primers: AK072707, 5′-CGTCGAACAGCCTGAGTACA-3′ and 5′-ATCTGTTGGCCAGGTTCTTG-3′; AK069303, 5′-CCAAACTGGGACGTGTTTTC-3′ and 5′-TTCTGCAGCACCAACTTGTC-3′; AK062407, 5′-TTCTTTGATCGATCCCTTGC-3′ and 5′-TCCTCACGAACATCCAACAA-3′; AK065938, 5′-CAGTGGAAGAGCGGGTAGAG-3′ and 5′-TGTCATCGCTGTTTTCCTTG-3′; AK069611, 5′-TGGGCTTTACAAGGGAATTG-3′ and 5′-CAATGGCACAGAAGACTCCA-3′; AK072384, 5′-ACTCCACGAACGCATTTTCT-3′ and 5′-GTGATTGGAATCCCTTGTGC-3′; AK101854, 5′-CCCTTGTCAAATGGGAAGAA-3′ and 5′-ACCCTCGCTATGGCTGAAA-3′; AK058489, 5′-AACGGCGTCGAGTTCTAAAG-3′ and 5′-ATTCGTTCGAATGCAGGAAC-3′; AK073698, 5′-GCGAACTAGGCAACCTATGC-3′ and 5′-GAAAAATAGCACCGGAGTCG-3′; and K061681, 5′-ATGTGCCTGCTGATGACTTA-3′ and 5′-AACCTTTTGGACACCACGAG-3′. Real-time PCR was initiated by denaturing the cDNA at 95°C for 15 min, followed by 40 PCR cycles of 94°C, 15 s; 55°C, 30 s; and 72°C, 30 s. Their relative transcript abundance was detected with an Applied Biosystems 7000 Sequencer and SYBR Green PCR Master Mix (PE-Applied Biosystems, USA). Gene-specific primers were designed to work under the same experimental conditions (95°C for 10 min, followed by 40 cycles of 95°C for 1 min and 50°C for 30 s). This generated products of about 200 to 250 bp at the 3′UTR of each cDNA clone. Relative transcript abundance again was detected by an Applied Biosystems 7000 Sequencer and SYBR Green PCR Master Mix.

Isolation of Mitochondria and Measurement of Their Oxygen Uptake

Mitochondria were isolated by protoplast fraction and lysis, followed by differential centrifugation, essentially as described by de Pinto et al. (2000). Oxygen uptake was measured under darkness and according to our previous method (Goh et al., 2004). Mitochondria (50 µg protein) were added to 1.5 mL of a respiratory medium containing 210 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 5 mM potassium phosphate (pH 7.4), 3 mM $MgCl_2$, and 5 mg mL^{-1} bovine serum albumin in the presence of 2 µg of rotenone. Succinate (5 mM) was used as a respiratory substrate, and either ADP (0.5 mM) or FCCP (1.25 µM) was added to induce respiration. Oligomycin (2.5 µM) and antimycin (2 µg) were used for inhibiting oxygen uptake.

Protein Preparation

The leaves from rice seedlings were finely ground in liquid N_2 and lysed in a solution containing 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethyammonio]-1-propanesulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT), 2% v/v pharmalyte, and 1 mM benzamidine. Proteins were extracted for 1 h at room temperature with vortexing. Samples were then centrifuged at 15000*g* for 1 h at 15°C. The insoluble material was discarded and the soluble fraction was used for two-dimensional gel electrophoresis analysis. Protein levels were determined by the Bradford assay (Bradford, 1976).

Two-Dimensional Gel Electrophoresis

IPG dry strips were equilibrated for 12 to 16 h in a solution containing 7 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl) dimethyammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), and 1% pharmalyte, and were then loaded with 150 or 300 µg of sample. Afterward, isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit and an EPS 3500 XL power supply (Amersham Biosciences, Sweden) according to the manufacturer's instructions. The voltage was increased linearly from 150 to 3500 V over 3 h for the sample entry. Samples were then subjected to a constant voltage of 3500 V until focusing was completed after 96 kV h^{-1}. Prior to electrophoresis in the second dimension, strips were incubated for 10 min in equilibration buffer [50 mM Tris-Cl (pH 6.8), 6 M urea, 2% SDS, and 30% glycerol], first with 1% DTT and then with 2.5% iodoacetamide. These equilibrated strips were inserted onto SDS-polyacrylamide gels (20 to 24 cm, 10 to 16%). SDS-polyacrylamide-gel electrophoresis (-PAGE) was performed using a Hoefer DALT two-dimensional system (Amersham Biosciences) according to the manufacturer's instructions. The gels were run at 20°C for 1700 V h^{-1}. These 2-D gels were then silver-stained as described by Oakley et al. (1980), except that we omitted the fixing and sensitization steps with glutaraldehyde.

