Transcript levels of the nuclear-encoded respiratory genes in rice decrease by oxygen deprivation: evidence for involvement of calcium in expression of the alternative oxidase 1a gene

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Abstract **We investigated the effect of oxygen on the expressions of respiratory genes encoded in the nuclear and mitochondrial genomes of rice (*Oryza sativa* L.). Hypoxic treatment decreased the transcript levels of nuclear-encoded, but not mitochondrial-encoded respiratory genes. The effects of ruthenium red (an inhibitor of Ca^{2+} fluxes from organelles) and/or $CaCl_2$ on plants under hypoxic conditions suggested that Ca^{2+} is a physiological transducer of a low-oxygen signaling pathway for expression of the alternative oxidase 1a gene (*AOX1a*), but not for expressions of genes involved in the cytochrome respiratory pathway, in rice.**

Key words: Oxygen deprivation; Mitochondrion; Respiratory gene; Calcium; *Oryza*

1. Introduction

Most organisms depend on oxygen and have sensory systems to gauge oxygen availability. The response to oxygen deprivation (hypoxia or anoxia) of plants, as in yeasts and animals, is comprised of complicated biochemical and genetic programs that include the differential expressions of a large number of genes (see [1–3] for reviews). As a result of flooding, anaerobiosis rapidly represses the synthesis of pre-existing proteins and induces the synthesis of new anaerobic proteins [4–6]. The anaerobic proteins include enzymes involved in sugar phosphate metabolism, glycolysis and alcoholic fermentation (e.g. sucrose synthase, glyceraldehyde-3-phosphate dehydrogenase, aldolase, pyruvate decarboxylase and alcohol dehydrogenase (ADH)) [2]. In both maize and *Arabidopsis thaliana* seedlings, oxygen deprivation elevates the cytosolic Ca^{2+} ($[Ca^{2+}]_c$) level through intracellular Ca^{2+} flux, and ruthenium red (RR), which is thought to be a blocker of Ca^{2+} fluxes from organelles, represses induction of expressions of anaerobiosis-inducible genes such as *ADH* [7,8]. In maize, it has been suggested that the release of Ca^{2+} from mitochondria plays an important role in the elevation of $[Ca^{2+}]_c$ under anaerobic conditions and participates in the signaling of oxygen deprivation [9,10].

Biogenesis of respiration-competent mitochondria depends on the presence of oxygen, which is the final acceptor in the respiratory electron transfer chain. The components involved in the respiratory chain are encoded by both the nuclear and mitochondrial genomes in most eukaryotes. In *Saccharomyces cerevisiae*, it is known that oxygen regulates the expressions of most nuclear-encoded genes involved in the respiratory chain at the transcriptional level, and regulates the expressions of some mitochondrial-encoded respiratory genes at the translational level (see [11] for review). In plants, however, it is unclear whether the availability of oxygen affects the expressions of nuclear-encoded and mitochondrial-encoded respiratory genes. In this study, we demonstrate the regulation of expression of nuclear-encoded respiratory genes by oxygen in rice. Furthermore, we describe the involvement of $[Ca^{2+}]_c$ in the expression of a nuclear-encoded respiratory gene, alternative oxidase 1a (*AOX1a*), under anaerobic conditions.

2. Materials and methods

2.1. Plant material, growth conditions and treatments

Rice (*Oryza sativa* L. cv. Nipponbare) seedlings were grown in the light at 28°C for 7 days. For hypoxia treatment, aerobically-grown seedlings were submerged in the dark at 28°C for 24 h. The submerged seedlings were then transferred to aerobic conditions in the dark at 28°C for 24 h. For RR treatment, seedlings were submerged in water that contained 100 μM RR (Sigma Chemical, USA) and/or 5 mM $CaCl_2$ (Wako Pure Chemical Industries, Tokyo, Japan) in the dark at 28°C for 24 h. For RNA extraction, leaves of seedlings were harvested and immediately frozen in liquid nitrogen.

