Free Radical Research, February 2007; 41(2):191–199

informa
healthcare

Cytosolic NADP-isocitrate dehydrogenase of pea plants: Genomic clone characterization and functional analysis under abiotic stress conditions*

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Accepted by Professor J. Viña

(Received 11 August 2006; in revised form 21 September 2006)

Abstract
NADPH is an essential electron donor in numerous biosynthetic and detoxification reactions. In animal, yeast and bacteria, the NADP-dependent isocitrate dehydrogenase (NADP-ICDH), which catalyzes the production of NADPH, is being recognized as an essential component of the antioxidative defence mechanisms. In plant cells, there is little information on the antioxidant properties of NADP-ICDH. Using a pea cDNA λgt11 library, the full-length cDNA of a *NADP-ICDH* was obtained. In pea leaves, the analyses of activity, protein and transcript expression of NADP-ICDH under six different abiotic stress conditions (CL, continuous light, HLI, high light intensity, D, continuous dark, LT, low-temperature HT, high-temperature and W, mechanical wounding) revealed a differential regulation at transcriptional and post-translational level depending on the abiotic stress. The activity and protein expression of NADP-ICDH and catalase increased only under HLI but the NADP-ICDH transcripts were up-regulated by cold stress (70%) and W (40%). Under the same conditions, the transcript analysis of glutathione reductase (GR), monodehydroascorbate reductase (MDAR) and ascorbate peroxidase (APX), key components of the antioxidative ascorbate–glutathione cycle, showed similar inductions. These data indicate that in pea plants the cytosolic NADP-ICDH shows a differential response, at mRNA and activity level, depending on the type of abiotic stress and suggests that this dehydrogenase could have a protective antioxidant role against certain environmental stresses in plants.

Keywords: *Abiotic stress, cold stress, NADP-ICDH, environmental stress, pea, wounding*

Abbreviations: *APX, ascorbate peroxidase; CL, continuous light; D, continuous dark; GR, glutathione reductase; HLI, high light intensity; MDAR, monodehydroascorbate reductase; NADP-ICDH, NADP-dependent isocitrate dehydrogenase; W, wounding*

Introduction

NADPH is an essential electron donor in numerous enzymatic reactions, biosynthetic pathways and detoxification reactions. Thus, NAPDH is an essential 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 against oxidative damage. NADPH is also a cofactor of important enzymes of the reactive nitrogen species (RNS) and reactive oxygen species (ROS) metabolism, such as nitric oxide synthase (NOS) [1] and NADPH-oxidase [2], respectively. Cells have several sources of NADPH, including the two dehydrogenases of the

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*Sequence data from this article have been deposited in the EMBL/GenBank data libraries under accession numbers AY509880 for the *NADP-ICDH* complete cDNA and AY730588 for the genomic sequence of cytosolic *NADP-ICDH*.

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2007 Informa UK Ltd.
DOI: 10.1080/10715760601034055

pentose-phosphate pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase), the malic enzyme and the NADP-dependent isocitrate dehydrogenase (NADP-ICDH). In addition, photosynthetic organisms have ferredoxin-NADP reductase (FNR) [3].

NADP-ICDH (EC 1.1.1.42) catalyzes the oxidative decarboxylation of isocitrate to 2-oxoglutarate with the production of the reduced coenzyme NADPH [4]. This enzyme belongs to a multienzymatic family whose members are widely distributed in living organisms and is localized in different cell compartments including cytosol, mitochondria, chloroplasts and peroxisomes [4]. In higher plants, however, little information is available of the specific function of each ICDH isoform. The cytosolic NADP-ICDH has been suggested to be involved in the 2-oxoglutarate supply for ammonium assimilation, in the cycling, redistribution and export of amino acids during senescence [4,5] and in the cytosolic synthesis of NADPH [4]. The participation of cytosolic NADP-ICDH in the degradation of fatty acids has also been proposed [6].

In recent years, NADP-ICDH has been increasingly recognized as an essential component of the antioxidative defence mechanisms in animals [7–10], yeast [11] and bacteria [12]. However, in plant cells comparatively much less information is available on the protective role of NADP-ICDH against oxidative stress. In pea and pepper plants under Cd stress [13,14] and in olive plants under salinity [3], evidence was obtained indicating that NADP-dehydrogenases are key antioxidant systems against oxidative stress.

In this work, the genomic clone of cytosolic NADP-ICDH of pea leaves was characterized and using pea plants exposed to six different abiotic stress conditions which are known to induce oxidative stress in plants, including continuous light (CL), high light intensity (HLI), continuous dark (D), low-temperature (LT) and high-temperature (HT) and mechanical wounding (W), the involvement of leaf cytosolic NADP-ICDH in the mechanism of plant response to those abiotic stresses was studied.