Image Analysis

PDQuest software (version 7.0; BioRad, USA) was used to analyze our digitized images according to the manufacturer's protocols. The quantity of protein within each spot was normalized to the total valid spot intensity. Protein spots that exhibited greater than two-fold differences (as compared with the control spots) were selected for further analysis.

Enzymatic Digestion of Protein In-Gel

Protein spots were subjected to enzymatic in-gel digestion with modified porcine trypsin, in a manner similar to that described by Shevchenko et al. (1996). Tryptic peptides were recovered by combining the aqueous phases recovered from several extractions of gel pieces with 50% aqueous acetonitrile. The peptide mixtures were concentrated and de-salted with C18ZipTips (Millipore, USA). Peptides were then eluted in 1 to 5 µL of acetonitrile. An aliquot of this solution was mixed with an equal volume of a saturated

solution of α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile. Afterward, 1 μL of the mixture was spotted onto a target plate.

MALDI-TOF Analysis and Database Search

Protein analysis was performed using an Ettan Matrix-Assisted Laser Desorption Time-of-Flight (MALDI-TOF) mass spectrometer (Amersham Biosciences). Peptides were evaporated with an N_2 laser at 337 nm via delayed extraction. The peptide fragments were then accelerated with a 20 Kv injection pulse for time-of-flight analysis. Each spectrum was the cumulative average of 300 laser shots. The search program ProFound, developed by Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe), was used to identify proteins by peptide mass fingerprinting. Spectra were calibrated with trypsin auto-digestion ion peak m/z (842.510, 2211.1046) serving as internal standards.

Extraction and Measurements of ATP, ADP, and AMP

Frozen samples (approx. 0.5 g) were extracted by the method of Chen and Nose (2004). ATP was assayed according to the procedure of Lamprecht and Trautschold (1974) in a 2.5 mL reaction mixture containing 100 mM triethanolamine hydrochloride (pH 7.6), 4 mM $MgCl_2$, 2 mM glucose, 2 mM NADP, 2.8 units lactate dehydrogenase (EC 1.1.1.27), and 1.8 units hexokinase (EC 2.7.1.1). Both ADP and AMP were measured, as described by Jaworek et al. (1974), in a 2.5 mL reaction mixture containing 100 mM triethanolamine hydrochloride (pH 7.6), 1 mM PEP, 33.4 mM $MgSO_4$, 0.12 M KCl, 0.36 mM NADH, 24 units of lactate dehydrogenase, and 18 units of pyruvate kinase (EC 2.7.1.40). The ADP concentration was calculated as the difference in readings before and after the addition of pyruvate kinase. After this, 16 units of myokinase (EC 2.7.4.3) were added to measure the AMP level. Recoveries for adenylates were 95 to 102%. The adenylate energy charge (AEC) was calculated according to the formula of Atkinson (1968), i.e., AEC = [ATP] + 0.5[ADP]/[ATP] + [ADP] + [AMP].

RESULTS AND DISCUSSION

We set out to investigate whether loss of function of a single chloroplast gene would result in compensatory changes elsewhere in the plant. To test this possibility, we examined how a single-gene null ('knockout') mutation affects the abundance of cellular proteins. Recently, we have shown that the magnitude of current conducted through the plasma membrane outward-rectifying K^+ channel (K_{out} channel) is greater in wild-type rice mesophyll cells than in chlorophyll-deficient cells. This is because of the knockout of a Mg-chelatase H subunit (*chlh*) chelatase (Jung et al., 2003; Goh et al., 2004), which is involved in chlorophyll biosynthesis (Larkin et al., 2003). The *oschlh* mutant exhibits a chlorina phenotype in which photosynthetic electron flow is impaired, thereby signaling that it should not photosynthetically produce ATP in the cells. This smaller whole-cell current in the *oschlh* knockout 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 (Saga et al., 1992). Thus, the ion channel density within the plasma membrane may affect the K_{out} current in mutant cells (Homann and Thiel, 2002). However, we cannot exclude the possibility that this observed decrease in current is caused by an inactivation of channels in the plasma membrane of the mutant cells (Homann and Thiel, 2002; Goh et al., 2004). This may result from metabolic compensation via intra-cellular communication, such as by regulating the number of K^+ channels in the plasma membrane to reduce the metabolic requirements in cytosolic ATP and NAD(P)H, due to a lack of photosynthetic activity in the mutant cells.

