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Influence of irradiation on cyanide-resistant respiration and *AOX1* multi-gene family expression during greening of etiolated rice seedlings

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Abstract

We investigated the differential expression of *AOX1* multi-gene family and the regulation of alternative respiratory pathway during initial greening development in leaves of rice (*Oryza sativa* L.) seedlings. After exposing the dark-grown rice seedlings to continuous irradiation, total respiration (V_t), capacity of alternative pathway (V_{alt}), and their ratio (V_{alt}/V_t) increased with the greening of leaves. In this process, *AOX1c* transcript increased under constant irradiation, while *AOX1a* and *AOX1b* transcripts were hardly detected. Thus *AOX1c* in rice presents a similar expression pattern as *AOX2* does in many dicotyledonous species during greening development. Compared with the rapid increase of cyanide-resistant respiration in the presence of photon energy, CO_2 fixation was not observed until 8 h after the onset of irradiation. The AOX inhibitor salicylhydroxamic acid (SHAM; 1 mM) inhibited 67.3 % of cyanide-insensitive oxygen uptake in dark-grown leaves and 69.4 % of it in leaves grown under irradiation. Dark-grown plants pre-treated with SHAM were then irradiated for 12 h. SHAM did not obviously modify photosynthetic CO_2 fixation rate on a chlorophyll (Chl) content basis in both leaves and simultaneously isolated chloroplasts. Hence during initial greening steps of the plants, the induction of alternative pathway and *AOX1* expression by irradiation is not directly linked with carbon assimilation of photosynthesis. The application of SHAM partially limited Chl production in rapidly greening leaves, indicating that Chl synthesis in the process of greening might be medicated to some extent by alternative respiratory pathway.

Additional key words: chlorophyll; chloroplast; CO_2 fixation; inhibitors; *Oryza*; oxygen uptake; salicylhydroxamic acid.

Introduction

Cyanide-resistant respiration is connected with the presence in the respiratory chain of an additional terminal oxidase—alternative oxidase (AOX, a key enzyme of alternative respiratory pathway). AOX branches from the main respiratory chain at the level of ubiquinone and catalyses the four-electron reduction of oxygen to water (Millenaar and Lambers 2003). This respiratory pathway produces heat instead of ATP in appendix tissue during flowering of *Suaromatum guttatum*, a thermogenic plant (Vanlerberghe and McIntosh 1994).

AOX is present in all angiosperms, many algae and some fungi also contain the genetic capacity to express this respiratory pathway (Vanlerberghe and McIntosh 1997). By the use of techniques such as cDNA library and polymerase chain reaction (PCR), AOX is encoded by a small family of nuclear genes among a wide variety of non-thermogenic monocotyledon and eudicotyledon plants such as tobacco (*Nicotiana rustica* L.; Vanlerberghe and McIntosh 1994, Whelan *et al*. 1995), *Arabidopsis* (Saisho *et al.* 2001, Clifton *et al.* 2005), soybean (*Glycine max*; McCade *et al.* 1998), and wheat (*Triticum aestivum* L.; Takumi *et al.* 2002). Differential expression of the *AOX* multi-family genes in responses to developmental cues and environmental perturbations raises a possibility that AOXs in plants have different roles (Considine *et al.* 2002, Clifton *et al.* 2005).

The irradiation-dependent expression of mitochondrial genes suggests that there is a close association between photosynthesis and mitochondrial metabolism (Svensson and Rasmusson 2001). Irradiation induces alternative respiratory pathway and changes the expression of AOX genes (Obenland *et al.* 1990, Finnegan *et al.* 1997, Ribas-Carbo *et al.* 2000). In previous reports, alternative oxidase activity can be induced from a low

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amount when the tissue becomes photosynthetically active or has a higher saccharide content (Azcón-Bieto *et al.* 1983, Lennon *et al*. 1995). Recently, Clifton *et al*. (2005) revealed that the compounds that affect chloroplast function increased expression of *AOX* gene. All this suggests that AOX activation in light is associated with photosynthetic metabolites. The reducing power generated in chloroplasts through photosynthesis may be exZted as malate to the cytosol and used in mitochondria to stimulate the activity of alternative oxidase (Day and Wiskich 1995, Padmasree and Raghavendra 1999, Van Lis and Atteia 2004). Based on this hypothesis, an activation of alternative pathway in light could be of great benefit to dissipation of excess reduced equivalents for chloroplasts, or for optimization of photosynthetic carbon assimilation (Hoefnagel *et al*. 1998, Svensson and Rasmusson 2001, Padmasree *et al.* 2002, Raghavendra and Padmasree 2003, Bartoli *et al.* 2005).

