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ORIGINAL PAPER

Quantitative data on the contribution of GSH and Complex II dependent ascorbate recycling in plant mitochondria

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Abstract The reduction of dehydroascorbate, the oxidized form of ascorbate plays important role in the maintenance of sufficient level of ascorbate. In plant mitochondria two DHA reducing mechanisms, the GSH-dependent and the mitochondrial electron transfer chain dependent ascorbate recycling have been characterized. Although both pathways have been extensively studied quantitative information about the electron fluxes from one or another direction for the reduction of DHA is not known. The cellular, mitochondrial glutathione pools and mitochondrial DHA reducing capacity was measured in BSO treated and control tobacco cells. While BSO caused dramatic decrease of cellular GSH content the difference was much smoother at mitochondrial level. The difference in DHA reduction capacity was even smoother affirming the existence of alternative, non-GSH dependent DHA reducing mechanism(s) in plant mitochondria. On the base of the parallel determination of mitochondrial GSH content and ascorbate production upon DHA addition, GSH (consumption) is responsible for the ~ 20 % of ascorbate production. Almost 90 % enhancement of ascorbate production could be provoked by the addition of Complex II substrate succinate which could be almost totally prevented by the concomitant addition of malonate or TTFA. On the base of these results, the importance of mitochondrial Complex II compared to GSH-dependent mechanisms in mitochondrial ascorbate recycling has been underestimated so far.

Keywords Ascorbate · Glutathione · Plant mitochondria · Ascorbate recycling · Respiratory chain

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Introduction

Ascorbate metabolism is linked to the mitochondrion by multiple ways in plant cells. Except of the last step of Smirnoff–Wheeler pathway, the major biosynthetic route of ascorbate is localized in the cytosol (Wheeler et al. 1998). However, L-galactono-1,4-lactone dehydrogenase (GLDH), the enzyme catalyzing the ultimate step, is attached to the so-called "membrane arm" of Complex I, uses oxidized cytochrome c as the only electron acceptor and has a role in the assembly of Complex I (Schertl et al. 2012; Szarka et al. 2013). Furthermore, the electron flow through complex I has a regulatory role on the ascorbate biosynthesis capacity (Millar et al. 2003).

From its site of synthesis, ascorbate is transported to the different subcellular compartments (Horemans et al. 2000), where ascorbate is being used in different reactions. Due to these reactions, different redox forms are generated; monodehydroascorbate (MDHA) and the fully oxidized dehydroascorbate (DHA).

The role of ascorbate recycling (i.e. reduction of its oxidized forms) in the maintenance of a sufficient level of ascorbate is generally accepted. Ascorbate regeneration from its oxidized forms should occur in a relatively short time otherwise it would be lost because of the opening of the instable lacton ring of DHA (Winkler 1987). This hydrolysis of DHA irreversibly yields 2,3-diketogulonate

in a reaction that is highly pH-dependent, bicarbonate-promoted and possibly catalyzed by a plant enzyme (Parsons and Fry 2012).

The existence of the elements of the GSH-dependent ascorbate recycling pathway (Foyer-Halliwell-Asada cycle) in plant mitochondria (Jimenez et al. 1997; Chew et al. 2003) suggested that these organelles play an important role not only in the synthesis of ascorbate but also in the regeneration of ascorbate from its oxidized form(s). This role was reinforced by the description of the mitochondrial transport of the oxidized form, DHA (Szarka et al. 2004) and by the elucidation of the role of mitochondrial electron transfer chain in DHA reduction (Szarka et al. 2007). On the base of the effect of different respiratory substrates and inhibitors the presumptive location of DHA reduction is Complex II (Szarka et al. 2007).

Up to date the Foyer-Halliwell-Asada cycle and the Complex II dependent DHA reduction have been characterized in plant mitochondria. However, any quantitative data on the contribution of the certain pathways to DHA reduction is not known. In this study, information was gained by applying inhibitors of these two DHA reducing pathways about the contribution of these pathways to DHA reduction in plant mitochondria.

Materials and methods

Materials

Buthionine sulphoximine (BSO), glutathione (GSH), KCN, 4-morpholinepropanesulfonic acid (MOPS), mannitol, monochlorobimane, Murashige and Skoog medium were obtained from Sigma. Percoll was purchased from GE Healthcare. All other chemicals were of analytical grade.