2.2. Extraction of total RNA and Northern hybridization

Rice total RNA was extracted by the standard sodium dodecyl sulfate/phenol method [12]. Electrophoresis of RNA and Northern hybridization were performed by the method of Saisho et al. [13]. The intensities of signals obtained by Northern blot hybridization were quantified using a Lumino-image analyzer LAS-1000 (Fujifilm, Japan) and Image Gauge software (Fujifilm, Japan). Each blot was hybridized only with the specific probe and equal loading of RNA was confirmed by an rRNA blot that was included in the same experimental set. The experiments were repeated two more times and the same results were obtained.

3. Results

3.1. Effects of hypoxia on steady-state levels of mRNA encoding genes for the respiratory chain

Unlike the mitochondria of animals and yeasts, mitochondria of higher plants contain two quinol-oxidizing pathways: one is the cyanide-sensitive cytochrome pathway, and the other is an alternative pathway that is not sensitive to cyanide (see [14] for review). The terminal oxidases of these pathways

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Abbreviations: RR, ruthenium red; $[Ca^{2+}]_c$, cytosolic Ca^{2+}

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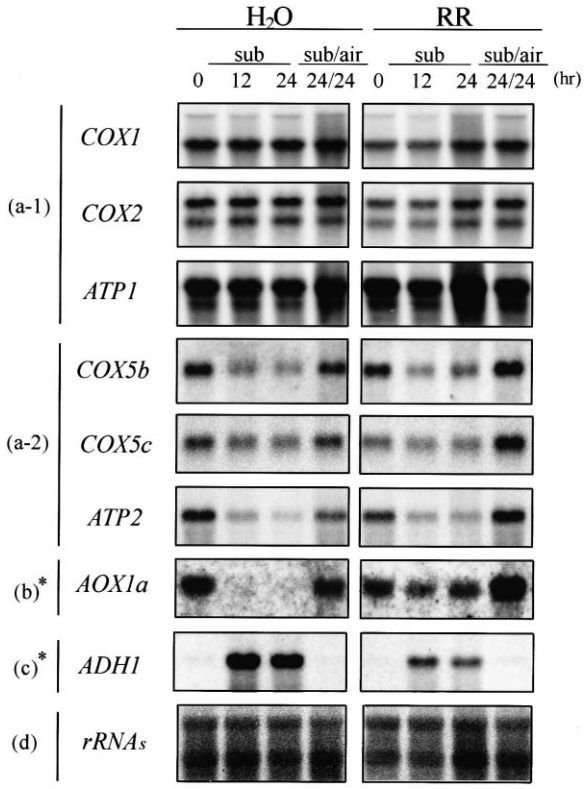

Fig. 1. Effect of oxygen deprivation on the expressions of mitochondrial-encoded and nuclear-encoded genes of rice. 7-day-old aerobically-grown rice seedlings were submerged in water (H_2O) or in 100 μM RR for 12 and 24 h (sub 12 and sub 24), and then, 24 h submerged seedlings were transferred to aerobic conditions where they were kept for 24 h (sub/air 24/24). Total RNA (5 μg) from each treatment was used to determine the transcript levels by Northern hybridization. Gene types (a-1), (a-2), (b), (c) and (d) are explained in Table 1. Asterisks indicate genes whose expressions were influenced by RR treatment.