When dark-grown soybean cotyledons were irradiated, a significant increase of alternative pathway has been observed as soon as during first hours of irradiation (Ribas-Carbo *et al.* 2000). However, unlike cotyledons of soybean and other dicotyledonous species, leaves of monocots are neither storage tissues nor main photosynthetic organs in the initial steps of plant growth (Harris *et al*. 1986). Thus, an attention to the initial greening of monocotyledon plants contributes to further understanding of the relationship between irradiation (or photosynthesis) and alternative pathway. Unfortunately, the effect of irradiation on alternative pathway in greening monocotyledon plants is studied only rarely.

Moreover, based on the studies of molecular distinction among alternative oxidases from plant species, *AOX1* is known for its induction by stress stimuli in many tissues and is present in both monocots and dicots, but *AOX2* is usually constitutive or developmentally expressed in dicots and is absent from the genomes of all monocot species examined to date (Considine et al. 2002). In etiolated soybean cotyledons, the *AOX3* transcript was most abundant while the expressions of *AOX2* and *AOX1* were much less. But after irradiation, the increase of AOX activity is accompanied by the increased *AOX2* transcript abundance and the *AOX3* transcript abundance is decreased (Finnegan *et al*. 1997). Escobar *et al*. (2004) suggest that *Arabidopsis AOX2* (analogous to soybean *AOX3*) transcript abundance is repressed after irradiation but other members of AOX genes would be enhanced by irradiating. The expression pattern of *AOX1s* in greening monocot species is still unclear.

Presently, more work is needed to obtain a more detailed picture of the alternative respiratory pathways and its relative events in light, especially concerning different plant species with different physiological features. Three genes that encode AOX have been isolated from nuclear genes of rice (*Oryza sativa* L.) (Saika *et al.* 2002). Based on phylogenetic analysis, these *AOX* genes were designated as *AOX1a*, *AOX1b*, and *AOX1c*. They accumulate differently at low temperature or in various organs (Saika *et al.* 2002). These genes would allow us to investigate the differential expressions of *AOX1* genes and possible changes of alternative pathway detected in greening monocot species. Moreover, gradual evolution of photosynthetic apparatus in greening leaves of monocots (Baker 1984) makes it possible to elucidate the relationship between greening and AOX pathway.

Materials and methods

Plants and treatment: Rice (*Oryza sativa* L. sd.301) seeds (Luoyang Academy of Agricultural Sciences, China) were treated with 1 % NaOCl for 10 min and swollen in distilled water for 10 h with continuous bubbling of air at the normal temperature. These rice seeds were germinated and grown under complete darkness or with photoperiod of 12 h (were performed using white fluorescent tubes) at 26±1 °C for 10 d.

Salicylhydroxamic acid (SHAM), an inhibitor of AOX activity, has been used in AOX studies in intact tissues (Chivasa *et al*. 1997, Chivasa and Carr 1998, Naylor *et al.* 1998). To determine the appropriate concentration of SHAM, we mimicked a titration experiment similarly to a most recent work (Bartoli *et al.* 2005). The cytochrome *c* pathway was inhibited with 1 mM KCN, whereas the AOX pathway was inhibited with 0.1–10.0 mM SHAM. Both respiratory inhibitors were supplied for 4 h through the cut tips of leaves attached to the plant that were grown under darkness or with photoperiod of 12 h. Thereafter, apical leaves were cut into 2 cm long segments, placed in the oxygen electrode chamber, and respiration measured as described below.

According to the titration experiment, 1 mM SHAM was used in the subsequent experiments. As described by Bartoli *et al*. (2005), parts of 9-d-old etiolated seedling leaves (attached to the plant) were supplied with 1 mM SHAM (1 M stock solution freshly prepared in methoxyethanol) for 4 h through the cut tips of leaves and maintained at dark at 26±1 °C. Thereafter, these rice seedling leaves pre-treated with SHAM were transferred from darkness to continuous 12 h of irradiation (performed with “white” fluorescent tubes). Control experiments showed that supplements of the solvent alone to leaves had no effect on any of the measured experimental parameters (data not shown). The plants kept under darkness were controls.