Materials and methods

Plant material and growth conditions

Pea (*Pisum sativum* L., cv. Lincoln) seeds were obtained from Royal Sluis (Enkhuizen, Holland). Seeds were surface sterilized with 3% (v/v) commercial bleaching solution for 3 min and then were washed with distilled water and germinated in vermiculite for 3–4 days under growth chamber conditions (24–18°C, day–night temperature; 80% relative humidity). Healthy and vigorous seedlings of 2–3 weeks were selected and exposed to different stress conditions: (a) CL. Plants were continuously illuminated for 48 h at 190 $\mu E\ s^{-1}\ m^{-2}$ [15]; (b) HLI. They were irradiated for 4 h at 1189 $\mu E\ s^{-1}\ m^{-2}$, using a lamp GE 300 W-230 V PAR 56/WFL (General Electric). To avoid heating of plants, a Petri dish (19 cm diameter) containing cold water was placed 4 cm above plants and water was replaced every 30 min [15]; (c) D. Plants were kept in darkness in a growth chamber for 48 h [16]; (d) W. Leaves were injured by clicking them with a striped-tip forceps. Then, plants were maintained under the initial growth conditions for 4 h and damaged leaves were collected and analysed after this time [17]; (e) LT. Plants were grown for 48 h at 8°C [18]; and (f) HT. Plants were sequentially exposed to 30°C for 1 h, 35°C for 1 h and finally, 38°C for 4 h [19].

Preparation of leaf crude extracts

All operations were carried out at 0–4°C. Leaves were frozen in liquid N_2 and then ground to a powder in a mortar with a pestle and 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 DTT, 0.2% Triton X-100 and 10% glycerol (1:4, w/v). Homogenates were filtered through two layers of Miracloth and centrifuged at 28,000*g* for 30 min. Aliquots of supernatants were immediately used for the assays.

Enzyme assays

Catalase activity was determined according to the method of Aebi [20]. NADP-ICDH activity was determined spectrophotometrically by recording the reduction of NADP at 340 nm [5]. The assay was performed at 25°C in a reaction medium (1 ml) containing 50 mM HEPES, pH 7.6, 2 mM $MgCl_2$, 0.8 mM NADP and the reaction was initiated by the addition of 10 mM 2R,3S-isocitrate. One milliunit of activity was defined as the amount of enzyme required to reduce 1 nmol NADP min^{-1} at 25°C.

SDS-PAGE, Western blot and protein determination

SDS-PAGE was carried in 10% acrylamide slab gels. For Western blot analysis, proteins were electroblotted to PVDF membranes with a semi-dry Trans-Blot cell (BioRad). After transfer, membranes were used for cross-reactivity assays with polyclonal antibodies against pea ICDH [21] at a 1:4000 dilution. For immunodetections, an affinity-purified goat anti-(rabbit IgG)-horseradish peroxidase conjugate (BioRad) was used and an enhanced chemiluminescence method with luminol was employed [15]. The chemiluminescence was detected with a photographic film (Hyperfilm; Amersham Pharmacia Biotech). Protein concentration was determined with the Bio-Rad Protein Assay (Hercules, CA), using bovine serum albumin as standard.

cDNA library screening and isolation of the NADP-ICDH *genomic clone*

The *NADP-ICDH* cDNA was cloned by PCR from a cDNA library constructed in bacteriophage λgt11 from pea leaf poly(A) RNA (kindly supplied by Dr G. Creissen and Dr P. M. Mullineaux, UK). A first PCR allowed the amplification of a 860 bp ICDH fragment employing two degenerated primers (ICP 1F, ICP 2R) (see Table I) designed for conserved regions of the ICDH protein sequence of different species. The PCR mix contained 1.5 mM $MgCl_2$, 1 × PCR buffer, 1 U of Taq polymerase (Roche) and 1 μM of each primer in a final volume of 20 μl. Reactions were carried out in the Hybaid thermocycler. A first step of 2 min at 94°C was followed by 35 cycles of 30 s at 94°C, 30 s at 53°C and 90 s at 72°C. The ICDH fragment was sub-cloned in the vector pGEM-T-easy (Promega) and confirmed by sequencing. In order to get the complete cDNA, two specific primers were designed within the cloned fragment. A second PCR was done in the same conditions but using the following pairs of primers: ICP 3F/gt11-R and ICP 4R/gt11-F (see Table I). The obtained fragments were also sub-cloned in the vector pGEM-T-easy (Promega) and confirmed by sequencing. Overlapping sequences permitted the reconstruction of the complete cDNA. To confirm this sequence, amplification of the complete *ICDH* cDNA was carried out with two specific primers (ICP5-Hind-F/ICP6-Sal-R) (see Table I) localized at each end and containing an enzyme restriction site to facilitate subsequent cloning. The PCR product was confirmed by sequencing.