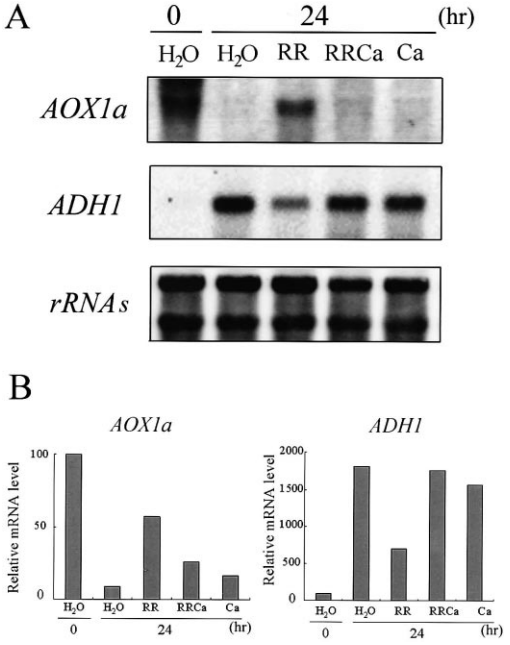

Fig. 2. Ca^{2+} prevents RR repression of hypoxic gene expression in rice seedlings. Aerobically-grown rice seedlings were submerged in water (H_2O), 100 μM RR (RR), both 100 μM RR and 5 mM $CaCl_2$ (RRCa) and 5 mM $CaCl_2$ (Ca) for 24 h. (A) Northern hybridization using *AOX1a*, *ADH1* and rRNA genes as probes. (B) Quantification of relative mRNA levels of *AOX1a* and *ADH1* shown in A. All mRNA levels were normalized to the rRNA level and are presented as a percent of that observed at 0 h in H_2O.

are cytochrome *c* oxidase (COX) and AOX, respectively. The components involved in the cytochrome pathway are encoded by both the nuclear and mitochondrial genomes. In contrast, AOX is composed of a homodimer and is encoded by a multigene family in the nuclear genome [13–16]. To investigate whether the availability of oxygen affects the expressions of nuclear-encoded and mitochondrial-encoded respiratory genes in rice, in this study, three mitochondrial-encoded genes (*COX1*, *COX2* and *ATP1*) and three nuclear-encoded genes (*COX5b*, *COX5c* and *ATP2*) for the cytochrome pathway and one nuclear-encoded gene (*AOX1a*) for the alternative pathway were chosen as probes for Northern hybridization (Table 1). As controls, the anaerobiosis-inducible gene *ADH1* and the genes for 25S/17S rRNAs were used (Table 1). 7-day-old aerobically-grown seedlings of rice were submerged in water for 24 h. After 24 h of the hypoxic treatment, the submerged seedlings were transferred to aerobic conditions for 24 h. Total RNA was isolated from seedlings submerged for 0, 12 and 24 h (Fig. 1, lanes 0, sub 12 and sub 24, respectively) and from aerobically recovered seedlings (Fig. 1, lane sub/air 24/24),

Table 1
Genes examined in this study

Gene and protein names	Accession number	Reference
(a) Genes involved in the cytochrome respiratory pathway		
(a-1) Mitochondrial-encoded		
COX1	X15990	[18]
COX2	X01088	[19]
ATP1 (F_1F_0-ATP synthase α subunit)	D50567	[20]
(a-2) Nuclear-encoded		
COX5b	D85381	[21]
COX5c	AB027123	[22]
ATP2 (F_1F_0-ATP synthase β subunit)	D10491	[23]
(b) Genes involved in the alternative respiratory pathway		
AOX1a	AB004864	[24]
(c) Genes involved in the alcoholic fermentation		
ADH1	X16296	[25]
(d) Genes for ribosomal RNA		
25S/17S rRNAs	M16845	[26]

