DOI: 10.1007/s10535-012-0244-6 BIOLOGIA PLANTARUM 56 (4): 705-710, 2012

Cytosolic NADP-isocitrate dehydrogenase in *Arabidopsis* leaves and roots

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Abstract

NADP-dependent isocitrate dehydrogenase (NADP-ICDH) catalyses the production of NADPH, which is an essential component in the cellular homeostasis. In *Arabidopsis,* the kinetic parameters (K_m and V_{max}) of cytosolic NADP-ICDH were different in leaves and roots. *In vitro* applied H_2O_2 did not affect the NADP-ICDH activity in either organ, however, the reduced glutathione inhibited the activity in leaves but not in roots. On the other hand, *S*-nitrosoglutathione (a NO donor) and peroxynitrite depressed NADP-ICDH activity in leaves and roots.

Additional key words: hydrogen peroxide, nitric oxide, peroxynitrite, reactive nitrogen species, *S*-nitrosoglutathione.

Introduction

NADPH is a key cofactor in cellular redox homeostasis, being an indispensable electron donor in numerous enzymatic reactions, biosynthetic pathways, and detoxification processes. Thus, besides being used in the photosynthetic Calvin-Benson cycle, NADPH is a necessary reducing equivalent for the regeneration of reduced glutathione (GSH) by glutathione reductase (GR) and for the activity of the NADPH-dependent thioredoxin system, two important cell antioxidants. NADPH is also a cofactor of nitric oxide synthase (NOS; Corpas *et al.* 2009a) and NADPH oxidase (NOX; Sagi and Fluhr 2006). Moreover, the chloroplastic Tic62 (a component of the translocon at the inner envelope of chloroplasts, Tic complex) and NADPH thioredoxin reductase C (NTRC) have been shown to require NADPH for their activity (Spinola *et al.* 2008, Stengel *et al.* 2008).

NADP-dependent isocitrate dehydrogenase (NADP-ICDH; EC 1.1.1.42) catalyses the oxidative decarboxylation of isocitrate to 2-oxoglutarate with the production of the reduced coenzyme NADPH (Gálvez and Gadal 1995). In the cells of higher plants, NADP-ICDH activity has been detected and characterized in the different subcellular compartments including the cytosol, mitochondria, chloroplasts and peroxisomes (Attucci *et al.* 1994, Gálvez *et al.* 1995, Rasmusson and Møller 1998, Corpas *et al.* 1999b, Leterrier *et al.* 2007, Pascual *et al.* 2008, McCarthy-Suárez *et al.* 2011). Significant progress is being achieved in understanding how the NADP-ICDH is involved in nitrogen metabolism, in redox regulation, and in response to oxidative stress (Scheible *et al.* 2000, Hodges *et al.* 2003, Valderrama *et al.* 2006, Mateos *et al.* 2009, Mhamdi *et al.* 2010, Sulpice *et al.* 2010). However, the specific function of this enzyme in each subcellular compartment in physiological and stress conditions remains unclear. In animal cells, NADP-ICDH is reportedly required under oxidative stress conditions because it supplies NADPH needed for GSH production (Maeng *et al.* 2004). Additionally, it has been shown that both cytosolic and mitochondrial NADP-ICDHs are susceptible to inactivation by peroxynitrite ($ONOO^-$; Yang *et al.* 2002). For plant cells, no similar information is available. Thus, the present study, using *Arabidopsis thaliana* as a model plant, examines the *in vitro* effects of GSH, H_2O_2, NO, and $ONOO^-$ on the NADP-ICDH activity in roots and leaves.