The complete NADP-ICDH genomic clone was isolated by PCR on genomic DNA from pea leaves (extracted by Qiagen DNA extraction kit) using specific primers designed from the cDNA sequence (accession number AY509880). The PCR mix contained 1 × PCR buffer 3 (Boehringer-Expand Long template PCR system): 50 mM Tris–HCl, pH 9.2, 14 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$, 20% (v/v) DMSO, 1% (v/v) Tween 20, 200 μM each dNTPs, 250 ng genomic DNA, 300 nM ICDHpea-3F primer, 300 nM ICDHpea-4R primer (see Table I) and 0.25 U of Taq DNA polymerase/*Pwo* mix (Boehringer). Amplification was done with one denaturation cycle at 94°C for 2 min, 10 cycles of 20 s at 94°C, 30 s at 60°C and 4 min at 68°C, 20 cycles of 20 s at 94°C, 30 s at 60°C and 4 min at 68°C with a time increment of 10 s per cycle, followed by a final cycle of extension at 68°C for 10 min. The PCR reaction was loaded on a 0.8% agarose gel. The visualized band (at about 4 kb) was cut and extracted from the gel. The purified fragment was cloned into the pBluescript-KS cut by *SmaI* and sequenced.

Table I. Oligonucleotides used for cDNA cloning and semi-quantitative RT-PCR analysis. F, forward oligonucleotide; and R, reverse oligonucleotide. In oligonucleotides (R) = A or G; (Y) = C or T; and (K) = T or G. The oligonucleotides ICP1-F and ICP 4-R were used for the SQ-PCR analysis. Underlined sequences correspond to enzyme restriction sites.

Name	Oligonucleotide sequences (5′–3″)	Accession No
NADP-ICDH		AY509880
ICP 1F	ATGTGGA(R)(R)AGTCCAAA(Y)GGG	
ICP 2R	GC(W)AG(R)TCCTT(K)GTCAT(Y)TTTCC	
ICP 3F	TGCCCTGATGGGAAGACTATTG	
ICP 4R	ATGCCCATACATAACCTCCTT	
ICP5-Hind-F	ATGAAGCTTCATTCCAGAAAATC	
ICP6-Sal-R	ACCGTCGACTCCAATCTCATCAC	
ICDHpea-3F	CTTCTTCCGATCACCACCAT	
ICDHpea-4R	AAGCACAACCCATGCTATCC	
Phage lambda		
gt11-F	GGTGGCGACGACTCCTGGAGCCCG	
gt11-R	TTGACACCAGACCAACTGCTAATG	
Actin II		X68649
ACT-SQ-F	AATGGTGAAGGCTGGATTTG	
ACT-SQ-R	AGCAAGATCCAAACGAAGGA	
Cytosolic *GR*		X98274
GR1-F	ACAGTATTTGGTGGGCAAGC	
GR1-R	AAACTGCGCTTTCGTAGCTC	
Cytosolic *APX*		X62077
APX-F	TCCTTTCGGAACAATTAAGC	
APX-R	TCCTTCTCACCAGTCAACAA	
MDAR		AY662655
MDAR-SQ-F	AAAGGGACAGTTGCTGTTGG	
R-MDH	ACGGGAATAGAAGTATGGAAGG	
Catalase		X60169
CAT-F	ATCCCTATCTTCTGCTCC	
CAT-R	GACATACACCGATTTTCC	

RNA isolation and semiquantitative reverse transcription-PCR

Total RNA was isolated from pea leaves using the Trizol reagent kit according to the manufacturer's instructions. About 2 μg of total RNA from leaves was used as a template for the reverse transcriptase (RT) reaction. It was added to a mixture containing 5 mM $MgCl_2$, 1 mM dNTPs, 0.5 μg oligo (dT_{23}) primers, 1 × RT-buffer, 20 U Rnasin ribonuclease inhibitor and 15 U AMV reverse transcriptase (Promega, Madison, WI). The reaction was carried out at 42°C for 40 min, followed by a 5 min step at 98°C and then by cooling to 4°C.