Respiration of leaves: The apical 2 cm of leaves were detached and cut into six pieces with a razor blade. Pieces were transferred into an air-tight cuvette. The total respiration rate (V_t) and the capacity of cyanide-resistant respiration (V_{alt}) were measured at 26 °C using a Clark-

type electrode (Institute of Plant Physiology & Ecology, Chinese Academy of Sciences) by the method of Bingham and Farrar (1989).

Preparation of special probes for *AOX1a*, *AOX1b*, and *AOX1c*: Probes were made by PCR. Primer 1 and Primer 2 were used to obtain a specific probe for *AOX1a*, Primer 3 and Primer 4 for a specific probe for *AOX1b*, and Primer 5 and Primer 6 for a specific probe for *AOX1c*. The rice total DNA was extracted from the leaves as template. PCR with *Taq* DNA polymerase (*Sangon*, China) was carried out for 37 cycles, each consisting of 30 s at 95 °C, 30 s at 45 °C, and 1 min at 72 °C. The products were extended at 72 °C for 5 min and held at 4 °C for 1 min. The PCR products were cloned into the Pgt (Fig. 1) Vector and transformed into the DH5α-strain. Nucleotide sequences of the inserts were determined by using the automatic DNA sequencer (*Beckman*, USA). The DNA sequencing data of PCR production were analyzed with the *chromas1.45* software.

P1 : 5`-GATGTTTGTCTACTGCCGAGGATTT-3`

P2 : 5`-ATGTAGTATATATAACTCAGCTGCC-3`

P3 : 5`-TCATCATTCATCAACGGGCGATGC-3`

P4 : 5`-TGTGCACGGGTCAGCCAACGGCCA-3`

P5 : 5`-CTGAAGAAATCTTACGGCGG-3`

P6 : 5`-CCAAACAGATAACAGGACGC-3`

Extract for the total RNA and Northern hybridization: Total RNA was extracted with Total RNA *Trizol* Extraction Kit (*Sangon*, China). Total RNA was quantified using the UV-VIS spectrophotometer *Tu-1800* (*Purkinje General*, China). The integrity of RNA was checked by loading in per lane on 0.8 % agarose–1 × TAE gel, separated by electrophoresis. Equal loading of the RNA (based on OD_{260}) was confirmed by ethidium bromide staining. Northern hybridization was performed with the ECL DNA Labelling and Detection Kit (*Enzo Diagnostics*, UK) according to the manufacturer's instructions.

Leaf CO_2 fixation rate: Apical 2-cm parts of leaves were detached and cut into pieces with a razor blade. The capacity of carbon fixation of the leaves was monitored as HCO_3-dependent O_2 evolution by the methods reported by Padmasree and Raghavendra (1999).

Chlorophyll (Chl) contents were determined following the method of Arnon (1949) with some modification. The apical 2 cm of leaves were cut into small pieces and 100 mg of such masses were placed into a mortar and crushed thoroughly with a pestle. Ten cm^3 of 80 % acetone was added to allow the tissue to be thoroughly homogenized and centrifuged at 4 000 rpm for 5 min to obtain the supernatant. Absorbance of the Chl solution was recorded at 663 and 645 nm wavelengths by spectrophotometer. 80 % acetone solution served as control.

Chloroplast isolation: The apical 2-cm pieces of leaves were ground in buffer containing 50 mM HEPES, pH 7.6, 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 5 mM ascorbic acid, and 0.05 % (m/v) bovine serum albumin using a homogenizer. The homogenate was filtered through a 20-μm pore size nylon mesh and centrifuged at 3 000×*g* for 5 min. The pellet was suspended in wash medium (50 mM HEPES, pH 7.6 and 330 mM sorbitol), loaded in a solution consisting of 35 % (v/v) *Percoll* in wash medium, and centrifuged at 2 500×*g* for 5 min. The pellet containing intact chloroplasts was used to measure CO_2 fixation rate by the methods of Lurie (1977).

Statistical analysis: Results are presented as the mean ± standard deviation. Data were analyzed using the Kruskall-Wallis one-way analysis of variance test. $p<0.05$ was considered statistically significant.

