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Spatial distribution of cytosolic NADP$^+$-isocitrate dehydrogenase in pine embryos and seedlings

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Summary Cytosolic NADP$^+$-linked isocitrate dehydrogenase (NADP$^+$-IDH) is considered the main enzyme catalyzing the production of 2-oxoglutarate for amino acid biosynthesis in plants. We characterized a full-length cDNA encoding the cytosolic NADP$^+$-IDH in the gymnosperm *Pinus pinaster* Ait. The deduced amino acid sequence exhibited high homology with available sequences in angiosperms. Genomic analysis indicated that only one gene, or two genes with a high degree of homology, encodes the protein in *P. pinaster*. Cytosolic NADP$^+$-IDH is up-regulated during embryo germination concomitantly with glutamine synthetase. Immunohistochemical analysis of germinating seeds and young seedlings showed a broad spatial pattern of NADP$^+$-IDH expression. The protein was detected in vascular tissues of germinating embryo and seedling organs, as well as in other cellular types and tissues, including parenchyma and epidermal cells. The spatial pattern of NADP$^+$-IDH expression in the embryo and seedling organs did not coincide with the reported spatial patterns for other key enzymes of nitrogen metabolism. Treatment of seedlings with phosphinothricin, an inhibitor of glutamine synthetase (GS), differentially affected GS and NADP-IDH in cotyledons. In response to herbicide treatment, GS was up-regulated in 0.5-cm-long cotyledons, whereas NADP$^+$-IDH was up-regulated in 1.5-cm-long cotyledons, suggesting that 2-oxoglutarate is required to overcome the herbicide effect in tissues with a high demand for glutamate. The results indicated that cytosolic NADP$^+$-IDH is a housekeeping enzyme that has not undergone functional specialization during evolution. Its spatial pattern in pine tissues suggests that it facilitates metabolism in different ways depending on the characteristics of the particular tissue and cellular type.

Keywords: immunohistochemistry, pine NADP$^+$-isocitrate dehydrogenase cDNA, Pinus pinaster, primary development.

Introduction

The synthesis of 2-oxoglutarate provides a key link between carbon and nitrogen metabolism in plants. The oxoacid is the primary carbon skeleton for the assimilation of inorganic nitrogen into amino acids through the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle. This cycle comprises key enzymes in nitrogen metabolism that catalyze the synthesis of glutamate from ammonia, 2-oxoglutarate, ATP and reducing power (Ireland and Lea 1999). Glutamate is an important nitrogen donor in the biosynthesis of other amino acids and nitrogen compounds, and has a possible role in signal transduction (Forde and Lea 2007). The supply of 2-oxoglutarate is a critical step in glutamate biosynthesis. The oxoacid can be produced by several reactions catalyzed by aminotransferases, the mitochondrial NAD$^+$-isocitrate dehydrogenase associated with the Krebs cycle, and by NADP$^+$-linked isocitrate dehydrogenase (NADP$^+$-IDH), which comprises a number of isoenzymes in different subcellular compartments including cytosol, chloroplast, mitochondria and peroxisome. The cytosolic NADP$^+$-IDH isoenzyme represents more than 90% of total IDH activity in all plant organs (Chen and Gadal 1990*a*, Chen 1998, Palomo et al. 1998), and is assumed to be mainly responsible for 2-oxoglutarate production when large quantities of glutamate are required, as occurs during development when the production of new structures demands a net supply of nitrogen donors, glutamate and glutamine. Although the central role of cytosolic NADP$^+$-IDH isoenzyme was proposed more than a decade ago (Chen and Gadal 1990*b*), there is little evidence to confirm its role in plants.