Plant material

Nicotiana tabacum L. cv. Bright Yellow-2 cell suspension culture was propagated in Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with KH_2PO_4 (5.5 mM), K_2HPO_4 (1.15 mM), sucrose (88 mM), 2,4-dichlorophenoxiacetic acid (0.9 μM), thiamine-HCl (30 μM), and myo-inositol (0.55 mM) at pH 5.8. Cells were cultured in a rotary shaker (130 rpm) at 27 °C in the dark. Weekly 1.4 ml of cells was transferred to 100 ml of fresh medium in 500-ml flasks. After 6 days the cell culture reached the stationary phase.

Isolation of mitochondria

Mitochondria were isolated from BY2 tobacco cells by differential and density-gradient centrifugation according to Szarka et al. (2004). Finally, mitochondria were carefully suspended in MOPS-KOH buffer (300 mM mannitol, 1 mM EDTA, 10 mM MOPS, pH 7.2). The enrichment of the mitochondrial fraction and the integrity of mitochondria were checked by the measurement of cytochrome c oxidase activity of the fraction as described by Szarka et al. (2004).

Incubation of mitochondria and measurement of metabolites

Freshly purified BY2 mitochondria were used in all experiments. Mitochondria were incubated in a buffer containing 300 mM mannitol, 1 mM EDTA, 10 mM MOPS, pH 7.2 at room temperature (22–25^{O}C). Furthermore, the reaction medium contained catalytic amount of P_i. Incubations were terminated by the addition of 0.3 volume of 25 % (w/v) metaphosphoric acid in case of ascorbate measurements and methanesulphonic acid in case of GSH measurements.

GSH determination

Total cell homogenates and mitochondrial sample were derivatized with 1 mM monochlorobimane (mBCl). Added mBCl reacts specifically with thiol groups, producing a highly fluorescent thioether. The separation of derivatized GSH was performed by gradient HPLC analyses with a Waters 2690 separation module and Waters 2475 fluorescence detector at excitation wavelength of 395 nm and emission wavelength of 477 nm. The separations were carried out on a Teknokroma Nucleosil 100 C18 column (average particle size 5 μm, 25 cm × 4.6 mm). A 30-min separation protocol was used employing the following linear gradients of 0.25 % (v/v) aqueous acetic acid, NaOH, pH 3.9 (solvent A) and methanol (solvent B): 0 min at 92 % (v/v) solvent A; 10 min at 85 % (v/v) solvent A; 30 min at 85 % (v/v) solvent A. The flow rate was 1 ml/min.

Ascorbate determination

Ascorbate and dehydroascorbate levels were measured from the supernatants by reverse phase HPLC. The isocratic analyses were carried out with a Waters 2690 separation module and Waters 2487 dual λ absorbance detector at 254 nm. The separations were carried out on a Teknokroma Nucleosil 100 C18 column (average particle size 5 μm, 25 cm × 4.6 mm).

Other methods

Protein concentration of mitochondria was determined using the BioRad protein assay solution with bovine serum

albumin as a standard, according to the manufacturer's instructions.

DHA was prepared from ascorbate by the bromine oxidation method as described in Del Bello et al. (1994). Freshly prepared DHA was used immediately.

All data are expressed as mean ± S.D. Statistical analysis was carried out using Student's *t* test.

Results

BY2 tobacco cells were treated by the gamma-glutamylcysteine synthetase (GSH synthesis) inhibitor BSO to determine the contribution of GSH to the reduction of DHA in plant mitochondria. The BSO treatment caused marked decline of the cellular GSH content (Fig. 1a). The almost 90 % decrease in cellular GSH did not influence the viability of the cells as it was determined by trypan-blue exclusion staining. The dye excluded from more than 95 % of both the BSO treated and non-treated cells. As it was expected the GSH content of the mitochondria from BSO treated cells decreased by significantly lower extent (89 vs. 38 %) (Fig. 1 a, b).

