Journal of Plant Physiology 174 (2015) 75–84

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Physiology

Light regulation of mitochondrial alternative oxidase pathway during greening of etiolated wheat seedlings

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ARTICLE INFO

Article history:
Received 5 June 2014
Received in revised form 21 August 2014
Accepted 5 September 2014
Available online 20 October 2014

Keywords:
Alternative oxidase
Respiration
De-etiolation
Gene expression
Triticum aestivum

ABSTRACT

This study deals with effects of de-etiolation (48 h) of spring wheat (*Triticum aestivum* L., var. Irgina) seedlings on differential expression of *AOX1* genes, levels of AOX protein and the alternative respiratory pathway (AP) capacity. As a result of exposure to continuous irradiation of dark-grown wheat seedlings, the respiratory activity and AP capacity in leaves significantly increased during the first 6 h of studies. Expression of *AOX1a* was up-regulated by light and proved consistent with changes in the AP capacity. Effects on expression of *AOX1c* were less pronounced. Immunoblot analysis showed three distinct bands of AOX with molecular weights of 34, 36 and 38 kDa, with no significant changes in the relative levels during de-etiolation. The lack of a clear correlation between AOX protein amount, *AOX1a* expression, and AP capacity suggests post-translational control of the enzyme activation. The *AOX1a* suppression and a decrease in the AP capacity correlated with the sugar pool depletion after 24 h of the de-etiolation, which may mean a possible substrate dependence of the AOX activity in the green cells. More efficient malate oxidation by mitochondria as well as the higher AOX capacity during the first 6 h of de-etiolation was detected, whereas respiration and AOX capacity with exogenous NADH and glycine increased after 6 and 24 h, respectively. We conclude that AOX plays an important role during development of an actively photosynthesizing cell, and can rapidly adapt to changes in metabolism and photosynthesis.

Introduction

De-etiolation, which occurs in plants after their transition from growth in the dark to that in the light, is usually accompanied by substantial physiological, biochemical, and morphogenetic changes. The changes include those in seedling morphology and physiology, triggered by the light-regulated expression of numerous genes. The most prominent visual aspect of the process is a substantial increase in green color (greening) of tissues due to chlorophyll formation and chloroplast development. This phenomenon has been thoroughly studied, including the signaling regulation of expression of nuclear and plastidic genes coding for chloroplast proteins (López-Juez, 2007; Kravtsov et al., 2011). On the other hand, only scarce information is available on effects in mitochondria and mitochondrial activity during greening. Moreover, the greening process is considered to be a suitable model for studies on regulatory aspects of respiration in a developing photosynthesizing cell and on the role of mitochondrial respiratory chain components during formation of the photosynthetic machinery.

Mitochondrial electron transport chain (mETC) in plants is more complicated and dynamic compared with ETC in mammalian mitochondria. The mETC in higher plants consists of two pathways for electron transport from ubiquinone (Q) to O_2: the phosphorylating cytochrome pathway (CP) and the cyanide-insensitive alternative pathway (AP) that branches off the CP at the level of ubiquinone (Vanlerberghe and McIntosh, 1997). The enzyme catalyzing

Abbreviations: AP, alternative respiratory pathway; AOX, alternative oxidase; BHAM, benzhydroxamic acid; COX, cytochrome oxidase; CP, cytochrome respiratory pathway; MDH, malate dehydrogenase; SDH, succinate dehydrogenase; NDin and NDex, rotenone-insensitive internal and external type II NADH dehydrogenases respectively; mETC, mitochondrial electron transport chain; PIB, post-illumination CO_2 burst; PR, photorespiration; R_D, dark respiration; SHAM, salicylhydroxamic acid; TCA, tricarboxylic acid; V_{alt} and V_{cyt}, capacity of alternative and cytochrome pathway of respiration, respectively; V_t, total respiration measured as O_2 uptake rate.

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http://dx.doi.org/10.1016/j.jplph.2014.09.016
0176-1617/

respiration through AP is alternative oxidase (AOX), occupying the inner mitochondrial membrane, and is encoded by a small group of nuclear genes. The AP bypasses proton-pumping complexes III and IV in the mETC, therefore alternative respiration is considered an energetically wasteful process. However, AP plays an important role in cells. The electron flow through the AP is not limited by an adenylate control, which is advantageous for survival of plants when the main CP is restrained by some other factors (including the overflow of reductants) (Millenaar and Lambers, 2003). The AP is believed to execute some fine tuning of the reduction level of ubiquinone, and, more globally, of the redox balance during the mitochondrial electron transport, thus limiting the formation of superoxides (Maxwell et al., 1999; Millenaar and Lambers, 2003).

Recently, more evidences were reported pointing out to the importance of AOX pathway in the process of optimization of photosynthesis, and protection of cells against photoinhibition (Krömer et al., 1993; Raghavendra and Padmasree, 2003; Yoshida et al., 2008, 2011; Dinakar et al., 2010; Zhang et al., 2010). AP is considered to play a role in the dissipation of excess reductants produced in chloroplasts and exported to mitochondria (Padmasree and Raghavendra, 1999; Yoshida et al., 2011; Zhang et al., 2010). Light can contribute to the regulation of AOX through some direct photoreceptor control and indirect photosynthesis-dependent variations in metabolites, influencing the AOX transcript levels and affecting the abundance and post-translational modification of protein (summarized in Rasmusson and Escobar, 2007; Noguchi and Yoshida, 2008; Igamberdiev et al., 2014).