Received 2 September 2011, *accepted* 11 November 2011.
Abbreviations: GR - glutathione reductase; GSH - reduced glutathione; GSNO - S-nitrosoglutathione; NADP-ICDH - NADP-dependent isocitrate dehydrogenase; NOS - nitric oxide synthase; NOX - NADPH oxidase; $ONOO^-$ - peroxynitrite; PVDF - polyvinyl difluoride; RNS - reactive nitrogen species; ROS - reactive oxygen species; SIN-1 - 3-morpholinosydnonimine.
Acknowledgements: M.L. appreciated a JAE-Doc contract from CSIC. This work was supported by grants from Ministry of Education and Science (BIO2009-12003-C02-01 and BIO2009-12003-C02-02). Special thanks are given to Mr. C. Ruíz-Torres for his excellent technical support.
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Materials and methods

Arabidopsis thaliana L. wild-type plants were grown on soil plus *Vermiculite* (1:3) in a growth chamber at temperature of 22 °C, 16-h photoperiod and irradiance of 100 - 120 μmol m^{-2} s^{-1} for 15 d. Then, they were grown individually in a hydroponic system with an aerated basal nutrient solution (Cellier *et al.* 2004) for one month, before flowering. The nutrient solution was renewed twice a week.

Leaves and roots of 6-week-old plants were frozen in liquid N_2 and ground to a powder in a mortar with a pestle. The powder was suspended in homogenizing medium containing 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM $MgCl_2$, 2 mM dithiotreitol, 0.2 % (v/v) *Triton X-100* and 10 % (v/v) glycerol (1:4, m/v). Homogenates were centrifuged at 13 000 *g* for 20 min. Aliquots of soluble fraction were immediately used for the assays. All operations were carried out at 0 - 4 °C.

NADP-ICDH activity was determined spectrophotometrically (*Beckman DU-640*, Culter, USA) by recording the reduction of NADP at 340 nm (Leterrier *et al.* 2007). The assay was performed at 25 °C in a reaction medium (1 cm^3) containing 50 mM HEPES, pH 7.6, 2 mM $MgCl_2$ and 0.8 mM NADP. The reaction was started by the addition of 10 mM 2*R*,3*S*-isocitrate. One unit (U) of activity was defined as the amount of enzyme required to reduce 1 μmol NADP min^{-1}. For the determination of dependence on temperature, the reaction medium was pre-incubated with the plant samples for 5 min at the indicated temperature in the spectrophotometer before adding the 2*R*,3*S*-isocitrate.

The kinetic data were analysed by the non-linear-regression method described by the Michaelis-Menten equation $v = V_{max}$ [S] /(K_m + [S]). This nonlinear plot was constructed with the aid of a computer program (*Hyper32,* University of Liverpool, UK). For kinetic studies the range of D-isocitrate concentrations was 0.0025 - 2.5 mM at saturating concentration of 0.8 mM NADP. In the case of NADP kinetic analysis, the range of NADP concentrations was 0.01 - 0.8 mM at saturating concentration of 2.5 mM of D-isocitrate.

The NADPH content in *Arabidopsis* roots and leaves were determined according to Tamoi *et al.* (2005). Briefly, samples were frozen in liquid N_2 and ground in a mortar with a pestle. The powder was suspended in a homogenizing medium (1/4; m/v) of 0.1 M KOH prepared in 50 % ethanol. The extracts were centrifuged at 25 000 *g* for 10 min and supernatants were used for the NADPH assay. The reaction mixture contained 100 mM HEPES-KOH (pH 8.0), 0.5 mM EDTA, 2.5 mM glucose 6-phosphate (G6P), 1.66 mM phenazine ethosulfate (PES), 0.42 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), and an extract or a standard solution corresponding to 0 - 150 pmol of NADPH in a final volume of 1 cm^3. The reaction was started by the addition of 0.7 units of G6P dehydrogenase and the absorbance at 570 nm was determined since the rate of reduction of MTT is proportional to the concentration of NADPH.

SDS-PAGE was performed on 10 % polyacrylamide slab gels. For Western-blot analysis, proteins were electroblotted onto polyvinyl difluoride (PVDF) membranes with a semi-dry *Trans-Blot* cell (*Bio-Rad,* Hercules, CA, USA). After transfer, PVDF membranes were used for cross-reactivity assays with a polyclonal antibody against pea NADP-ICDH (Chen *et al.* 1989) at a 1:6 000 dilution. For immunodetection, an enhanced chemiluminescence method using luminol (Corpas and Trelease 1997) was conducted using affinity purified goat anti-(rabbit IgG)-horseradish peroxidase conjugate (*Bio-Rad*). The chemiluminescence was detected with a *Gel Doc EZ* (*Bio-Rad*).