Results

Cyanide-resistant respiration changes during the greening of dark-grown rice seedling leaves: Total respiration rate (V_t) of 10-d-old etiolated leaves increased from 4.67 before irradiation to 9.17 μmol(O_2) kg^{-1}(FM) s^{-1} after 12 h of irradiation (Table 1). The AP (alternative pathway) capacity (V_{alt}) increased from 0.50 to 3.33 μmol(O_2) kg^{-1}(FM) s^{-1} (Table 1). V_{alt}/V_t, calculated with the average values of V_{alt} and V_t, was approximately 10.7 % before irradiation. An increase in V_{alt}/V_t was observed when etiolated leaves were irradiated for only 1 h (near 17.2 %), indicating mitochondrial electron partitioning through the alternative pathway is very sensitive to the transition from dark to light. After 12 h of irradiation, V_{alt}/V_t reached around 36.4 % (Table 1). These results suggest a relative contribution of the alternative pathway to total respiration in greening process.

Expression of rice *AOX1* multi-gene family during the greening of dark-grown rice seedling leaves: The specific probes for *AOX1a*, *AOX1b*, and *AOX1c* were used to investigate the effect of irradiation on rice *AOX1* expression. The steady-state mRNA of *AOX1a*, *AOX1b*, and *AOX1c* were almost undetectable in etiolated rice seedling leaves. During continuous 12 h of irradiation, *AOX1c* transcript increased with the time of irradiation (Fig. 1). Combining this with the results shown in Table 1, irradiation increased the *AOX* transcript resulting in an enhancement in the capacity of AOX pathway, and AOX

pathway in greening is regulated by radiant energy, at least in part by changes in *AOX1c* abundance.

But both *AOX1a* and *AOX1b* transcripts were hardly observed in this process (Fig. 1). *AOX1b* gene is located about 1.9 kb upstream of the *AOX1a* gene, and its expression is almost the same as those of rice *AOX1a* in various organs or at low temperature (Saika *et al.* 2002). In this case, *AOX1a*/*AOX1b* and *AOX1c* are regulated differentially by radiant energy.

Table 1. Changes of total respiration rate (V_t) and capacity of alternative pathway (V_{alt}) [μmol(O_2) kg^{-1}(FM) s^{-1}], and V_{alt}/V_t (the ratio of capacity of alternative pathway to total respiration rate) [%] during continuous 12 h of irradiation or under continuous 12 h of darkness as control. These are individual samples taken during six independent experiments. Time indicates hours after starting irradiation. Means ± SD. V_{alt}/V_t was calculated from the average of V_t and V_{alt} experiments carried out separately.

Time [h]	V_t		V_{alt}		V_{alt}/V_t	
	Irradiation	Dark	Irradiation	Dark	Irradiation	Dark
0	4.67±0.33	4.67±0.33	0.50±0.17	0.50±0.17	10.71	10.71
1	4.83±0.33	4.33±0.17	0.83±0.17	0.50±0.33	17.24	11.53
2	5.00±0.33	4.33±0.17	1.00±0.33	0.67±0.33	20.00	15.38
4	6.33±0.17	4.83±0.50	1.67±0.33	0.33±0.17	26.31	6.90
6	7.67±0.67	4.50±0.67	2.17±0.17	0.67±0.33	28.26	14.81
8	8.50±0.67	4.83±0.04	2.67±0.33	0.50±0.17	31.37	10.34
10	8.84±0.50	4.83±0.50	3.17±0.17	0.67±0.33	35.85	13.79
12	9.17±0.50	4.17±0.50	3.33±0.33	0.67±0.17	36.36	16.00

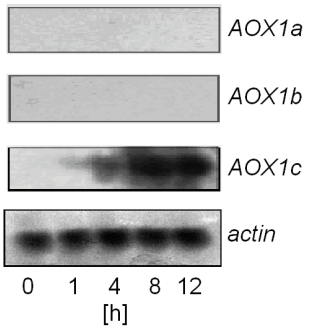

Fig. 1. *AOX1a* , *AOX1b,* and *AOX1c* transcript levels during continuous 12 h of irradiation (0, 1, 4, 8, and 12 h).

AOX inhibition: In these experiments, *in vivo* V_{alt} was reduced by 22–85 % when leaves were fed with 0.1–10.0 mM SHAM (Fig. 2). Treatment with 1 mM SHAM inhibited cyanide-insensitive oxygen uptake by 67.3 % in dark-grown leaves (10-d-old) (Fig. 2). Although green leaves might have higher capacity of AOX pathway than dark-grown ones, 1 mM SHAM inhibited cyanide-insensitive oxygen uptake by 69.4 % in 10-d-old leaves grown with photoperiod of 12 h. Therefore, 1 mM SHAM was used in the subsequent experiments to determine the possible function of AOX in greening plants.