To assess the effect of decreased GSH content in mitochondria on DHA reduction, the appearance of the reduced form, ascorbate, was monitored after the addition of oxidized form DHA (1 mM) to plant mitochondrial suspension. A clear decrease could be observed in the DHA reduction capacity of mitochondria isolated from BSO treated cells compared to mitochondria from non-treated cells (Fig. 2). Although this decrease was significant, but its rate was even lower than the rate of GSH decrease in the mitochondria from BSO treated cells (15 vs. 38 %) (Figs. 1b, 2). This observation clearly implies that non-GSH dependent reduction(s) of DHA also exist in plant mitochondria.

The increase of ascorbate and the decrease of GSH content in tobacco mitochondria were determined parallel to gain quantitative information on the connection of DHA reduction (ascorbate production upon DHA addition) and GSH consumption. Hence, the appearance of ascorbate and the disappearance of GSH were monitored after the addition of DHA (1 mM) to plant mitochondrial suspension. In this case, the production of ascorbate is also exceeded the consumption of GSH (GSH consumption reached only 20 % of ascorbate production) (Fig. 3 a, b) supporting the existence of non-GSH dependent DHA reducing reaction(s).

Beyond the GSH-dependent Foyer-Halliwell-Asada cycle the respiratory electron transfer chain coupled DHA reduction has been described in plant mitochondria up to date. Thus in the next turn of experiments the effects of respiratory substrate and inhibitors were investigated on ascorbate production (upon DHA addition). Since it was found that only complex II has a role in the reduction the complex II substrate succinate was chosen to provoke and the complex II inhibitors malonate and TTFA were chosen to inhibit the succinate provoked DHA reduction. In accordance with our previous results (Szarka et al. 2007) succinate addition stimulated the ascorbate production by 90 % (Fig. 4). This stimulatory effect could be totally suspended by both complex II inhibitors (Fig. 4). Hence, quantitative information could be gained on the contribution of both currently known DHA reducing mechanisms in plant mitochondria.

Discussion

It is widely known and generally accepted that mitochondria are capable for the reduction of DHA taken up (Szarka et al. 2004) or generated locally by the mitochondria (Szarka et al. 2012).

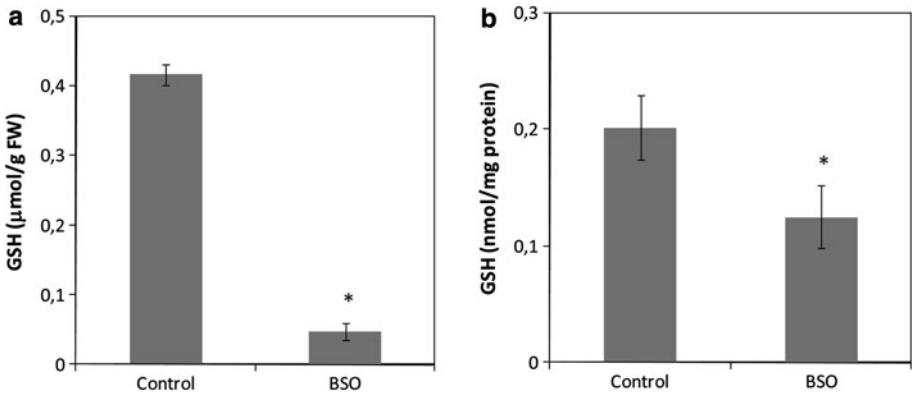

Fig. 1 Cellular (**a**) and mitochondrial glutathione (**b**) content of control and BSO treated BY2 tobacco cells. Mitochondria were isolated from both control and BSO treated BY2 tobacco cells. Cellular or mitochondrial glutathione (GSH) was derivatized with monochlorobimane. Monochlorobimane glutathione conjugate assessed by HPLC as described in Materials and methods. Data are expressed as mean ± S.D. *Significant difference with respect to control ($P < 0.05$)