The growth of our oschlh mutant seedlings was comparable to that of the wild type under illumination. In the absence of sucrose, fresh weights for 8-d-old seedlings were 0.193 ± 0.006 (wild type) and 0.075 ± 0.003 g (mutant) (data not shown). When these plants were grown in the dark (Fig. 1A), their fresh weights were similar for 8 d (Fig. 1B), implying that mitochondria in the mutant were active. Interestingly, the addition of 3% sucrose, which stimulates

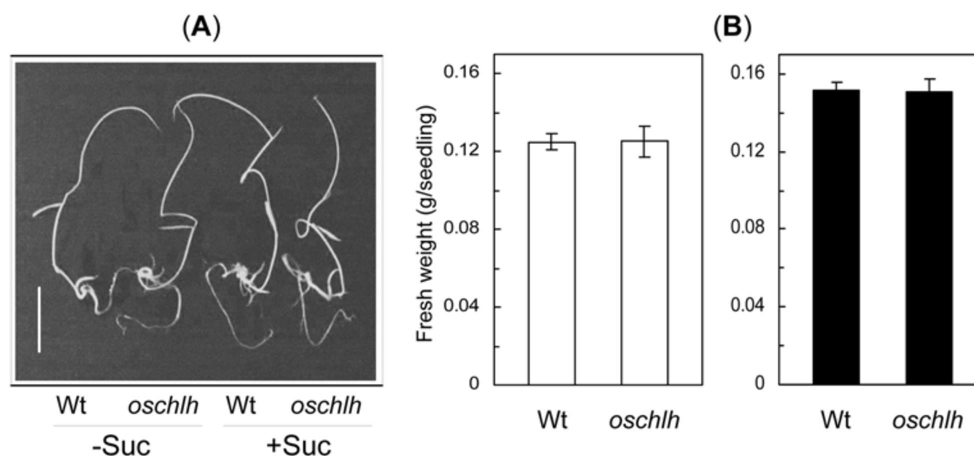

Figure 1. Growth of *oschlh* mutants in dark. (A) Intact 8-d-old seedlings of wild type and *oschlh* mutant in presence or absence of 3% sucrose. Bar = 1.4 cm. (B) Growth analysis of *oschlh* mutant seedlings in darkness. Fresh weights were measured at 8 d after germination. Values are expressed as mean ± SE (n=20 for wild type and n=10 for *oschlh* mutants). White, - sucrose (-Suc); Black, + sucrose (+ Suc).

Table 1. Quality control by RT-PCR analysis.

Accession No.	Putative function	Fold change
Potassium channel:		
AK072707	potassium channel beta subunit (KOB1)	-1.00
AK069303	outward-rectifying potassium channel (KCO1)	-0.25
AK062407	potassium channel protein (ZMK2)	-0.25
AK065938	potassium channel protein (KAT1)	+3.25
Mitochondria:		
AK069611	mitochondrial phosphate transporter	+1.50
AK058489	putative mitochondrial dicarboxylate carrier	+1.50
AK073698	malate dehydrogenase (MDH)	+0.75
AK072384	mitochondrial ATP synthase 6KD subunit	+33.0
AK101854	mitochondrial processing peptidase alpha-chain precursor	+6.25
AK061681	mitochondrial F1-ATPase	+1.50

Fold-change differences for 10 typical cDNAs that were apparently expressed physiologically and differentially in the mutant seedlings were confirmed by real-time PCR. Negative and positive ratios indicate down-regulation and up-regulation, respectively, in the mutant.

the generation of ATP by mitochondrial respiration (Journet et al., 1986) led to much greater growth enhancement (approx. 25%) in both wild-type and oschlh mutant seedlings. We speculate that this resulted from higher levels of mitochondrial activity in the latter.

Because coordination of gene expression between the three genomes (plastid, mitochondrial, and nuclear) is of crucial importance for plant cells, the mitochondria are thought to be significantly regulated by organellar crosstalk and signaling. To verify these gene expression data, we performed real-time PCR analysis of four typical K^+ channels that might be involved in the ATP-sensitive K^+ current as well as five mitochondrial genes that might be related to ATP production (Table 1). Results previously obtained by our physiological analysis (Goh et al., 2004) were in agreement (up- or down-regulation) with those obtained here. Interestingly, the potassium channel protein KAT1 (AK065938) was up-regulated when other detectable channels were negative in the mutant seedlings. This indicates that organellar crosstalk is associated with beneficial interactions for the metabolism of the whole cell. Our results suggest that this mutation affects whole-cell redox homeostasis and gene expression, and influences metabolic pathways that use