Further, the samples were incubated with different compounds including H_2O_2 as oxidizing agent, reduced glutathione (GSH) as reducing agent, *S*-nitrosoglutathione (GSNO) as NO donor and 3-morpholinosydnonimine (SIN-1) which generates peroxynitrite and mediates process of tyrosine nitration (Chaki *et al.* 2009). Samples were incubated in the dark at 25 ºC for 5 min with increased concentration (0 - 5 mM) of H_2O_2, at 25 ºC for 30 min with increased concentration (0 - 5 mM) of GSH, at 25 ºC for 30 min with increased concentration (0 - 5 mM) of GSNO or at 37 ºC for 1 h with increased concentrations (0 - 5 mM) of SIN-1 (*Calbiochem*, Madrid, Spain). In all cases, the solutions were made up fresh before being use.

Microarray data analyses for NADP-ICDH genes were retrieved from the *Arabidopsis* electronic fluorescent pictograph [eFP] browser at www.bar.utoronto.ca (Winter *et al*. 2007).

The protein content was determined with the *Bio-Rad* protein assay using bovine serum albumin as the standard. The statistical significance between means was determined by Student's *t*-test.

Results

The *Arabidopsis* database shows that its genome contains three NADP-ICDH genes *At1g65930*, *At5g14590* and *At1g54340* coding for cytosolic, chloroplastic/mitochondrial and peroxisomal NADP-ICDH, respectively. Expression analyses of these genes in roots and leaves using microarrays from public database (www.bar.utoronto.ca) demonstrate that the cytosolic NADP-ICDH (*At1g65930*) has the highest expression in both organs (roots and leaves).

The analysis of the total NADPH content in roots and leaves of healthy *Arabidopsis* plants was 12.1 and 7.8 nmol (NADPH) mg^{-1}(protein), respectively. There-

fore, the NADPH content is 1.5-fold higher in the roots than in the leaves. In both organs the antibody allowed the detection of NADP-ICDH monomers and dimers, the monomer band being more prominent in the roots while the dimer band was more prominent in the leaves (Fig. 1).

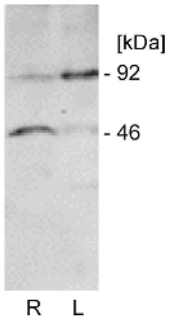

Fig. 1. Immunoblot analysis of *Arabidopsis* root (R) and leaf (L) samples probed with an antibody against pea NADP-ICDH (1:6 000 dilution). For each sample, 20 μg proteins were loaded.

The apparent K_m value for isocitrate was calculated using DL-isocitrate as a substrate. Considering that it contained 50 % D-isocitrate, the estimated K_m for D-isocitrate was 42 μM for the leaf enzyme and 239 μM for the root enzyme. The apparent K_m values for NADP were 4.4 and 61.6 μM for the leaf and root enzymes, respectively. In leaves, the NADP-ICDH enzyme registered 5.7-fold lower K_m for isocitrate and 14-fold lower for NADP. On the other hand, the V_{max} for both isocitrate and NADP was 3-fold higher in roots than in leaves. In the same way, the catalytic efficiency (V_{max}/K_m) for both substrates was also higher in the leaf enzyme (Table 1).

Table 1. Kinetic parameters of NADP-ICDH activity in roots and leaves of *Arabidopsis* plants. Kinetic parameters were determined by using a non-linear-regression analysis program using Eadie-Hofstee plot. Maximal velocity (V_{max}) is expressed as nmol(NADPH) mg^{-1}(protein) min^{-1}, Michaelis constant (K_m) as μM and catalytic efficiency is expressed as V_{max}/K_m. Data are means ± SEM of three independent experiments.