Amplification of *actin II* cDNA from pea (accession number X68649) was chosen as a control. *NADP-ICDH*, *actin II*, cytosolic glutathione reductase *GR*, monodehydroascorbate reductase (*MDAR*), ascorbate peroxidase (*APX*) and catalase cDNAs were amplified by the polymerase chain reaction (PCR), as follows: 1 μl of the produced cDNA diluted 1/20 was added to 250 μM dNTPs, 1.5 mM $MgCl_2$, 1 × PCR buffer, 1 U of Ampli Taq Gold (PE Applied Biosystems) and 0.5 μM of each primer (see Table I) in a final volume of 20 μl. Reactions were carried out in the Hybaid thermo-cycler. A first step of 10 min at 94°C was followed by 28–33 cycles (depending of the gene) of 30 s at 94°C, 30 s at 60°C and 45 s at 72°C. The expected amplified PCR products were 535 bp for *ICDH* (using the oligonucleotides ICP1F and ICP 4R, see Table I), 321 bp for *GR*, 381 bp for *MDAR*, 435 bp for *APX*, 496 bp for *actin II* and 711 bp for catalase. Then, they were detected by electrophoresis in 1% agarose gels and staining with ethidium bromide.

Quantification of the bands was performed using a Gel Doc system (Bio-Rad Laboratories, Hercules, CA) coupled with a high sensitive CCD camera. Band intensity was expressed as relative absorbance units. The ratio between each specific gene and *actin II* amplification was calculated to normalize for initial variations in sample concentration. Means and standard deviations were calculated after normalization to *actin II*.

Sequence analysis, databases searches, subcellular localization predictions and primers design

Blast searches were made in the National Center for Biotechnology Information internet site (http://www.ncbi.nlm.nih.gov/). Alignments were performed using OMIGA (2.0) and CLUSTAL W v.1.8 (http://www.infobiogen.fr/services/analyseq/cgibin/clustalw_in.pl). Primers design was done with OMIGA or with PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Localization predictions were realized with PSORT Prediction and iPSORT Prediction (http://psort.nibb.ac.jp). The theorical molecular mass and pI was estimated using the http://www.expasy.org/cgibin/ pi_tool.

Data analyses

Phylogenetic analyses were conducted using MEGA version 3.0 (http://www.megasoftware.net/) using the unrooted neighbour-joining of 13 amino acid sequences of plant NADP-ICDHs. The predicted candidate gene products were aligned using CLUSTAL W and the tree was done with MEGA. Accession number for the proteins referred to in Figure 2 are NP_175836, NP_196963 and NP_176768 for Arabidopsis; BAA34113 and BAA34112 for carrot; CAA53300 for potato; AAD37810 and NP_917313 for rice; AAC64182 and S33612 for soybean; T04355 and CAA54912 for tobacco; and AAU44341 for pea.

To estimate the statistical significance of differences the data were analysed by Student's *t*-test.

Results

Isolation and characterization of pea cytosolic NADP-ICDH *cDNA and its genomic clone*

To isolate the pea NADP-ICDH cDNA, a λgt11 cDNA library was used. The size of the full-length cDNA sequence represented 1565 nucleotides long, with a 5′-unstranslated region of 78 nucleotides, an open reading frame of 1239 nucleotides for the entire protein coding region and a 3′ non-coding region of 383 nucleotide with a polyA tail in the end. This is the first cDNA described for this enzyme in *Pisum sativum* (GenBank accession number AY509880). The nuclear gene of this *NADP-ICDH* was also obtained. Its genomic organization comprises 15 exons and 14 introns, giving a total length of 3543 nucleotides (4004 nucleotides with the two UTR regions). In all cases, the exon–intron boundaries are conformed to the splice acceptor/donor consensus sequences, AG and GT (GenBank accession number AY730588). The structure of the pea gene and the *Arabidopsis NADP-ICDH* gene family which are found in chromosome 1 and 5 are shown in Figure 1. In all cases, the genes have 15 exons and 14 introns. At1g65930 has 3203 nucleotides and codifies a protein (NP_176768) which seems to be cytosolic. At1g54340 has 3306 nucleotides and codes for a protein (NP_175836) which could be peroxisomal, and At5g14590 has 3437 nucleotides and codifies a protein (NP_196963) which seems to be localized in the chloroplasts. All genes are quite similar in length.