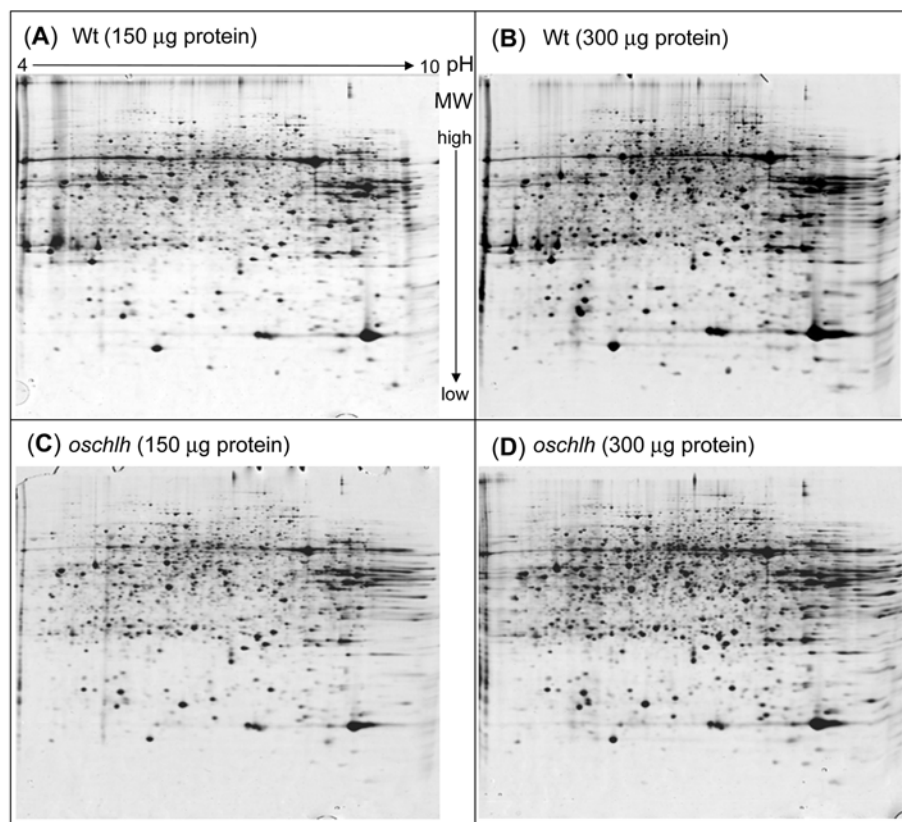

Figure 2. 2-D patterns of protein extracts from leaves of 8-d-old rice seedlings of wild type (upper panels) and *oschlh* mutant (lower panels). Gels were loaded with 150 μg (**A** and **C**) or 300 μg (**B** and **D**) of protein. (**E**) Full 2-D image of *oschlh* mutant protein expression pattern (300 mg loaded). Arrows indicate variations in expression of another pair of genes due to single mutation. (**F**) and (**G**) show examples of genetic differences between wild type (**F**) and *oschlh* mutant (**G**). These were compared with those in Figure 2B and D. Arrow indicates Rubisco (LSU), which was expressed at similar levels in wild type (Wt) and mutant. (**H**) and (**I**) present typical captured images, showing protein that was decreased (**H**) and protein that was increased (**I**) in *oschlh* mutant, relative to control. a) modified image by image analysis program (PDQuest), b) Wt (300 μg protein loaded), c) mutant (300 μg), d) Wt (150 μg), e) mutant (150 μg). Arrows indicate 1313 and 6215 spots, respectively. All experiments were highly reproducible, with $R^2 = 0.92$ for wild type and 0.89 for mutant.