Substrate	Parameter	Roots	Leaves
D-isocitrate	V_{max}	134.8 ± 1.4	42.6 ± 0.2
	K_m	239.0 ± 6.0	42.0 ± 2.4
	V_{max}/K_m	0.6	1.0
NADP	V_{max}	154.9 ± 1.2	58.5 ± 1.6
	K_m	61.6 ± 0.7	4.4 ± 0.1
	V_{max}/K_m	2.5	13.3

NADP-ICDH activity was measured in root and leaf samples after 5 min of pre-incubation at temperatures from 25 to 60 ºC (Table 2). In general, the activity increased when the temperature was raised. The activity in the roots increased progressively until reaching at maximum at 45 ºC; after which the activity declined. On the other hand, the activity in leaves increased progressively to a maximum at 60 ºC. In all cases, the activity was much higher in the roots than in the leaves.

Given that NADP-ICDH could provide NADPH to several pathways involved in ROS and RNS metabolism such as ascorbate-glutathione cycle, NOX or NOS, the effects of different compounds such as GSH, H_2O_2, S-nitrosoglutathione (GSNO) as NO donor, and peroxynitrite ($ONOO^-$) in the NADP-ICDH activity were evaluated (Table 3). H_2O_2 did not appear to significantly alter NADP-ICDH activity in roots or leaves. The NADP-ICDH activity was not affected by GSH in roots but in leaves the activity fell approximately by 42 %

Table 2. Effect of temperature on NADP-ICDH activity [nmol(NADPH) mg^{-1}(protein) min^{-1}] in *Arabidopsis* root and leaf samples. Data are means ± SEM of five independent experiments. *Asterisk* indicates significant difference at $P < 0.05$ from values at 25 ºC.

Temperature [ºC]	Roots	Leaves
25	288.9 ± 2.7	16.8 ± 0.1
35	507.3 ± 10.3*	29.5 ± 0.1*
40	537.7 ± 10.1*	41.8 ± 0.3*
45	602.5 ± 27.9*	49.1 ± 0.2*
50	517.4 ± 19.3*	55.4 ± 0.8*
60	378.3 ± 20.7*	79.6 ± 1.7*

Table 3. Effect of H_2O_2, GSH, SIN-1 and GSNO on NADP-ICDH activity [%] in *Arabidopsis* root and leaf samples. See Material and methods for details. Data are means ± SEM of five independent experiments. *Asterisk* indicates significant difference at $P < 0.05$ from control values.

Compound	Conc. [mM]	Roots	Leaves
H_2O_2	0.0	100.0 ± 0.6	100.0 ± 2.8
	0.1	100.0 ± 0.4	100.0 ± 3.1
	0.5	90.3 ± 0.5	100.0 ± 2.1
	1.0	89.5 ± 0.6	100.0 ± 2.0
	5.0	80.2 ± 1.7*	97.6 ± 3.4
GSH	0.0	100.0 ± 1.1	100.0 ± 1.0
	0.1	100.0 ± 0.5	92.5 ± 1.1
	0.5	100.0 ± 0.8	66.5 ± 1.9*
	1.0	100.0 ± 1.1	66.6 ± 2.5*
	5.0	99.5 ± 1.2	60.1 ± 1.8*
SIN-1	0.0	100.0 ± 1.0	100.0 ± 2.2
	0.1	100.0 ± 0.6	95.8 ± 2.1
	0.5	97.3 ± 0.9	92.7 ± 2.5
	1.0	87.3 ± 1.0	78.4 ± 3.4*
	5.0	56.0 ± 1.1*	56.1 ± 2.0*
GSNO	0.0	100.0 ± 1.0	100.0 ± 0.7
	0.1	100.0 ± 1.5	100.0 ± 1.2
	0.5	99.1 ± 0.5	81.3 ± 2.3*
	1.0	97.1 ± 1.3	73.3 ± 2.5*
	5.0	62.7 ± 1.8*	29.5 ± 1.8*

at a concentration higher than 0.5 mM. The compound 3-morpholinosydnonimine (SIN-1) is a peroxynitrite donor which can mediate protein nitration (Chaki *et al.* 2009). Thus, NADP-ICDH activity in roots and leaves was clearly inhibited around 44 % with 5 mMSIN-1. On the other hand, in roots and leaves, inhibition increased with the GSNO concentration. The maximum inhibition 37 % in roots and 71 % in leaves was at the highest GSNO concentration.