In herbaceous plants, cytosolic NADP$^+$-IDH is associated with nitrogen metabolism (Hanning and Heldt 1993), and its up-regulation along with that of other enzymes involved in nitrogen assimilation has been observed in tobacco plants with low nitrate reductase activity (Scheibe et al. 1997). In tomato fruits, cytosolic NADP$^+$-IDH is associated with the accumulation of glutamate and other derived amino acids during the ripening process (Gallardo et al. 1995); and a role in recycling and distribution of amino acids during senescence has also been proposed in other Solanaceae species (Fieuw et al. 1995, Masclaux et al. 2000). Information about NADP$^+$-IDH in woody plants is scarce. In purified mitochondria from *Picea abies* (L.) Karst., NADP$^+$-IDH activity was half that of NAD$^+$-IDH activity (Cornu et al. 1996). In pine, cytosolic

$NADP^+$-IDH is estimated to represent about 90% of total IDH activity present in embryos and seedlings (Palomo et al. 1998). Cytosolic GS and $NADP^+$-IDH activities both increase during germination of pine embryos, but the increase in cytosolic $NADP^+$-IDH activity during differentiation of pine hypocotyls is unrelated to the activities of GS, GOGAT and Rubisco (Palomo et al. 1998). These data suggest that $NADP^+$-IDH has other, as yet unknown, functions in plant development (Palomo et al. 1998). Recently, several ESTs corresponding to cytosolic $NADP^+$-IDH have been detected in libraries prepared from cambial tissue in woody plants, including poplar and pine (Sterky et al. 2004, Cantón et al. 2004), and the enzyme has been located in the vascular tissue of *Eucalyptus* seedlings (Boiffin et al. 1998). The molecular characterization of $NADP^+$-IDH has been carried out in a few angiosperms (Hodges et al. 2003). The functional enzyme is always referred to as a homodimeric protein, and a small nuclear gene family encodes $NADP^+$-IDH isoenzymes. These facts have led to its use as a marker in population studies based on allozyme analyses (Murphy et al. 1996), and in the elucidation of plant phylogeny in *Saltugilia* spp. (Weese and Johnson 2005).

Apart from the importance of the $NADP^+$-IDH-catalyzed reaction in plant metabolism, little is known about the enzyme, including the conservation of its primary structure between gymnosperms and angiosperms, the cellular types in which the protein is mainly expressed, the factors affecting its biosynthesis and activity, and the use of its reaction products in processes other than glutamate biosynthesis. We describe the analysis of cytosolic $NADP^+$-IDH from maritime pine (*Pinus pinaster* Ait.). Its spatial expression pattern during seed germination and early seedling growth is revised according to the new information recently reported for other key nitrogen enzymes in pine seedlings (Cánovas et al. 2007 and references therein). Our results suggest that cytosolic $NADP^+$-IDH has been conserved during evolution as a basic component of cell metabolism in higher plants.

Materials and methods

Plant material and growth conditions

Pinus pinaster seeds from Sierra Bermeja (Málaga, Spain) were provided by the Consejería de Medio Ambiente (Junta de Andalucía). Seeds were imbibed in deionized water for 24 h with continuous aeration and then germinated. Seedlings were grown as described previously (Cánovas et al. 1991), with no external nitrogen supplied. Seedlings at two developmental stages—cotyledons of 0.5 and 1.5 cm in length—were sprayed with 25 mM phosphinothricin (PPT) and samples collected 8 h after the herbicide treatment. Seedlings sprayed with water served as controls.

Sequence analysis

Random sequencing of a cDNA library from differentiating xylem of *P. pinaster* (Cantón et al. 2004) allowed the identification of three clones each with a sequence similar to that of $NADP^+$-IDH from angiosperms. The cDNA inserts were subcloned into the pSK vector and then sequenced. All cDNA inserts corresponded to the same gene product, and the largest one contained a 1736 nt full-length cDNA encoding $NADP^+$-IDH. The sequence was deposited in Genbank as Accession no. AY344584. Sequence alignments and phylogenetic analyses were performed with the CLUSTAL X program (Higgins et al. 1996). The program Boxshade (www.ch.embnet.org) was used to draw the alignment of protein sequences.

Protein extraction, SDS-PAGE and Western-blot analysis

Total soluble proteins were extracted as described earlier (Gallardo et al. 1999). Protein was estimated by the method of Bradford (1976) with bovine serum albumin as a standard. Proteins were analyzed by SDS-PAGE according to the procedure of Laemmli (1970) followed by Coomassie blue staining. Cotyledonary proteins were fractionated by SDS-PAGE (10% polyacrylamide gels) followed by Western blot analysis with antisera raised against pine $NADP^+$-IDH (Palomo et al. 1998) and GS (Cantón et al. 1996).