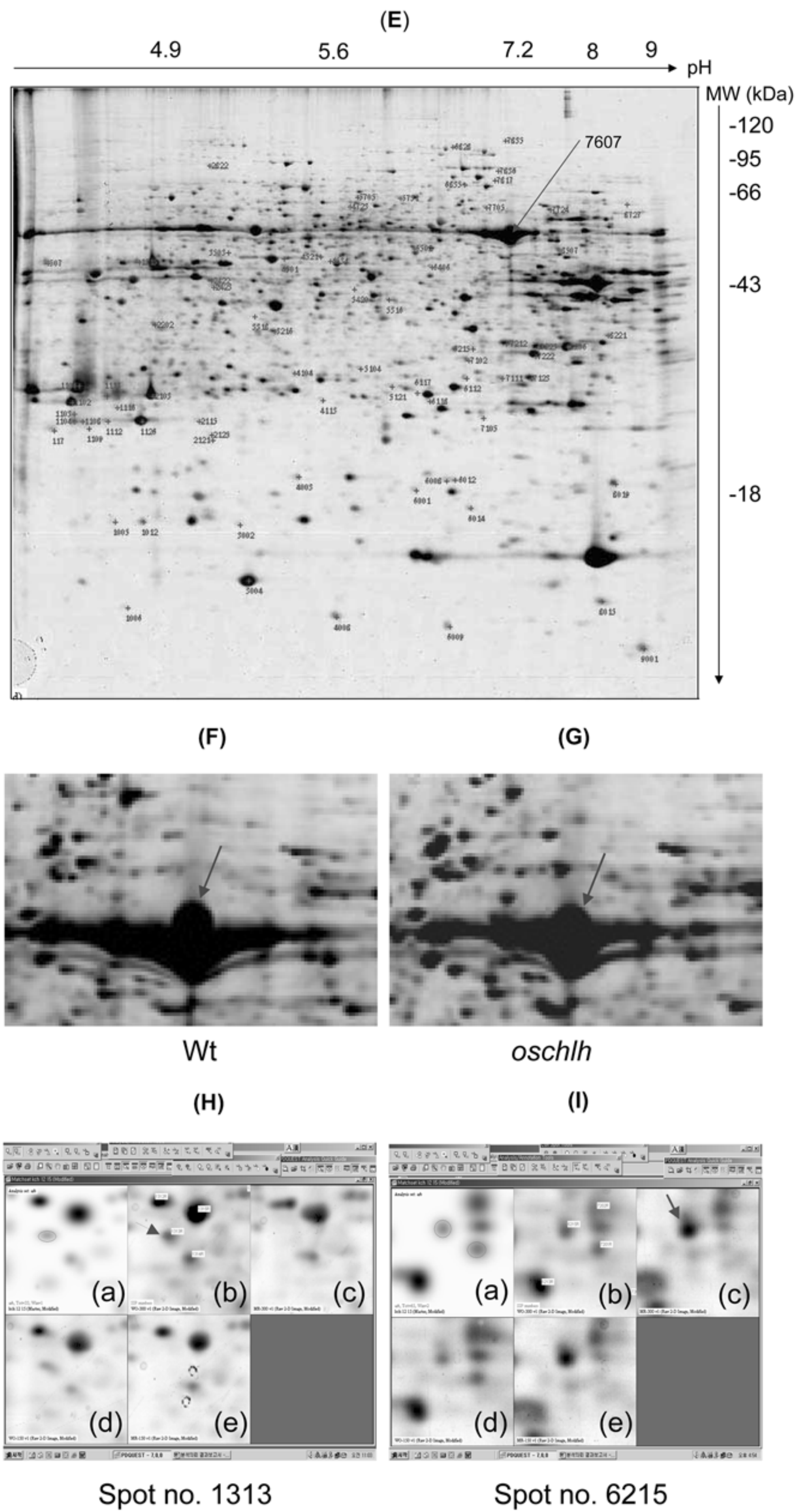

Figure 2. Continued.

common key metabolites, e.g., ADP/ATP, NAD(P)H, triose-P, and hexose-P (Oh and Kaplan, 2000). Therefore, we conclude that, because the mutant has a reduced cytosolic ATP pool (Goh et al., 2004), it readjusts the number of channels at the plasma membrane and mitochondrial organization to sustain the balance of energy required for normal cellular

Table 2. Identification of proteins differentially expressed in chlorophyll-deficient mutant rice (*oschlh*) seedlings.

Spot no.	Protein[a]	Calculated MW/pI	Mass (KDa)	Sub-cellular localization[b]	Fold change[c] Wt/Mutant
0117[d]	Hypothetical protein	23.6/4.3	46.0	n.d.	>-6
0507	α-Amylase precursor	47.0/4.3	48.6	cytoplasmic	-8
1102	AP004270 NID	26.3/4.2	28.8	cytoplasmic	>+17
1005	Unidentified	16.4/4.6	n.d.	n.d.	>-3
1006	Probably small nuclear ribonucleoprotein G	12.1/4.7	19.1	nuclear	-3
1103	Unidentified	25.0/4.3	n.d.	n.d.	>-5
1108	Unidentified	4.3/4.5	n.d.	n.d.	-7
1112[d]	OSJNBb0062B06.18	24.4/4.5	113.4	cytoplasmic	-5
1116	RNase S-like protein	25.8/4.7	29.0	extracellular	+2.7
1126	Thylakoid luminal protein	24.4/4.8	211.4	chloroplast	>+4.1
1313[d]	Nidogen-2 precursor	38.2/4.7	35.3	plasma membrane	>-3
1515	Actin	47.2/4.8	42.1	cytoplasmic	-2
2202	Putative thiamine biosynthetic enzyme	36.0/4.9	37.2	cytoplasmic	-3
3002	ATPase a-subunit	16.3/5.2	29.4	chloroplast	-3
3318	Putative RNA-directed RNA polymerase alpha	37.2/5.3	137.8	chloroplast	+2.5
4003	Rieske Fe-S precursor	19.5/5.5	24.2	mitochondria	-4
4008	WD-40 repeat protein MSI2	11.7/5.6	55.6	nuclear	-6
4104	OSJNBb0017I01.8	29.6/5.4	28.0	chloroplast	+6
4207	Unidentified	36.2/5.5	n.d.	n.d.	-2
4501	RICAAMYB NID	47.9/5.4	48.4	vacuolar	+2.5
4534[d]	OSJNBa0004L19.3	47.6/5.6	103.4	n.d.	+4
5705	P0022F10.12 protein (malic enzyme)	65.2/5.8	65.8	cytoplasmic	>-2
5731	Putative mutator-like transposase	64.8/6.2	110.6	cytoplasmic	-3
6001	Rubisco (LSU)	18.5/6.3	53.4	chloroplast	-4
6008	Rubisco (LSU)	19.3/6.6	50.1	chloroplast	-4.7
6009	NADP-specific glutamate dehydrogenase	11.2/6.6	46.2	chloroplast	-21
6012	B1065E10.16	19.2/6.7	23.7	nuclear	-2.5
6112	Rubisco (LSU)	29.0/6.8	52.0	chloroplast	-2.5
6117	AF047444 NID	27.3/6.4	29.2	chloroplast	+2.3
6215	Transposon MAGGY gag-pol gene homologues	32.8/6.9	172.1	cytoplasmic	+2.2
6828	Putative cytosolic tRNA-Ala synthetase	100.4/6.7	75.9	cytoplasmic	+2
7212	Rubisco (LSU)	33.3/7.2	49.9	chloroplast	+2
7705	Ferredoxin-nitrite reducta (EC 1.7.7.1)	61.9/7.0	66.7	cytoplasmic	-3
7817	TNP2-like protein	75.2/7.1	110.8	nuclear	+4.5
7835[d]	Aspartic protease inhibitor 9	105.9/7.2	22.5	cytoplasmic	+2.1
7836	Unidentified	81.1/7.1	n.d.	n.d.	>+4
8015	Rubisco (SSU)	12.3/8.1	19.7	chloroplast	-1.9
8019	Hemoglobin 1 (rHb1)	19.0/8.3	18.5	cytoplasmic	-4
8507	Rubisco (LSU)	50.1/7.6	53.4	chloroplast	-2
8727	Putative gag-pol polyprotein	63.3/8.5	142.2	chloroplast	+2.5
9001	WRKY DNA-binding protein 66	10.4/8.9	24.1	nuclear	>-14