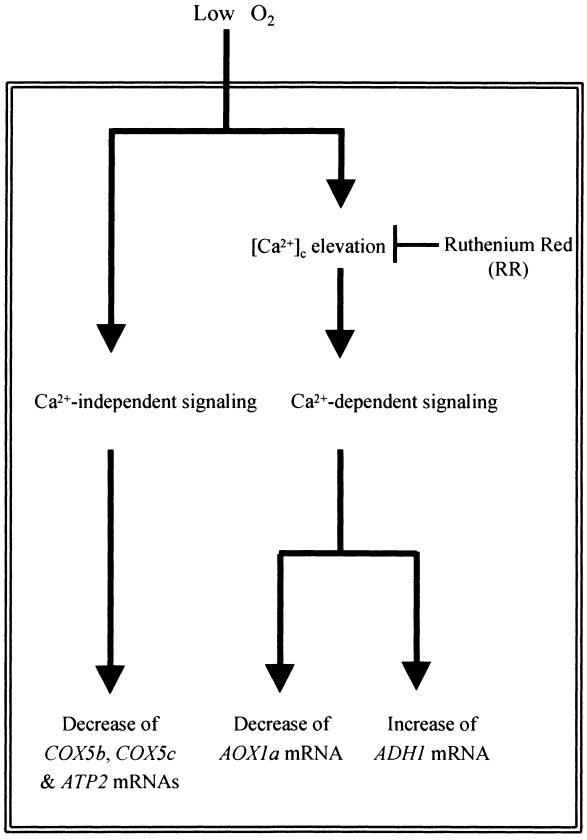

Fig. 3. A model for low-oxygen signaling pathways in rice. One is a Ca^{2+}-dependent pathway (right) and the other is a Ca^{2+}-independent pathway (left). RR blocks elevation of the $[Ca^{2+}]_c$ level.

and Northern hybridization was performed using the probes listed in Table 1. At first, the hypoxia-inducible *ADH1* gene was used to verify the low-oxygen status in the seedlings in each experiment. In response to the hypoxic treatment, the *ADH1* mRNA level dramatically increased (Fig. 1, left panel (H_2O)), confirming that the submerged seedlings were suffering from oxygen deprivation. Under these conditions, transcript levels of the mitochondrial-encoded *COX1, COX2* and *ATP1* genes were constant, regardless of the presence or absence of oxygen (Fig. 1). In contrast, the amounts of mRNA of the nuclear-encoded respiratory genes (*COX5b*, *COX5c*, *ATP2* and *AOX1a*) decreased after 12 and 24 h of oxygen deprivation (Fig. 1). When the 24 h submerged seedlings were allowed to recover in air for 24 h, we observed a recovery in the mRNA levels of the nuclear-encoded respiratory genes in these seedlings (Fig. 1). Quantification of transcript levels revealed that (1) *AOX1a* mRNA decreased by 10% compared with the mRNA levels under aerobic conditions, (2) *COX5b* and *ATP2* mRNAs decreased by 30–40%, and (3) *COX5c* mRNA decreased by 60% after both 12 and 24 h of oxygen deprivation. When the aerobically-grown seedlings were treated in the dark under aerobic conditions for 12 and 24 h as a control, the transcript levels of the genes examined were constant (data not shown).

3.2. RR suppresses the decrease in the steady-state level of *AOX1a* mRNA under hypoxic conditions

As mentioned in Section 1, it has been proposed that the expression of *ADH1* in maize cells is regulated by $[Ca^{2+}]_c$, which might be released from mitochondria under anoxia [7,9,10]. Here, it was of interest to examine whether the decreases in the amounts of mRNAs of the nuclear-encoded respiratory genes in rice are also regulated by oxygen deprivation-induced Ca^{2+} elevation. RR is thought to block Ca^{2+} fluxes from organelles [17], and treatment of maize cells by RR suppressed induction of ADH1 under submerged conditions [7]. We investigated the effect of RR on the expressions of the respiratory genes after 12 and 24 h of hypoxia. Treatment of rice seedlings with 100 μM RR under hypoxic conditions resulted in a steady-state level of *ADH1* mRNA that was 40–50% of the level induced in seedlings in water (without RR) (Fig. 1, right panel (RR) and Fig. 2). Thus, RR inhibits the induction of expression of *ADH1* by oxygen deprivation in rice, as it does in maize. As for the respiratory genes, RR dramatically blocked anaerobic repression of *AOX1a* mRNA (Fig. 1, right panel), whereas it had no significant effect on the transcript levels of nuclear-encoded genes (*COX5b*, *COX5c* and *ATP2*) and mitochondrial-encoded genes (*COX1, COX2* and *ATP1*) involved in the cytochrome pathway. On the other hand, addition of RR to rice seedlings did not affect the expressions of the genes examined here under aerobic conditions (data not shown). These results indicated that, under hypoxia, RR affects the expressions of *AOX1a* as well as *ADH1*, but not the expressions of the other cytochrome respiratory genes.