Nucleic acid isolation and analysis

Genomic DNA was prepared according to Dellaporta et al. (1983). Total RNA was isolated as described by Chang et al. (1993). Digestion of DNA and Southern and Northern blotting were carried out as described by Ausubel et al. (2008). Blots were hybridized with ^{32}P-labeled pine $NADP^+$-IDH full-length cDNA by the random-primer method. For Northern blot analysis, two probes were used for hybridization, a 975-bp fragment produced by digestion with BamHI, corresponding to the 5′ region of the $NADP^+$-IDH cDNA, and a 282-bp probe produced by NcoI and XhoI digestion and corresponding to the 3′ non-translated sequence of the cDNA. Prehybridizations and hybridizations were performed according to Church and Gilbert (1984) at 65 °C. After hybridization, membranes were washed at 65 °C in 0.1× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7) and 0.1% SDS in the case of Northern blots, and at 65 °C in 0.5× SSC, 0.1% SDS in the case of Southern blots before exposure.

Plant tissue preparation for microscopy and immunohistochemical localization

Pine tissues were fixed and embedded in paraffin as described by Cantón et al. (1999). The embedded tissues were sectioned at 10 μm thickness, and fixed on poly-L-lysine-coated glass slides. For light microscopy, the paraffin was removed with histoclear (National Diagnostic, Atlanta, GA) and the sections hydrated and washed in phosphate buffer saline (PBS, 3 times for 10 min). To inhibit endogenous peroxidase activity, sections were incubated for 20 min in 1% (v/v) hydrogen peroxide (H_2O_2). After PBS washing (3 times for 10 min), sections were incubated in 2% (v/v) normal goat serum and 0.3% (v/v) Triton X-100 in PBS at room temperature for 1 h, to block non-specific binding of the antibodies and permeate the tissues, respectively. To detect $NADP^+$-IDH polypeptides, the sections were incubated for 18 h with antibodies purified against the protein and diluted 1:200. After extensive washing

in PBS, the sections were incubated in a solution of biotinylated goat anti-rabbit immunoglobulin G (IgG; Sigma) diluted 1:500 for 1 h, washed again in PBS, and incubated in ExtrAvidin peroxidase (Sigma) diluted 1:2000 for another hour. Immunolabeling was revealed with 0.05% (w/v) diaminobenzidine (Sigma), 0.05% (w/v) nickel ammonium sulfate, and 0.03% (v/v) H_2O_2 in PBS. All steps were carried out at room temperature with gentle agitation. The sections were then thoroughly washed in PBS, dehydrated in ethanol, cleared in xylene, and cover-slipped with DPX (BDH, Poole, U.K.). Digital photographs were taken with a Nikon DXM1200 digital camera attached to a Nikon microscope (Model eclipse E800).

Results

Molecular characterization of pine NADP+-IDH

Sequencing of a maritime pine cDNA library from differentiating xylem (Cantón et al. 2004) revealed the presence of three clones having a sequence similar to angiosperm NADP+-IDH. All cDNA inserts corresponded to the same gene product, and the largest contained a 1736 nt full-length cDNA encoding NADP+-IDH (Accession no. AY344584). The deduced amino acid sequence is shown in Figure 1. Comparison of the pine nucleotide and amino acid sequences with other angiosperms NADP+-IDHs revealed a high amino acid similarity of 83.5–89.8% (mean of 87%) and nucleotide identity (mean value of 73%) to other cytosolic NADP+-IDHs; however, homology to peroxisomal, mithochondrial and chloroplastic NADP+-IDH isoenzymes was lower (means of 78.9 and 63.5% at the amino acid and nucleotide level, respectively, data not shown). Alignment with selected cytosolic NADP+-IDH amino acid sequences (Figure 1A) indicated that the conserved residues in the pine sequence included those involved in binding to substrates (Mg^{2+}-isocitrate and NADP+), as well as serine-96, a highly conserved residue among prokaryotic and eukaryotic NADP+-IDHs that is involved in the regulation of enzymatic activity by phosphorylation in bacteria (Hurley et al. 1991). These data indicate that the pine sequence corresponded to a cytosolic NADP+-IDH that is apparently expressed in developing pine xylem. A phylogenetic tree of cytosolic NADP+-IDHs is shown in Figure 1B. Sequences from species belonging to the same order such as Poales and Fabales shared a similarity index of 90% or higher. Relatively high values were also observed among the sequences from species belonging to different orders, with the exception of *P. pinaster*, *Arabidopsis thaliana* L. and *Cucumis sativus* L., which were grouped in the same branch and showed similarity indices of less than 90% with other cytosolic NADP+-IDHs (Figure 1B). These data suggest that few changes in the primary structure of cytosolic NADP+-IDH have occurred during the evolution of vascular plants.