[a]Protein identification via peptide mass fingerprinting was carried by the Mascot search program (http://www.matrixscience.com/cgi/search_form.pl?FORMVER= 2&SEARCH=PMF). Probability-based mowse score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 57 are significant ($p < 0.05$) (Tang et al., 2000).
[b]Predictions of sub-cellular localization site(s) were retrieved from a matching list by the program ProSLP (http://www.ccbb.re.kr/ ccbb/proslp/proslp.jsp).
[c]Fold change: ratio between wild type and mutant. Negative values in fold change indicate down-regulation; positive values indicate up-regulation in the mutant, relative to wild type. Protein expression of both wild type and mutant was compared as percentage when 300 mg of protein was loaded.
[d]The identification level of these proteins was probable, in that they had a high reproducibility in our experimental system. n.d., not detected.

responses. Thus, a single recessive-gene mutation may profoundly influence other physiological functions in mutant and transgenic plants. This suggests to us that mitochondrial metabolism is activated in the mutant as well.

We used proteomic analysis (Thiellement et al., 1999; Rakwal and Agrawal, 2003) to evaluate the overall effects of the *oschlh* knockout mutation. The protein expression profile revealed approximately 1850 spots in reference 2-D gels of wild-type seedlings (Fig. 2A). Micropreparative gels were loaded with 150 or 300 μg of protein and representative 2-D gels (Fig. 2A-D). Comprehensive proteomic analyses led to the identification of 75 spots that were differentially expressed; these were implicated in a variety of cellular functions (Fig. 2E). In contrast, the Rubisco large subunit (EC 4.1.1.39) (spot no. 7607) was not significantly different between wild-type and mutant seedlings (Fig. 2F, G). Of the 75 spots that were differentially expressed, 15 were up-regulated and 26 were down-regulated by greater than two-fold in the mutant seedlings ($P < 0.05$). Sub-cellular localization of 34 of these proteins was predicted by the ProSLP program (Table 2); 13 were localized in the chloroplasts, 12 were cytoplasmic, 5 were nuclear, 1 was expressed in vacuoles, 1 was extracellular, 1 was localized in the plasma membrane, and 1 was found in the mitochondria. The transposon MAGGY gag-pol gene homologue (Spot no. 6215) was up-regulated (Fig. 2H), whereas the nidogen-2 precursor (Spot no. 1313), which works as an organizer of the basement membrane in vertebrates and invertebrates (Kohfeldt et al., 1998; Schymeinsky et al., 2002), was down-regulated in the mutant (Fig. 2I). This implies that a single-gene *oschlh* mutation affects both the photosynthetic activity of chloroplasts and the physiological functions or phenotypic properties of other organelles.