The deduced amino acid sequence for the pea ICDH protein consisted of 412 amino acid residues with a theoretical molecular mass of 45,946 Da and a pI of 6.20. The total number of negatively charged residues (Asp + Glu) was 57 and the positively charged residues (Arg + Lys) was 53. The instability index (II) was computed to be 21.72 which classifies the pea ICDH as a stable protein [22]. Its protein sequence showed a 89–91% identity to the NADP-ICDH of

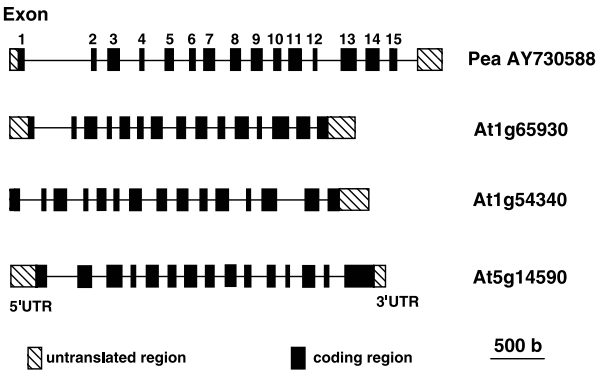

Figure 1. Genomic organization of the pea NADP-ICDH gene (AY730588) and the *Arabidopsis* NADP-ICDH gene family. The structure of the genes is shown with exons indicated by black boxes and introns shown as black lines. Untranslated regions are shown by dashed boxes. Exon–intron regions are drawn at scale. At1g65930 has 3203 nucleotides and codes for the protein NP_176768. At1g54340 has 3306 nucleotides and codes for NP_175836. At5g14590 has 3437 nucleotides and codes for NP_196963.

Glycine max (Q06197), *Medicago sativa* (S28423) and *Daucus carota* (BAA34112).

The phylogenetic tree of the deduced protein of pea NADP-ICDH (Figure 2) associates this protein with other cytosolic NADP-ICDHs which are well separated from the group of isoforms with a targeting signal for mitochondria and chloroplasts, and from the group of NADP-ICDHs with a peroxisomal targeting signal type 1 (PTS1) which is a SKL motif in the C-terminus of its sequence [23].

Activity and protein expression of NADP-ICDH and catalase in plants under abiotic stress conditions

The analysis of the enzymatic activity and protein expression of NADP-ICDH in leaves of pea plants under six different abiotic stress conditions, including CL, HLI, CD, W, LT and HT, is shown in Figure 3, panel A. Under these conditions, plants exposed to HLI showed an enzymatic activity 43% higher than the control and this enhancement was only 20% in the case of plants subjected to CL and CD. This significant increase of the NADP-ICDH activity was correlated with the higher protein expression observed by immunoblotting. The activity of catalase, a characteristic antioxidant enzyme, was increased (43%) by HLI but was not significantly affected by the other stress conditions (Figure 3, panel B).

Transcript analysis of NADP-ICDH*, catalase and the ascorbate-glutathione cycle enzymes in plants under abiotic stress conditions*

In leaves of pea plants exposed to different abiotic stress conditions the transcript analysis, by semiquantitative RT-PCR, of NADP-ICDH, catalase and three

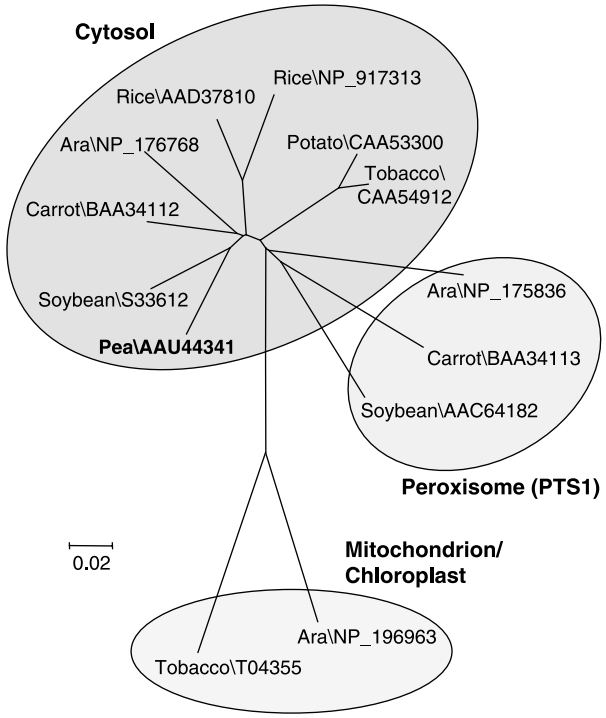

Figure 2. Phylogenetic tree of diverse plant NADP-ICDHs. The tree was calculated on the basis of the amino acid sequences by the neighbour joining method using MEGA3 and the alignment was done using ClustalW. Prediction of protein localization in cell compartments was made through iPSORT with PSORT Prediction programs. The horizontal bar represents 0.02 substitutions per nucleotide of the corresponding gene.

cytosolic enzymes of the antioxidative ascorbate–glutathione cycle, including GR, MDAR and PXA, was studied (Figures 3 panel B and 4).