Figure 1C shows the Southern blot analysis of pine genomic DNA following restriction digestion, with the full-length NADP+-IDH cDNA as probe. A few cross-hybridizing bands were observed when digestion was performed with several restriction enzymes. According to the restriction map of the cDNA (not shown), the pine cytosolic NADP+-IDH is encoded by a small family of one or two genes or by two alleles with high nucleotide identity.

NADP+-IDH is expressed in different cell types of P. pinaster embryos during germination

Analysis of NADP+-IDH expression indicated that NADP+-IDH mRNA is barely detectable in the embryos of dry seeds, or after 24 h of imbibition; however, once the embryonic radicle started to elongate, a peak of mRNA expression was detected (Figure 2A). Similarly, low amounts of NADP+-IDH protein were detected in dry and imbibed embryos, but increased abundance of the NADP+-IDH polypeptide signal was found in germinating embryos, paralleling the increased abundance of NADP+-IDH mRNAs (Figure 2A). The increased NADP+-IDH polypeptide signal during pine embryo germination was accompanied by an increased GS polypeptide signal (Figure 2A, cf. Palomo et al. 1998). Histological analysis of NADP+-IDH protein expression revealed a general increase in expression associated with germination as judged by the increased signal observed in imbibed embryos and in germinating embryo sections (Figures 2B–G). Increased intensity of the NADP+-IDH polypeptide signal was observed in the radicle of germinating embryos (Figure 2G) and in various tissues including the procambium, a thin strand of narrow cells corresponding to the embryonal vascular system, (Figures 2G and 2H), and in chlorophyll-containing cells of the palisade parenchyma of the cotyledons (Figure 2I). Negative controls showed no detectable endogenous peroxidase activity in dry mature embryos, imbibed embryos or germinated embryos (Figures 2B, 2D and 2F).

NADP+-IDH expression in different tissues of developing pine seedlings

The expression of the NADP+-IDH was analyzed by Northern and Western blots using the cDNA and specific antibodies as probes (Figure 3A). Based on a 975-bp probe corresponding to the 5′ region of the full-length cDNA, increased NADP+-IDH transcript abundance was highest in roots, high in hypocotyls, whereas low transcript levels were observed in cotyledons (Figure 3A). Similar results were observed when Northern blots where hybridized with either the full-length cDNA, or a 282-bp probe from the non-translated 3′ region of the cDNA (data not shown). The intensities of the signals for NADP+-IDH transcripts and polypeptides were closely correlated, with roots having the highest polypeptide signals.

Imumnohistochemical analysis indicated that the NADP+-IDH polypeptide signal was broadly distributed in all organs examined. In developing green cotyledons, the signal for NADP+-IDH polypeptide was localized in most cell types, including cells of the vascular bundles, and in parenchyma and epidermal cells (Figures 3B and 3C). In hypocotyls, the strongest signal was observed in parenchyma cells and vascular tissue, although the NADP+-IDH polypeptide signal was also detected in the epidermis and pith (Figures 3D, 3E, 4A and 4B).