Regardless of the mechanism by which mitochondrial metabolism is activated in the *oschlh* mutant, i.e., via direct or indirect inter-organellar cross talk, we analyzed mitochondrial activities to prove our suggestion. Mitochondria were isolated from both control (wild-type) and chlorina mutants (*oschlh* and tos17, as reported previously by Jung et al., 2003). These were compared for their capacity to oxidize succinate in a manner regulated by ADP (Fig. 3). In the control mitochondria, measurements of oxygen uptake indicated a control respiratory index of 1.8 (the ratio between the rate of oxygen uptake in the presence or absence of 0.5 mM ADP). In contrast, the mitochondria isolated from either the *oschlh* or the tos 17 chlorina showed a 30% increase because of the induction of succinate. More importantly, they exhibited normal sensitivity to the addition of ADP compared with the respiratory index, but higher oxygen consumption. Oligomycin inhibited oxygen uptake completely (data not shown).

Mitochondrial activation in chlorina mutants has also been observed in connection with oxygen evolution and CO_2 gas exchange on leaf tissue (Goh et al., 2004). For example, white leaves of albostrain barley, which has impaired chloroplast development, have enhanced transcript levels of mitochondrial genes, which is a consequence of their impaired plastids and not because of the nuclear albostrain allele (Hedtke et al., 1999; Emanuel et al., 2004). Based on that earlier work, we also determined the levels of

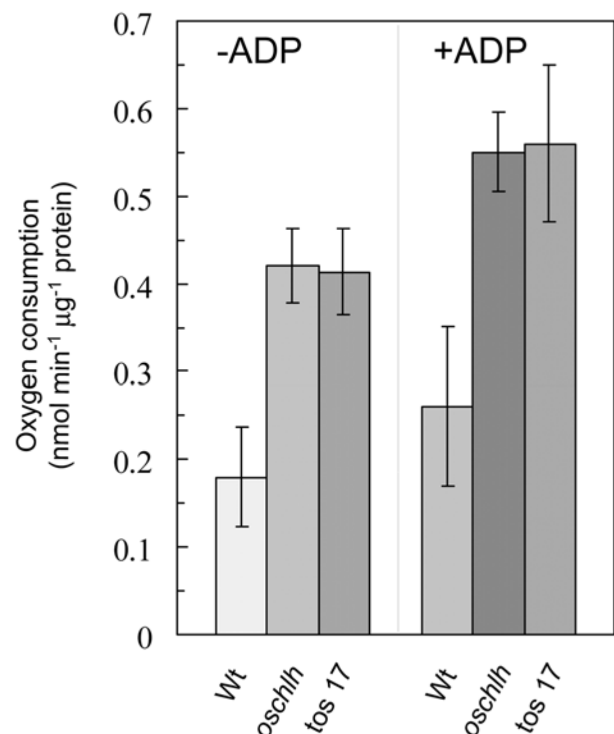

Figure 3. Oxygen uptake by mitochondria isolated from leaves, a result of externally added succinate. Isolated mitochondria (50 μg protein) were incubated in dark at 25°C in 1.5 mL of respiratory medium, and oxygen uptake was measured polarographically as function of time. As respiratory substrate, 5 mM succinate was added.

adenylates under darkness and light. ATP content increased in the former and decreased in the latter for both control (wild-type and heterozygous) and chlorina mutants (*oschlh* and tos 17) (Fig. 4A). The chlorina mutants showed somewhat high levels of ATP in the absence of glucose. However, glucose distinctly increased ATP levels in them, indicating higher mitochondrial respiration as reflected by their oxygen consumption (Fig. 3). ADP and AMP contents decreased in the dark and increased in the light (Fig. 4B, C). Because ATP rose under darkness and declined under light, AEC followed the same trend (Fig. 4D). As we reported previously (Goh et al., 2004), light influences ATP production in both wild-type and chlorina mutants. This might be explained by a photoreceptor-mediated transcriptional control (Escobar et al., 2004), but still in question is how the cellular mechanisms are regulated. Nevertheless, these results suggest that, in this case, a balance in cellular energy is involved in the process through which compensation is achieved.

Knowing how this cellular energy balance controls the interactions among chloroplasts, the nucleus, mitochondria, and plasma membrane is central to understanding how cell metabolism is modulated. Our current study used proteomic analysis of the rice *oschlh* knockout mutant to demonstrate that a chloroplast-targeted mutation affects other physiological functions in photosynthetic cells. We showed that, in response to a mutation within a single gene, cellular metabolism can adapt in a compensatory manner to reduce the consumption of common key metabolites. Mitochondrial proteins were not highly detected here due to the large