Influence of SHAM on Chl contents during greening of dark-grown rice leaves: Chl content was almost undetectable before irradiation, but it rapidly accumulated after the etiolated plants were irradiated (Fig. 3*A*). In the first 2 h of irradiation, Chl content increased to 61 mg kg^{-1}(FM). After 12 h of irradiation, the Chl content reached 353 mg kg^{-1}(FM).

Plants treated with SHAM were irradiated for 12 h. In the process of the first 2 h of greening, there was no significant difference in Chl content between the leaves pre-treated and untreated with SHAM. But during the following 4–12 h of irradiation, the Chl content was reduced in SHAM-treated greening plants, compared with the control (irradiated leaves untreated with SHAM). The decrease was most pronounced at 6 h of irradiation (about 82 % of control). After 12 h of irradiation, Chl content in the SHAM-treated plants was 91 % of that in control (barely significant difference with control).

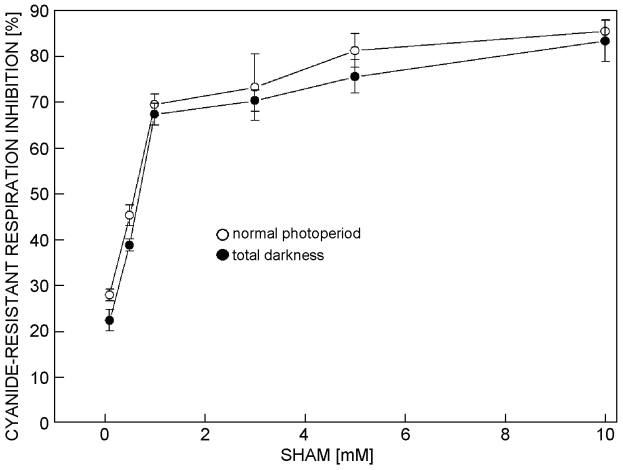

Fig. 2. *In vivo* inhibition of AOX activity in 10-d-old rice leaves treated with 1 mM KCN and SHAM from 0.1 to 10.0 mM. *Open and filled circles* correspond to leaves grown in normal photoperiod (12/12 h day/night) and total darkness, respectively. Oxygen uptake by leaves treated with 1 mM KCN in the absence of SHAM was denoted as the 100 % value for maximal AOX capacity. Means ±SD (*vertical bars*) of three independent experiments.

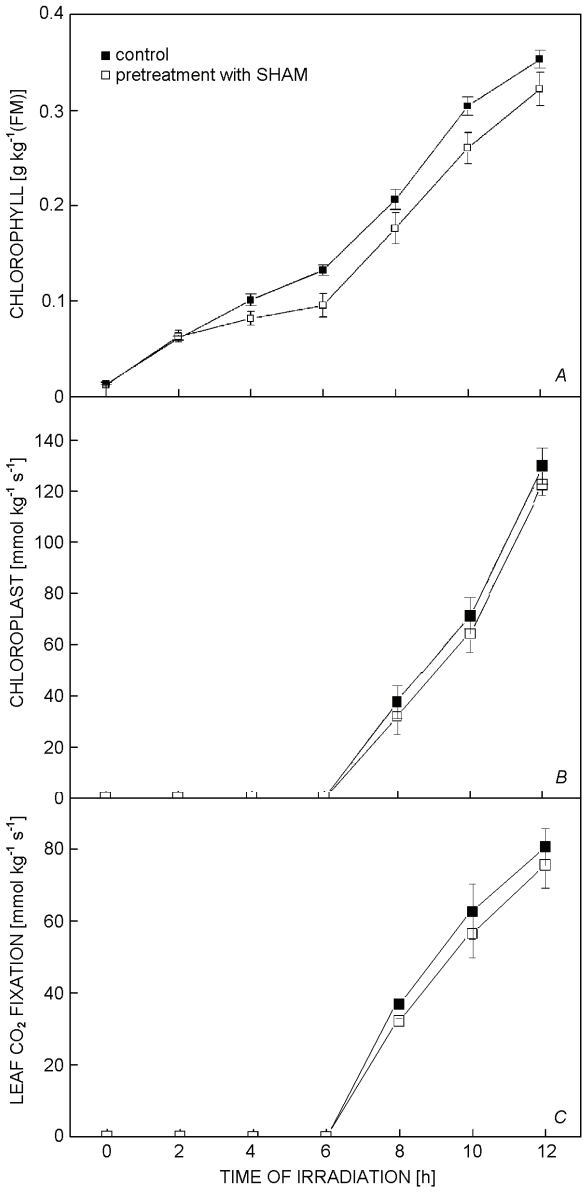

Fig. 3. The influence of SHAM on chlorophyll content (*A*) and carbon dioxide fixation rate in chloroplasts (*B*) or leaves (C) during continuous 12 h of irradiation in etiolated leaves treated with 1 mM SHAM (□) or the solvent (■; control) before irradiation. Individual samples taken during four independent experiments. Means ± SD.