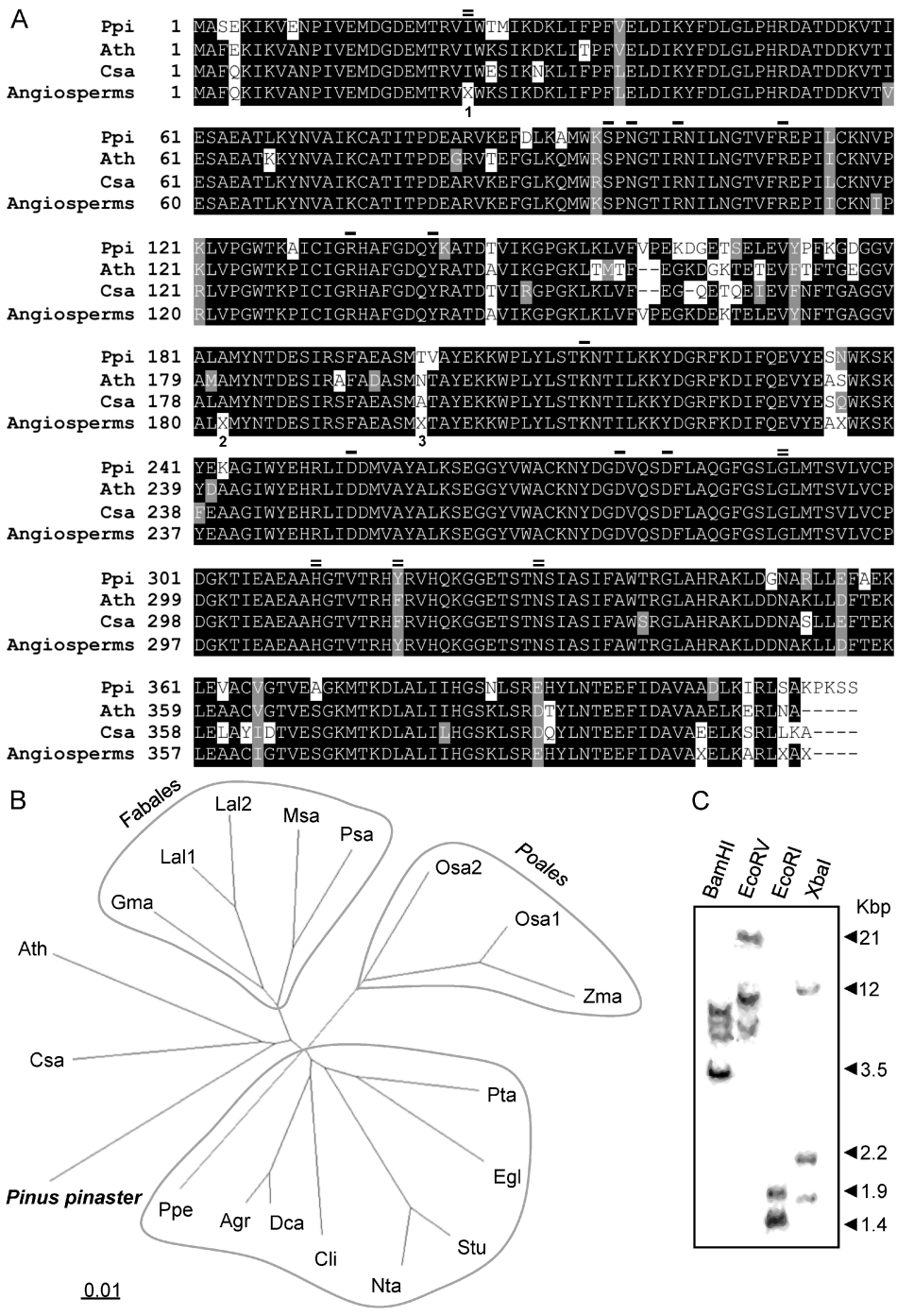

Figure 1. Primary structure, phylogenetic and genomic analyses of cytosolic *Pinus pinaster* NADP$^+$-IDH. (A) Amino acid sequence derived from the full-length cDNA encoding cytosolic pine NADP$^+$-IDH (Ppi, AY344584) and alignment with *Arabidopsis thaliana* (Ath, AY097340), *Cucumis sativus* (Csa, AJ437269) and a consensus sequence for cytosolic NADP$^+$-IDH from angiosperms based on the alignment of sequences considered in the phylogenetic tree (see below). Identical and similar residues are drawn with black or gray background, respectively. Conserved plant NADP$^+$-IDH residues implicated in Mg^{2+}-isocitrate and NADP$^+$ binding are underlined and double-underlined, respectively. A dash represents a gap introduced to optimize the alignment. Residues in the consensus angiosperm sequence indicated by X correspond to positions in which four or more different residues are found in the sequence from different species with the following exceptions: 1 indicates F or I; 2 indicates A or S; 3 indicates A, N or T. (B) Phylogenetic tree of plant cytosolic NADP$^+$-IDHs. The unrooted tree was constructed with the Clustal X programme (Higgins et al. 1996). Accession numbers: *P. pinaster* (Ppi), AY344584; *Apium graveolens* (Agr), Y12540; *Arabidopsis thaliana* (Ath), AY097340; *Citrus limon* (Cli), AF176669; *Cucumis sativus* (Csa), AJ437269; *Daucus carota* (Dca), AB019327; *Eucalyptus globulus* (Egl), U80912; *Glycine