The expression of *NADP-ICDH* was up-regulated 70, 40 and 30% by LT, W and HT, respectively. *GR* only was induced by cold stress (about 260%) but was down-regulated by HLI and continuous dark. On the other hand, *MDAR* was strongly induced (220%) by low temperature and wounding (200%). The cytosolic *APX* was up-regulated by all the stresses assayed, ranging from 150% by CL to 260% by cold stress. On the other hand, leaf catalase was not affected (Figure 3, panel B).

Discussion

Recent works have shown that NADP-ICDH is involved in the defence mechanisms against oxidative stress in bacteria [12], the malaria parasite *Plasmodium falciparum* [24], yeast [11,25] and animals. In these latter organisms, it has been proposed that one of the primary functions of NADP-ICDH is to control the redox balance and oxidative damages in cytosol and mitochondria by supplying NADPH to the antioxidant systems [7,10,26]. However, in plants the possible connection between NADP-ICDH and environmental/oxidative stress has been less explored. In this

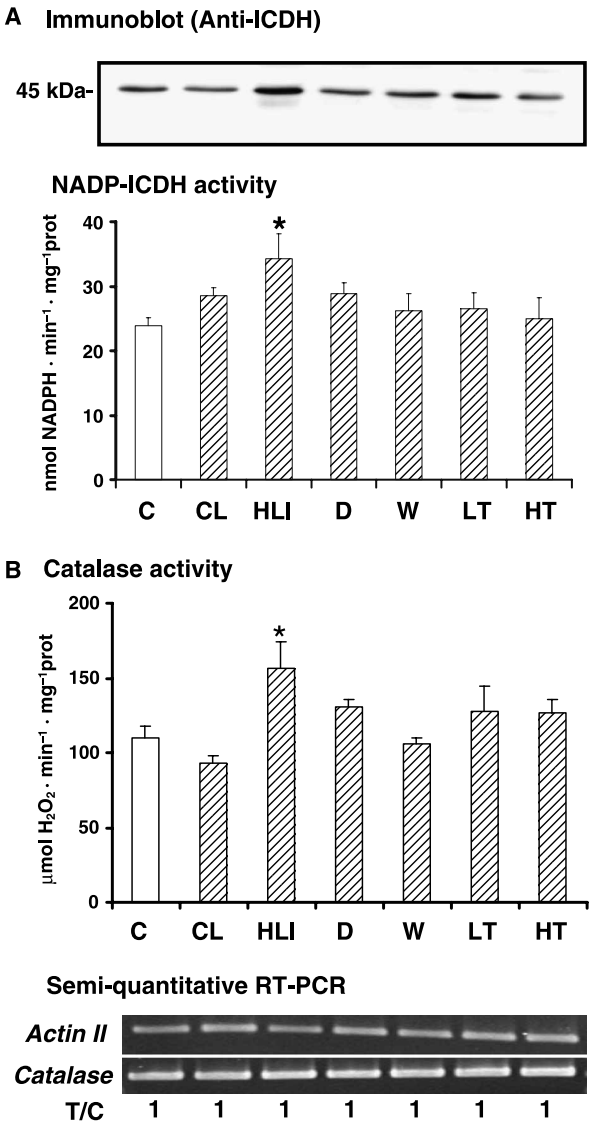

Figure 3. NADP-ICDH and catalase in leaf extracts from pea plants exposed to different stress conditions. Panel A, NADP-ICDH activity (25 µg protein) and immunoblot analysis of crude extracts (20 µg protein) probed with anti-pea NADP-ICDH (1:4000 dilution) showing an immunoreactive band with a molecular mass of 45 kDa. Panel B, catalase activity (25 µg protein) and a representative agarose electrophoresis gel of the semi-quantitative RT-PCR analysis of the amplification products of catalase transcripts visualized by ethidium bromide staining. T/C indicates the relative level of the *catalase* amplification product (T) over the *Actin II* internal control (C) after normalization to the control samples and expresses the change in folds with respect to the untreated control. Values are means of at least three different experiments. C, control; CL, continuous light; HLI, high light intensity; D, continuous dark; W, wounding; LT, low temperature; and HT, high temperature. Values are means of at least three independent experiments. *Differences from control values were significant at $P < 0.05$.