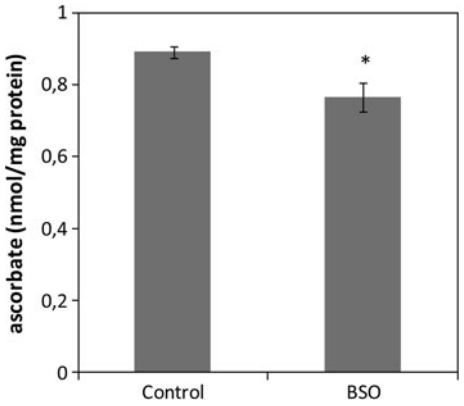

Fig. 2 The effect of BSO treatment on mitochondrial DHA reduction. Mitochondria were isolated from both control and BSO treated BY2 tobacco cells. Ascorbate production was initiated by the addition of 1 mM DHA. After 10 min of incubation, the reaction was stopped by the addition of metaphosphoric acid. The ascorbate content of each sample was determined by HPLC as described in Materials and methods. Data are mean ± SD. *Significant difference with respect to control ($P < 0.05$)

Various mechanisms have been suggested for intramitochondrial ascorbate recycling. The electrons can come from small molecular weight electron carriers such as glutathione (GSH) (Jimenez et al. 1997; Li et al. 2001) and lipoic acid (Xu and Wells 1996) or from the respiratory electron transfer chain (Li et al. 2002; Szarka et al. 2007). In the case of plant mitochondria only two of them, the GSH-dependent (Jimenez et al. 1997; Chew et al. 2003) and the mitochondrial electron transfer chain (mETC) dependent (Szarka et al. 2007) DHA reduction have been characterized. However, any quantitative information about the electron fluxes from one or another direction for the reduction of DHA is not known.

To determine the extent of GSH-dependent DHA reduction, BY2 tobacco cells were treated by the gamma-glutamylcysteine synthetase inhibitor BSO. The treatment caused a dramatic decrease in the cellular GSH content (Fig. 1a). However, this dramatic decrease did not influence the cell viability. The huge difference (90 % decrease) in GSH content of the treated cells was much smoother at mitochondrial level. The lower extent decrease (38 %) in mitochondrial GSH content is in accordance with previous results gained in BSO treated *Cucurbita pepo* (Zechmann et al. 2006) or *pad2-1* mutant *Arabidopsis* (Zechmann et al. 2008). These observations suggest a well-regulated mitochondrial GSH transport mechanism since GSH biosynthesis is localized in the cytosol and in the plastis (Szarka et al. 2012). The constant level of GSH in the mitochondria might be important in the protection of membrane intactness and helps to avoid cell death due to impairment of mitochondria (Fernández-Checa 2003). The difference in DHA reduction capacity of mitochondria between the BSO treated and control cells was even smoother (Fig. 2). This phenomenon affirms the existence of alternative, non-GSH dependent DHA reducing mechanism(s) in plant mitochondria. The contribution of mitochondrial GSH to DHA reduction was assessed by the determination of mitochondrial GSH content before and after (20 min) the addition of DHA to the mitochondrial fraction (Fig. 3a). The ascorbate production (upon DHA addition) was determined parallel (Fig. 3b). In the absence of any additional fuel (of metabolism) the pure GSH-dependence of DHA reduction in plant mitochondria could be assessed. The decrease in mitochondrial content was approximately one-fifth of ascorbate production (Fig. 3). Hence, GSH (consumption) is responsible for the ~ 20 % of ascorbate production (DHA reduction). The remaining DHA reduction can be occurred by other non-GSH dependent manner. The involvement of two of them is highly plausible, however, neither of them was investigated in plant mitochondria up to date. On the base of the mammalian analogy the role of thioredoxin reductase is