max* (Gma), L12157; *Lupinus albus* (Lal1), AB109113, and (Lal2) AB109114; *Medicago sativa* (Msa), M93672; *Nicotiana tabacum* (Nta), X77944; *Oryza sativa* (Osa1), AF155333, and (Osa2) AF155334; *Pisum sativum* (Psa), AY509880; *Populus tremula* × *P. alba* (Pta), DQ125944; *Prunus persica* (Ppe), AF367443; *Solanum tuberosum* (Stu), X75638; and *Zea mays* (Zma), BT019016. Sequences sharing a similarity index of 90% or higher are grouped. The scale bar indicates the estimated number of amino acid substitutions per site. (C) Southern blot analysis of NADP$^+$-IDH genes in *P. pinaster.* Twenty μg of genomic DNA isolated from pine cotyledons was digested with different restriction enzymes, *BamHI*, *EcoRV*, *EcoRI* and *XbaI*, and probed with the full-length NADP$^+$-IDH cDNA. Numbers on the right indicate size in kbp of lambda DNA markers.

In roots, the NADP$^+$-IDH polypeptide signal was mainly observed in the phloem (Figures 3F, 3G, 4C and 4D). Negative controls showed no endogenous peroxidase activity in cotyledons or hypocotyls (Figures 3B, 3D, 4A and 4C), whereas some staining was observed in parenchyma cells of roots (Figures 3F and 4C).

Phosphinothricin and NADP$^+$-IDH in pine seedlings

Up-regulation of NADP$^+$-IDH and GS activities in photosynthetic tissues have been reported in response to PPT, a structural analog of glutamate that inhibits GS activity, and thereby affects amino acid synthesis (Ávila et al. 1998, Pérez-García et