Chl formation is a determining factor in thylakoid development (Mahmudov and Abdullayev 2005), thus the decreased Chl accumulation by SHAM treatment indicates that AOX pathway might contribute to normal greening of plants.

Influence of SHAM on CO_2 fixation rate during greening of dark-grown rice seedlings: CO_2 fixation could not be detected in less than 6 h after the etiolated plants were irradiated. Until 8 h after the onset of irradiation, CO_2 fixation began, and the CO_2 fixation rate gradually increased with the duration of irradiation (Fig. 3*B*). After 12 h of irradiation, CO_2 fixation rate reached 6.22 mmol(CO_2) kg^{-1}(Chl) s^{-1}. Because SHAM decreased Chl content (Fig. 3*A*), lower CO_2 fixation rates (on the basis of leaf area or mass) were expected in SHAM-treated greening plants. But there was no statistical difference observed in CO_2 fixation rate between the leaves pre-treated and untreated with SHAM, when the rate was corrected to Chl basis (Fig. 3*B*).

Fig. 3*C* shows the development of CO_2 fixation rates in intact chloroplasts isolated from the greening leaves during 0–12 h of irradiation. Similarly in leaves, CO_2 fixation was detected only after 8 h of irradiation, and after 12 h reached about 9.92 mmol(CO_2) kg^{-1}(Chl) s^{-1}, which was less than 40 % of the rate obtained with chloroplasts from mature leaves. 1 mM SHAM was directly added into the isolated chloroplasts re-suspended in buffer containing sodium bicarbonate. SHAM had no significant effect on CO_2-dependent O_2 evolution of chloroplast.

Discussion

Our results showed that both V_{alt} and V_{alt}/V_t increased during the greening of dark-grown rice leaves (Table 1), indicating an increase in the mitochondrial electron fractionation by the alternative pathway in this process. This observation is consistent with the previous finding in greening soybean cotyledons (Ribas-Carbo *et al.* 2000).

After exposing the dark-grown rice seedlings to 12 h of continuous irradiation, Chl was rapidly accumulated. The application of SHAM partially limited Chl production in the greening leaves (Fig. 3*A*). We did not further investigate other potential targets of SHAM at this relatively low concentration. The effective inhibitions of AOX activity with 1 mM SHAM in our experiment (Fig. 2) are similar to the inhibitions (about 70 %) in well-irrigated plants and plants experiencing drought with the same SHAM concentration (Bartoli *et al.* 2005). One mM SHAM is sufficiently low to avoid the possible side-effects observed with higher amounts of this AOX inhibitor (Møller *et al.* 1988) or during a relatively long-term (hours) treatment (Bartoli *et al.* 2005). SHAM may increase the activities of NAD(P)H oxidase (Akerlund *et al.* 1987) and some peroxidases (Bingham and Stevenson 1995), which may occur in irradiated green tissue (Diethelm *et al.* 1990). However, if the increased activities of these enzymes were responsible for plant greening in this system, logically greening development might have increased in the presence of SHAM. Therefore, we suggested that this delayed Chl synthesis in the leaves pre-treated with SHAM was a result of the inhibition of AOX pathway. This means that Chl

synthesis in the process of greening might be partially medicated by alternative respiratory pathway.