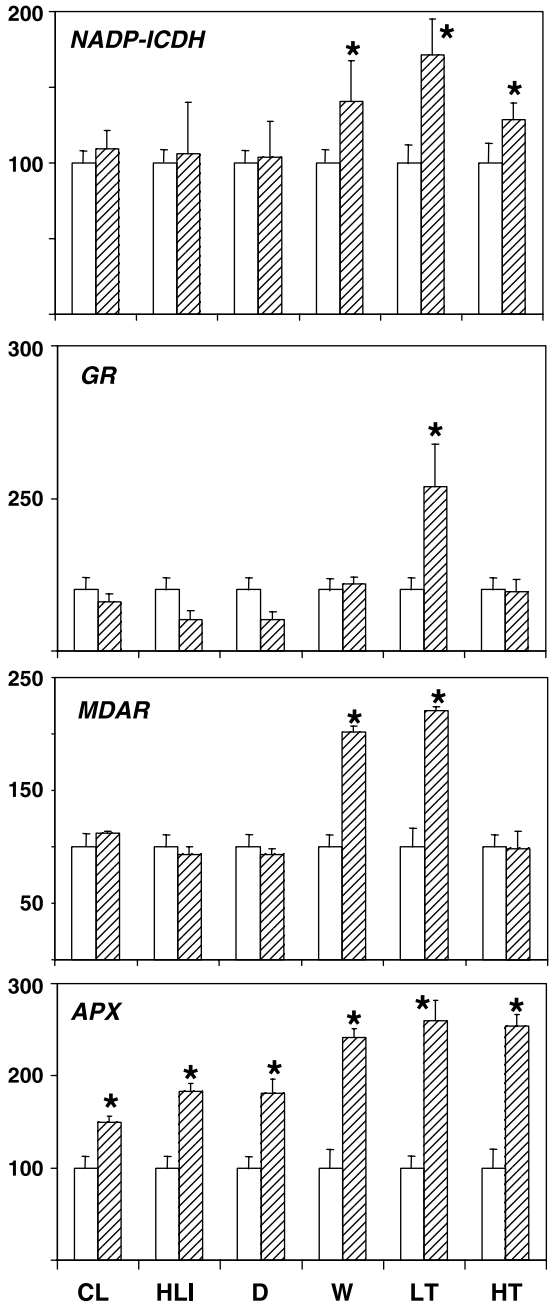

Figure 4. Transcript analysis of pea leaf *NADP-ICDH*, *GR*, *MDAR* and *APX* under six different abiotic stress conditions. Semi-quantitative RT-PCR was performed on total RNA isolated from leaves of plants subjected to different stress conditions. CL, continuous light; HLI, high light intensity; D, continuous dark; W, wounding; LT, low temperature; and HT, high temperature. The value of each enzyme transcript (hatched box) is expressed as percentage of the signal delivered by amplification of the *actin II* RNA, used as internal control (white boxes). The graphics represent the average results and SD obtained from at least three independent experiments and separate amplifications. *Differences from control values were significant at $P < 0.05$.

work, the molecular characterization of the cytosolic NADP-ICDH of pea leaves was carried out as a first step to study the response of this enzyme and other antioxidative enzymes to six different abiotic stress conditions, in order to know the possible involvement of plant NADP-ICDH in the cellular defence mechanisms against environmental stress conditions. In higher plants, the six different types of abiotic stress used in this study have been demonstrated to produce oxidative stress due to the overproduction of ROS

and/or the induction of imbalances in the antioxidative systems [27–29].

The cDNA of pea leaf NADP-ICDH was isolated and its genomic organization was described for the first time in pea plants. In *Arabidopsis,* three genes were found which coded for putative NADP-ICDHs, two in chromosome 1 and one in chromosome 5 (*At1g65930*, *At1g54340*, and *At5g14590*). In all cases, the gene had 15 exons. The comparative analysis of the intron positions revealed a strong conservation while the length of the introns was different. The length of the exons was exactly the same in all the *Arabidopsis* ICDHs and the pea enzyme, except for the first and last exons, and exon 8 (data not shown). This variation is likely to occur at the ends of proteins due to the targeting signals for the subcellular localization of the different isoforms.

The pea NADP-ICDH cDNA, isolated in this work, seems to code for the cytosolic isoform, and several reasons support this idea. First, the absence of any specific targeting signal in the cDNA and the cellular prediction obtained with the program PSORT Prediction. Second, the phylogenetic tree analysis showed three different groups (Figure 2) depending on their subcellular localization and the pea cDNA was in the group of the cytosolic isoforms.