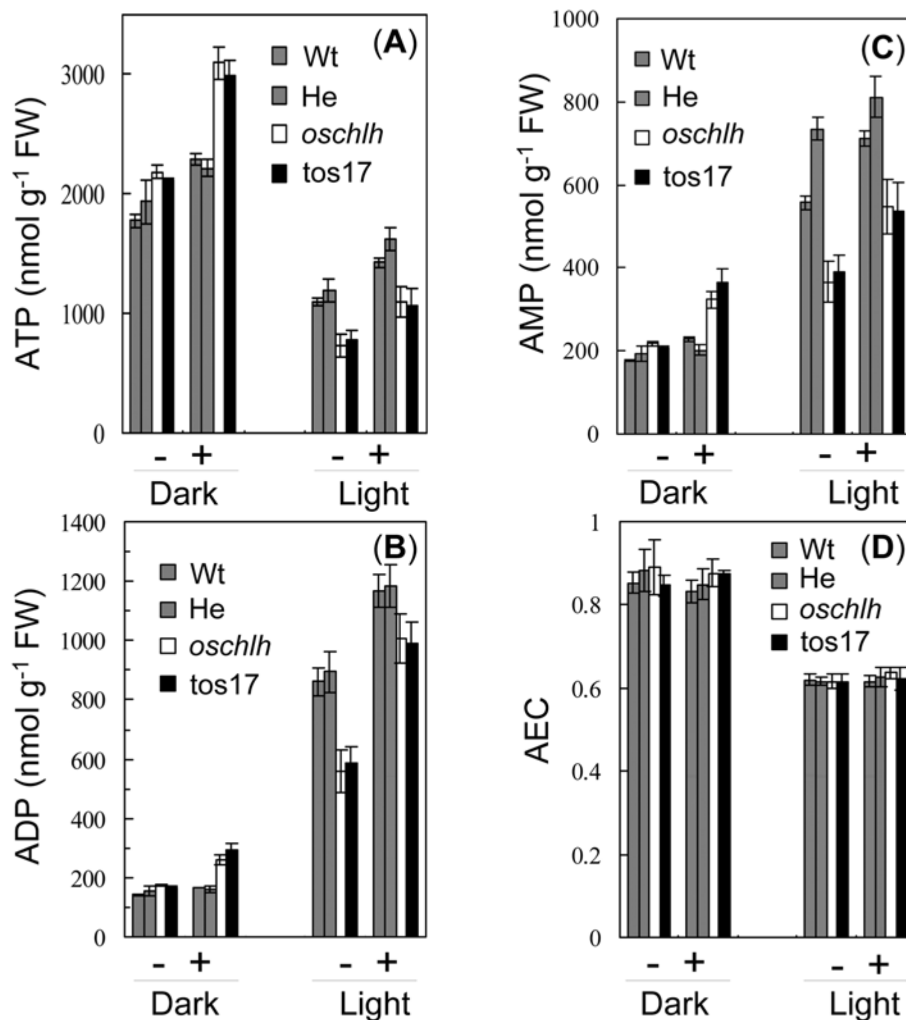

Figure 4. ATP (A), ADP (B), and AMP (C) contents and adenylate energy charge (AEC, D) in rice leaves. Wt, wild type; He, heterozygous; *oschlh* and tos17, chlorophyll-deficient rice mutants. – and + indicate absence and presence, respectively, of 3% sucrose in growth medium. Values are means of three experiments ± SE.

amounts of Rubisco and/or the limitation of methodological problems in protein isolation. Rubisco, the most abundant protein (about 50% of soluble protein) in leaf samples, can hinder high resolution of 2-DE because of restrictions in loading capacity on the IEF gel. Therefore, low-abundance proteins remain difficult to analyze (Kim et al., 2001) even though some methods have now improved the solubility of membrane proteins by employing reagents (Rabilloud et al., 1997). Here, fold-change differences in typical cDNAs that appeared to be physiologically and differentially expressed in the mutant seedlings were confirmed by real-time PCR (Table 1). Based on our current and earlier physiological results (Goh et al., 2004), we predict that this re-programming will affect the physiological functioning of other organelles during plant growth and development. The *ochlh* encodes Mg^{2+}-chelatase, which is involved in chlorophyll biosynthesis (Jung et al., 2003) but not directly associated with the regulation of membrane organization, movement of ion (K^+) currents across membranes, or K^+ concentrations within cells (Goh et al., 2004). We have now found that a mutation in a single gene can profoundly affect totally unrelated processes within a cell. Furthermore, under some circumstances, the cell is able to respond by activating systems capable of compensating for the loss of the protein product of the mutant gene. Such systems may involve stochastic events (Raser and O´Shea, 2004). We suggest, therefore, that the primary signal sensed in this complex of an intra-cellular signaling network is the cytosolic ATP concentration of the cell during growth and development of the *oschlh* rice mutant.

ACKNOWLEDGEMENTS

We wish to thank Professor Hetherington for his helpful comments on the manuscripts. This work was supported by grants from the Korea Research Foundation (KRF-2004-015-C00532) and BioGreen 21 (20050401-034-677-140-03-00), as well as partly an EBRC grant from KOSEF (R15-2003-012-01001-0), Gyeongsang National University, Korea.

Received May 1, 2007; accepted June 6, 2007.

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