In general, plant greening leads to an increase in the levels of the genes' transcription and proteins' synthesis related to the chloroplast formation and development (Bruick and Mayfield 1999). For this demand, glycolysis and the tricarboxylic acid (TCA) cycle may play a central role to provide carbon skeletons for the biosynthesis of amino acids, nucleic acids, and fatty acids in this sub-photosynthetic organism. Alternative oxidase may be a clutch that allows the TCA cycle to "spin off" carbon skeletons (Vanlerberghe *et al.* 1997, Mackenzie and McIntosh 1999). Some authors suggested that an enhanced providence of carbon skeletons *via* respiration might be more important than the cellular demand for ATP production for greening process (Gregory and Bradbeer 1975, Bradbeer 1981, Ribas-Carbo *et al.* 2000, Svensson and Rasmusson 2001). Our experiments provide some evidence to confirm the assumption that an enhanced AOX pathway might play a role in normal greening of plants. As well, since the delayed Chl synthesis due to SHAM treatment seemed relatively small (barely significantly different), compared with control, the AOX pathway could not be specifically contributing to greening development.

In etiolated soybean cotyledons, *AOX3* transcript was most abundant while the *AOX2* and *AOX1* transcripts were much less. After irradiation, the increase of AOX activity was accompanied with the increased amount of *AOX2* but the *AOX3* decreased (Finnegan *et al*. 1997). *AOX2* is usually constitutive or developmentally expressed in dicot species, but is absent from the genomes of all monocot species examined to date (Considine *et al.* 2002). We only detected an increase in the relative mRNA amounts of *AOX1c* gene, while the transcripts of *AOX1a* and *AOX1b* were hardly observable (Fig. 1). These results indicate that increase of cyanide-resistant respiration in greening rice is regulated, at least in part, by the *AOX1c* transcription. Rice *AOX1c* presents a similar expression pattern like that of soybean *AOX2*. Under low temperature, the steady-state mRNA levels of *AOX1a* and *AOX1b* increase, but *AOX1c* is not responsive to low temperature (Saika *et al.* 2002). *AOX1c* in monocot plants may be especially up-regulated by cell response to developmental cues, as *AOX2* does in many dicots.

In previous studies, the analysis of the pattern of onset of photochemical activities in greening plastids revealed a stepwise increase of enzymatic activity. Gyldenholm and Whatley (1968) reported that cyclic phosphorylation in isolated chloroplasts could be detected after 10-h irradiation while 15 h was needed for non-cyclic phosphorylation. Phung Nhu Hung *et al*. (1970), using barley, found non-cyclic phosphorylation coupled NADP reduction after 10 h of irradiation. The picture of photosynthesis development is clear, although the time of onset of each specific activity may vary, depending on the plant used and the irradiation regime to which it is subjected. The data presented in this paper, similarly as Lurie (1977) did, showed that CO_2 fixation was detected after 8 h of irradiation (Fig. 3). However, under the continuous 12-h irradiation, AP (Table 1) and the expression of *AOX1* gene (Fig. 3*A*) increased prior to the fixation of carbon dioxide in either leaves or isolated chloroplasts (Fig. 3). This indicates that the induction of AP and *AOX1* expression in light, at least before 6 h, is not directly linked with carbon assimilation of photosynthesis.

We found that addition of 1 mM SHAM into the isolated chloroplasts re-suspended in buffer did not affect the CO_2 uptake-dependent O_2 evolution of chloroplasts (Fig. 3*C*). Several researches also revealed that SHAM at low concentrations has no influence on photochemical electron transport activities in either mesophyll protoplasts or intact chloroplasts (Padmasree and Raghavendra 1999, Bartoli *et al.* 2005). These cases suggest that SHAM has no direct effect on the enzyme activities of chloroplasts. Padmasree and Raghavendra (1999) observed that SHAM restricted the carbon assimilation of mesophyll protoplasts that were isolated from fully expanded leaves of pea grown under natural photoperiod, suggesting that AOX pathway may be up-regulated by photosynthetic carbon assimilation. But in the present work, SHAM pre-treatment did not significantly decline leaf CO_2 fixation rate (on Chl basis) over the 12 h of greening (Fig. 3*B*), indicating that there is little interaction between AOX pathway and photosynthetic carbon assimilation in this process. This work combined the time-course analysis of onset of alternative pathway and photosynthesis suggesting that induction of AP by irradiation, at least during initial greening steps of rice, is not directly linked with carbon assimilation of photosynthesis.

Escobar *et al*. (2004) also found the light induction of the *nda1* and *ndc1* NAD(P)H dehydrogenase genes is independent of carbon status when etiolated *Arabidopsis* seedlings were irradiated, suggesting that influence of irradiation on respiratory electron transport chain might be controlled *via* photoreceptor-mediated transcriptional signals. Whether photoreceptor might also be involved in the induction of alternative pathway would certainly be needed to study for more information on the influence of irradiation on respiration.

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