To test the hypothesis that RR inhibits the expressions of *ADH1* and *AOX1a* by specifically blocking intracellular Ca^{2+} signaling, seedlings were treated for 24 h in the presence of 100 μM RR with 5 mM $CaCl_2$ under hypoxic conditions. $CaCl_2$, when supplied with RR at the same time, prevented the effect of RR on the expressions of *ADH1* and *AOX1a* (Fig. 2, RRCa). $CaCl_2$ itself did not affect gene expression under hypoxic conditions (Fig. 2, Ca). These results suggested that a release of Ca^{2+} from intracellular Ca^{2+} stores is a physiological transducer of oxygen deprivation signaling in rice, subsequently leading to a decrease of *AOX1a* mRNA and an increase of *ADH1* mRNA.

4. Discussion

The results presented here provide several new insights concerning regulation of genes involved in the respiratory chain in plants. Transcripts of nuclear-encoded respiratory genes, but not mitochondrial-encoded respiratory genes, were markedly reduced in rice seedlings during the first 12 h of hypoxia, and returning the seedlings to normal aerobic conditions resulted in recovery of their transcript abundance (Fig. 1). These results suggest that nuclear-encoded genes in rice that are involved in aerobic metabolic pathways such as respiration are down-regulated at the transcriptional or post-transcriptional level in response to low oxygen. This down-regulation may be because expressions of respiratory genes are not needed under hypoxia, which causes low efficiency of respiration.

Translation of mRNA is emerging as an important mode of gene regulation in plants. Bailey-Serres's group [5,6] demonstrated that, in maize, oxygen deprivation can result in the global repression of initiation of translation and selective translation of mRNAs that encode anaerobic proteins. Although nuclear-encoded respiratory genes in rice are down-regulated at the transcriptional or post-transcriptional level by hypoxia, it is possible that their expressions are re-

pressed even more at the translational level, as they are in maize.

On the other hand, the steady-state levels of the mRNAs of mitochondrial-encoded genes of the cytochrome respiratory pathway were not significantly influenced by oxygen deprivation. In yeast, expressions of several mitochondrial-encoded respiratory genes are regulated at the translational level in an oxygen-dependent manner, by some translational activators such as PET494 and PET111 (see [11] for review). Similarly, it is possible that the translational efficiency of the mitochondrial-encoded genes may be an important mode of oxygen regulation in plants.

Previously, Sachs and his colleagues [2] proposed a model for maize in which oxygen deprivation-induced changes in *ADH1* gene expression are preceded by $[Ca^{2+}]_c$ elevation. Our findings are consistent with this model and provide a new insight regarding the respiratory *AOX1a* gene. We suggest that hypoxia-induced elevation of $[Ca^{2+}]_c$, which might be preceded by a release of Ca^{2+} from intracellular stores, is involved in an early step in the oxygen signaling pathway for *AOX1a* and *ADH1* (Fig. 3). It is possible that RR (100 μM) also affects the calcium flux across the plasma membrane, and that the hypoxia-induced elevation of $[Ca^{2+}]_c$ originates from extracellular stores as well as intracellular stores [8]. In contrast to expressions of *AOX1a* and *ADH1*, expressions of the cytochrome respiratory genes were not affected by RR. The hypoxia-induced $[Ca^{2+}]_c$ elevation may not trigger a decrease in the mRNAs of these genes. Thus, the accumulation of *AOX1a* mRNA is Ca^{2+}-dependent but the accumulations of *COX5b*, *COX5c* and *ATP2* mRNAs are not. Further studies will be needed to understand the reason why expression of *AOX1a* is more tightly regulated than those of the other cytochrome respiratory genes under anaerobic conditions. In conclusion, our findings indicate that there are at least two pathways of oxygen signaling in rice: a Ca^{2+}-dependent pathway and a Ca^{2+}-independent pathway (Fig. 3), implying that there are multiple mechanisms/pathways involved in modulating the expression of nuclear-encoded genes under hypoxia in plants.

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