An increase in the AP activity was observed while using oxygen isotope fractionation during the greening phase in soybean cotyledons (Ribas-Carbo et al., 2000). Patterns of electron partitioning through AP changed dramatically during the first 12 h of greening which closely correlated with the time-course of change in the AOX gene expression earlier observed by Finnegan et al. (1997). The increased *AOX1c* expression and salicylhydroxamic acid (SHAM)-sensitive respiration during 12 h of greening in etiolated rice seedlings was reported (Feng et al., 2007). Patterns of light regulation of the *AOX1a* gene and cyanide-resistant respiration in Arabidopsis seedlings were reported during de-etiolation in different light (Zhang et al., 2010). Recently, we also found that the respiratory activity and AP capacity (maximal activity) increased during the greening in wheat seedlings (Garmash et al., 2013). The highest values of these parameters were detected in the period ranging from 4 to 12 h of continuous illumination, when prolamellar bodies got already converted into thylakoids but the photosynthetic machinery was not yet completely developed. All the changes in the AP capacity observed were accompanied by a heat emission in the dark, which proves AP to be an energy-dissipating system.

In the current paper, more detailed studies were performed on the engagement of AOX in the first leaf of wheat seedlings during their de-etiolation in order to learn more on the role of alternative respiration in metabolic activities of cells during a light-to-dark transition. In order to accomplish the tasks outlined, we analyzed parameters of mitochondrial respiration showing shifts in metabolism and electron transports via CP and AP, in regards to the development of the photosynthetic and photorespiratory activity in a greening leaf. For the first time ever, changes in expression of the wheat *AOX1* genes were studied from the standpoint of their responsiveness to light. We also analyzed whether the AOX protein levels are related to the *AOX* gene expression and AP capacity, respectively, and attempted to reveal possible reasons for discrepancy between these parameters. The data obtained shed more light on "the means by which AOX respiration is interacting with and aiding photosynthesis" (Vanlerberghe et al., 2009).

Materials and methods

Plant material and growth conditions

Plant material and culture condition: wheat (*Triticum aestivum* L., cv. Irgina) seeds were germinated in tap water. Three-day-old seedlings were transferred into 3-dm^3 boxes with a half-strength Knop medium and were grown in the dark for 2 days in a growth chamber (KBWF 720, Binder, Tuttlingen, Germany) at 23 °C and 70% relative humidity. Thereafter, the etiolated seedlings were exposed to continuous light (190 μmol(photon) m^{-2} s^{-1}, photosynthetic active radiation) at 21 °C and allowed to green for 48 h. Nutrient solution was changed every day. The light was produced by luminescent lamps (TL-D 30W, Philips, Amsterdam, The Netherlands).

All measurements were carried out on the first leaf blade, the top segment (1.5 cm) of which was removed and, from the rest, a segment of 2–3 cm in length was used in the experiment. The leaves exposed to light during 0, 1, 2, 4, 6, 12, 24, and 48 h were used. In a separate experiment, the seedlings after 6 h of the de-etiolation phase were put into dark for 18 h and thereafter again exposed to light for the next 24 h to analyze the light- and sugar-dependent character of the respiratory pathway capacity and patterns of gene expression of mitochondrial proteins.

CO_2 exchange (photosynthesis, dark respiration and photorespiration) measurements

The rates of net photosynthesis, the post-illumination respiratory burst reflecting the photorespiratory activity, and the dark respiration (R_D) rate were measured consistently using a portable photosynthesis system LCPro+ supplied with a compact Peltier heat transfer controller (ADC BioScientific Ltd., Great Amwell, Herforshire, England). Twenty leaves on intact plants from a midleaf region (4 cm long) were laid side by side in a 6.25 cm^2 leaf chamber under conditions with relative humidity of 60–70%, temperature of 22 °C and ambient CO_2 concentration.

Primarily, the rate of P_N in leaves exposed to 190 μmol(photon) m^{-2} s^{-1} was recorded. Secondary, a transient post-illumination CO_2 burst (PIB) was studied (Balaur et al., 2009, Huang et al., 2013). PIB shows CO_2 emission as a result of a higher rate of photorespiration (PR). The approach (Balaur et al., 2009), in our study, was applied to measure the CO_2 emission rate after the light being switched off during 20 min. The PIB was identified as a peak recorder deflection lasting for the first 4 min after the light switching off. Later on, the PIB gradually faded, and the remaining emission of CO_2 was attributed to the rate of R_D.

Isolation of mitochondria

Mitochondria were isolated from wheat leaves applying methods developed for wheat shoots (Vojnikov et al., 1984; Grabelnych et al., 2014), and using mediums proposed by Keech et al. (2005) for preparation of leaf mitochondria, with some modifications. All isolation steps were taken at 4 °C. 60 g of leaves was ground with a cold mortar and pestle in 240 ml of grinding buffer with pH 7.5 (pH 8.0 for isolation of mitochondria from leaves after 24 and 48 h of exposition to the light) containing: 0.3 M sucrose, 40 mM MOPS, 2 mM EDTA, 10 mM KH_2PO_4, 1 mM glycine, 1% (w/v) PVP-40, 50 mM ascorbate, 20 mM cysteine, and 0.5% BSA. The homogenate was filtered through a nylon mesh, and mitochondria were isolated by differential centrifugation: 3000 × *g* for 5 min and the resulting supernatant was centrifuged at 15,000 for 15 min. The pellet was suspended in 24 ml of washing buffer (0.3 mM sucrose, 10 mM MOPS and 10 mM KH_2PO_4, 0.5% (w/v) BSA, pH 7.5) and centrifuged at 15,000 × *g* for 14 min. The pellet was re-suspended in 1 ml of