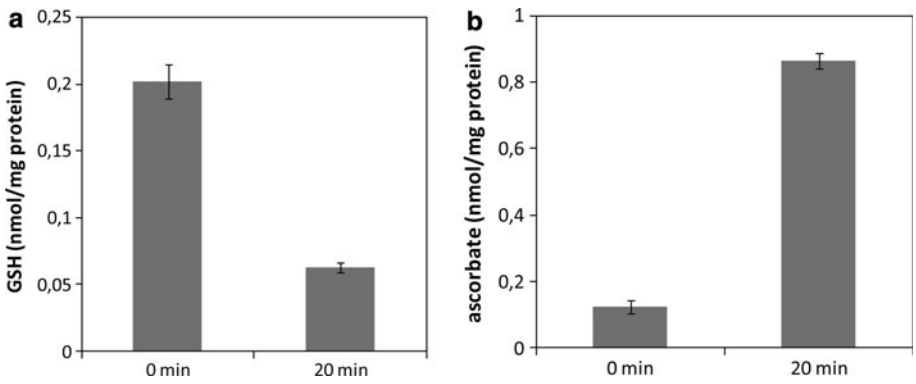

Fig. 3 The change in mitochondrial glutathione content (**a**) or ascorbate production (**b**) due to DHA addition. Freshly purified BY2 tobacco mitochondria (approximately 4 mg/ml protein) were incubated in the presence of dehydroascorbate for 20 min at room temperature. Samples were taken for glutathione and ascorbate determination at the indicated incubation times. Glutathione and ascorbate content of each sample was determined by HPLC as described in Materials and methods. Data are mean ± SD

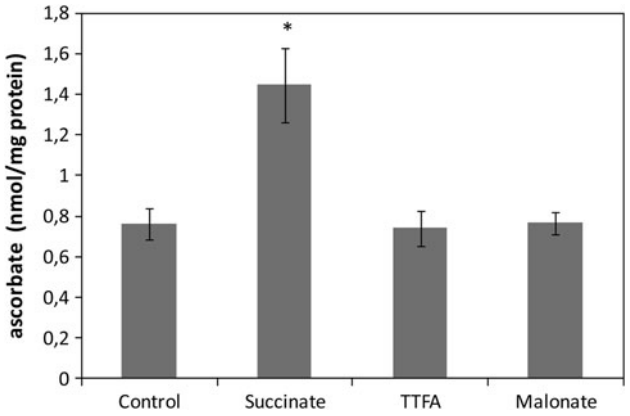

Fig. 4 The effect of Complex II substrate, succinate and inhibitors on mitochondrial DHA reduction. Freshly purified BY2 tobacco mitochondria (approximately 4 mg/ml protein) were pre-incubated in the presence of the indicated compounds for 10 min at room temperature. The inhibitors (TTFA, malonate) were added together with succinate. Reactions were initiated by the addition of 1 mM DHA. After 10 min of incubation, the reaction was stopped by the addition of metaphosphoric acid. The ascorbate content of each sample was determined by HPLC as described in Materials and methods. Data are mean ± SD. *Significant difference with respect to control ($P < 0.05$)

appeared since rat liver thioredoxin reductase was shown to catalyze the NADPH-dependent reduction of DHA with a K_m value of 0.7 mM (May et al. 1997). Similarly, rat liver mitochondria had the ability to reduce dehydroascorbic acid to ascorbic acid in an alpha-lipoic acid dependent manner. The alpha-lipoic acid dependent reduction was stimulated by factors that increased the NADH dependent reduction of alpha-lipoic acid to dihydrolipoic acid in coupled reactions. Pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase reduced DHA to ascorbic acid in an alpha-lipoic acid, coenzyme A, and pyruvate or alpha-ketoglutarate dependent fashion (Xu and Wells 1996).

According to our previous results only the Complex II of mETC has any contribution to DHA reduction in plant mitochondria (Szarka et al. 2007). Hence, the mETC dependent part of ascorbate production can be assessed by the supplementation of incubation medium with the Complex II substrate succinate and the Complex II inhibitors TTFA and malonate. The addition of succinate caused 90 % enhancement of ascorbate production of which could be almost totally prevented by the concomitant addition of malonate or TTFA (Fig. 4). Although succinate showed moderate inhibition on mitochondrial DHA uptake (Szarka et al. 2004), the addition of this complex II substrate resulted in marked elevation of ascorbate generation upon DHA addition (Fig. 4). Glucose, the competitive inhibitor of mitochondrial DHA uptake caused significant inhibition in DHA provoked ascorbate generation (Szarka et al. 2007). The observed boost in ascorbate generation due to succinate addition (Fig. 4) is definitively the result of higher electron donation which could compensate its possible inhibitory effect on mitochondrial DHA uptake.

On the base of these results the importance of mETC compared to GSH-dependent mechanisms in mitochondrial ascorbate recycling has been underestimated.

Necessarily both cases are artificial, non-physiological cases hence we should manage the data with caution. However, this way the contribution of the two presently known ascorbate recycling pathways in plant mitochondria could be assessed at first time.

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