Previous studies have shown that the cytosolic isoform of NADP-ICDH represents more than 90% of the total cellular NADP-ICDH activity [30,31]. Thus, in the abiotic stress conditions assayed the NADP-ICDH activity of crude extracts from pea leaves represents essentially the cytosolic isoform. Results described in this work showed the induction of NADP-ICDH activity by HLI, but the enzyme activity was not affected by the other environmental stresses. In *Mesembryanthemum crystallinum*, the induction of NADP-ICDH activity by salt stress was demonstrated [32] and in *Phaseolus vulgaris*, the heavy metals Zn and Cd can stimulate the activity of several dehydrogenases, including NADP-ICDH, glucose-6-phosphate dehydrogenase and malic enzyme [33]. The same effect was produced by Cd in leaves of pepper plants [14] and by salt stress in leaves of olive plants [3].

However, most studies have been focused on the analysis of NADP-ICDH in different plant tissues and during development [30,34,35] but little is known about the mRNA expression of NADP-ICDH under abiotic stress conditions. In our experimental conditions, a significant up-regulation of the NADP-ICDH transcripts was observed by LT, W and HT but the enzyme expression was not affected by the other stresses. This means that cytosolic NADP-ICDH has a differential regulation depending on the type of stress: a posttranslational regulation by HLI, when the enzyme activity and protein expression increased (Figure 3(A)); and a transcriptional regulation by LT, W and HT, as shown by the increase of the NADP-ICDH transcripts (Figure 4).

To explore the relationship between environmental stress and the induction of antioxidative enzymes, the activity and transcript expression of catalase, encharged with the removal of hydrogen peroxide, was also evaluated. The activity pattern of catalase was similar to that of NADP-ICDH, being significantly induced by HLI (Figure 3B). It has been reported that NADPH is bound to catalase and protects this enzyme from oxidative damage [36]. The results obtained in this work indicate that in pea plants illuminated with HLI NADP-ICDH could have a role in supplying NADPH to protect catalase activity.

The gene expression of three enzymes of the antioxidative ascorbate–glutathione cycle, GR, MDAR and APX, was also analysed. The cytosolic enzyme APX is involved in the direct removal of H_2O_2 and is considered one of the main antioxidative enzymes [37]. The expression analysis by semi-quantitative RT-PCR showed the upregulation of cytosolic APX in all the abiotic stresses studied which indicates that these abiotic conditions are oxidative stress producers. The induction of cytosolic APX has been reported in *Arabidopsis* by wounding [38], in spinach by high-light intensity and paraquat [39], in pea by drought and paraquat [40] and in rice by low temperature [41].

It is well known that LTs adversely affect plant growth and crop production [42]. Microarray studies on 7000 *Arabidopsis* genes under cold stress conditions has allowed identification of 53 cold stress-inducible genes [42], but the NADP-ICDH gene apparently was not included in this analysis. Results obtained in this work showed that the growth of pea plants at LT triggered the coordinate gene expression of cytosolic NADP-ICDH and the ascorbate–glutathione cycle enzymes, GR, MDAR and APX. The results obtained for GR are in agreement with those reported by Stevens et al. [43] in pea plants under cold conditions where the levels of the cytosolic GR transcript increased 2–3-fold.

In the stress by W, an up-regulation of the transcripts of NADP-ICDH, MDAR and APX was also observed. This suggests that the same signalling pathways could be operative in cold stress and W. Considering that wounding is a local physical damage and cold is a more general signal, there must be some common factors in both types of stress to generate a similar genomic response. Hydrogen peroxide has been shown to act as a second messenger for the induction of defence genes in response to wounding in tomato plants [44] and the induction of cytosolic APX in wounded *Arabidopsis* leaves has been reported [38]. The increase of the transcript of *APX* which catalyses the removal of H_2O_2 could indicate the involvement of this ROS in the up-regulation of *APX* transcription. The oxidative burst and the accumulation of H_2O_2 appears to be mediated by the activation of a membrane-bound NADPH-

oxidase complex [45] and this activation requires NADPH which could be supplied by NADP-ICDH.

In conclusion, in this work the full-length cDNA of a cytosolic NADP-ICDH from pea leaves was reported and the data obtained showed that this enzyme has a differential regulation depending on the type of abiotic stress. The molecular evidence obtained support the existence of a connection at transcriptional level between cytosolic NADP-ICDH and antioxidative enzymes induced by low temperature stress.

Acknowledgements

This work was supported by a Research Training Network grant of the European Commission (HPRN-CT-2000-00094) and the *Junta de Andalucía* (research group CVI 192). ML acknowledges a contract from the European Commission. The authors are grateful to Dr Pierre Gadal, Université de Paris-Sud, Orsay cedex, France, for his kind supply of the antibody to pea NADP-ICDH. We also thank Dr Phil M. Mullineaux, University of Essex, UK and Dr Gary Creissen, John Innes Centre, Norwich, UK, for the generous donation of the pea cDNA λgt11 library.

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