Discussion

NADP-ICDH is broadly distributed in plants, being encoded by multi-gene families. Within the cell, this enzyme could have different subcellular localizations, indicating that each isoenzyme is exposed to different environment and may participate in specific metabolic pathways, depending of the cell and organ type. It is generally accepted that this cytosolic isoenzyme is most abundant in the plant cells because it represents more than 90 % of the total activity (Chen *et al.* 1998, Gálvez and Gadal 1995) and the analysis of gene expression agree, since cytosolic NADP-ICDH showed the highest expression in both organs. Recently, in *Arabidopsis* leaves, it has been also corroborated that this is the most abundant cytosolic isoenzyme (Mhamdi *et al.* 2010). Given that this enzyme generates NADPH, which participates in different pathways (ascorbate-glutathione cycle, enzymatic nitric oxide generation, *etc*.), the goal of the present study is the assessment of regulation of cytosolic NADP-ICDH activity by different compounds involved in the metabolism of ROS and RNS, using *Arabidopsis thaliana* as the model plant.

The high NADP-ICDH specific activity found in *Arabidopsis* roots is consistent with the activity and protein-expression data reported in other plant species such as pea (Chen *et al.* 1988), alfalfa (Shorrosh and Dixon 1992), soybean (Udvardi *et al.* 1993), and pines (Pascual *et al.* 2008). However, under the assumption that the cytosolic NADP-ICDH represents more than 90 % of the total activity (Chen *et al.* 1988, 1989, Fieuw *et al.* 1995, Gallardo *et al.* 1995, Gálvez *et al.* 1995, Leterrier *et al.* 2007, Mhamdi *et al.* 2010), the kinetic parameters of this enzyme in *Arabidopsis* roots and leaves clearly differ, because the affinity of the enzyme for its substrates (isocitrate and NADP) was higher in leaves than in roots. This suggests a differential regulation that must be related to the specific physiological function in each organ. NADP-ICDH from leaves and roots differed also in thermal stability. It was higher in leaves than in roots within the temperature range studied and could be a protection mechanism, since leaves are usually exposed to high temperature fluctuations during the daytime and over the year. This could also be the reason why under denaturing conditions the dimer form is more prominent in leaves that in roots (Fig. 1). Similar behaviour has also been reported in purified cytosolic NADP-ICDH from pea roots and leaves (Chen *et al*. 1988).

NADP-ICDH catalyses the production of 2-oxo-glutarate, which is associated with the metabolism of amino acids and ammonia (Palomo *et al.* 1998, Gálvez *et al.* 1999, Hodges *et al.* 2003). However, this enzyme concomitantly generates NADPH, which contributes to the pool of NADPH produced during photosynthesis (Valderrama *et al.* 2006). This NADPH is needed for different metabolic pathways such as the ascorbate-glutathione cycle, thioredoxin system, superoxide radical ($O_2^{\cdot-}$) generated by NOX or NO generation through the L-arginine dependent NOS activity. This suggests a potential interdependency in the regulation of the NADPH content in the different organs with the metabolism of ROS and RNS. Thus, in both organs the NADP-ICDH activity is inhibited by GSNO (NO donor) and $ONOO^-$ (which results from the reaction of $O_2^{\cdot-}$ and NO). In relation to GSH and H_2O_2, it was observed that the NADP-ICDH activity in *Arabidopsis* roots was not affected whereas in the leaves it was inhibited by GSH. In this sense, irradiance could be a key factor, and some evidence supports this possibility. For example, in aquatic plant *Spirodela polyrhiza*, the cytosolic NADP-ICDH activity is stimulated by red and far-red radiation (Appenroth and Teller 2005). Therefore, it can be concluded that cytosolic NADP-ICDH activity in photo-synthetic and non-photosynthetic organs is differentially regulated.

In summary, the data reveal that the cytosolic NADP-ICDH activity in roots and leaves differs in biochemical characteristics and responses with respect to signalling molecules such as H_2O_2 and NO as well as $ONOO^-$. The NADPH generated by these enzymes is necessary either for GR activity in the ascorbate-glutathione cycle or for the L-arginine-dependent NO synthase activity (Corpas *et al.* 2009a,b). This supports the interconnection between ROS and RNS metabolism in plants.

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