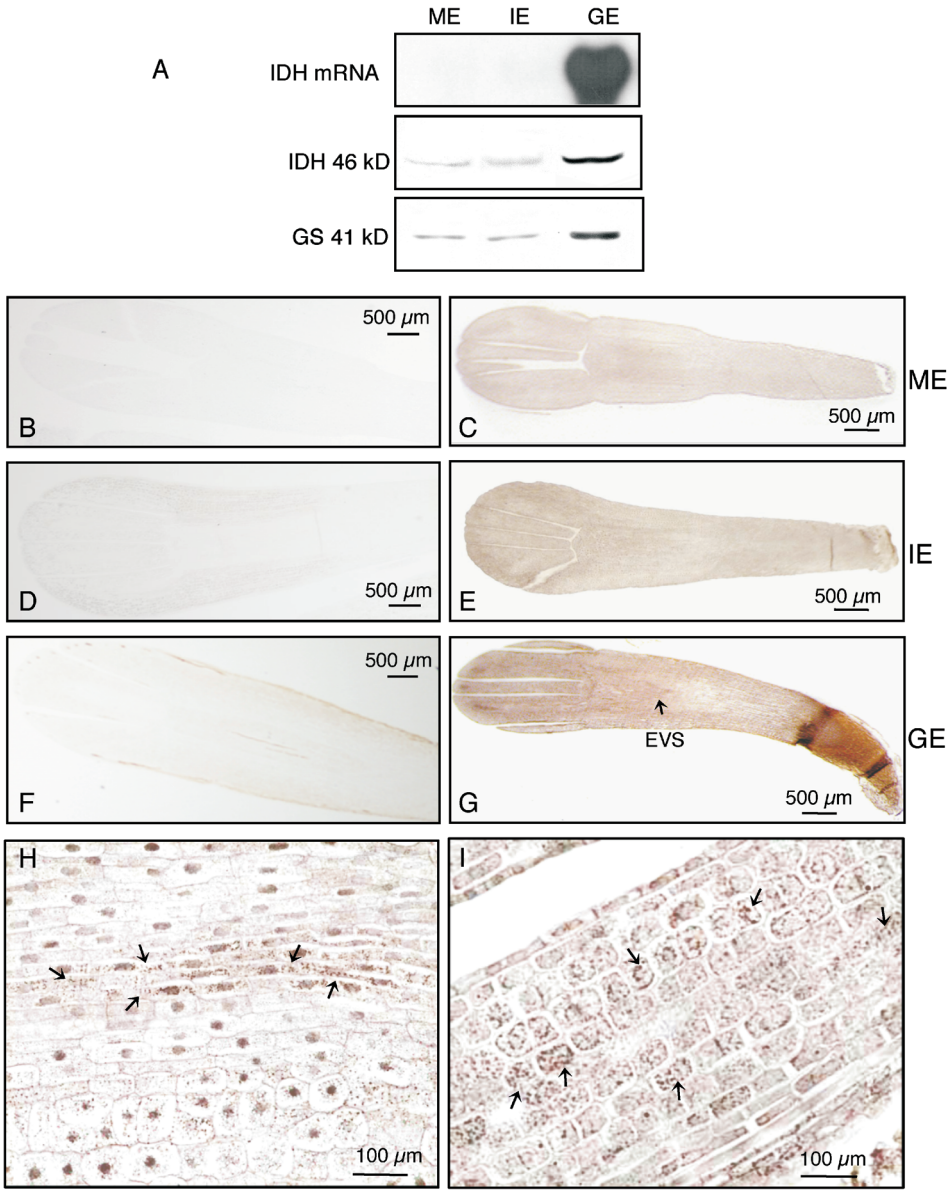

Figure 2. Expression analysis of NADP+-IDH in *Pinus pinaster* zygotic embryos. (A) Total RNA and protein-soluble extracts prepared from dry mature embryos (ME), embryos imbibed for 2 days (IE) and germinated embryos (GE). (B–G). Longitudinal tissue sections (10 μm thick) of dry (B and C, ME), imbibed (D and E, IB) and germinated (F and G, GE) mature zygotic embryos of *Pinus pinaster* probed with purified polyclonal NADP+-IDH antibodies (right panels) or with a pre-immune antiserum as control (left panels). The embryonal vascular system (EVS), forming a central cylinder of elongated cells showing strong labeling is indicated in germinated embryos (G). (H, I) Enlarged images of germinating embryos showing NADP+-IDH signals in the embryonal vascular system (H, arrows) and in cotyledonary cells of the palisade parenchyma (I, arrows).

al. 1998, Pascual et al. 2008). *Pinus pinaster* seedlings bearing cotyledons 0.5 or 1.5 cm in length were treated with 25 mM PPT (control seedlings received water) for 8 h, after which protein expression was analyzed by fractionation by SDS-PAGE and staining with Coomassie blue. We found a slight difference in the amounts of the large subunit of Rubisco (LSU) between cotyledons of 0.5 and 1.5 cm in length, with greater amounts in the 1.5-cm cotyledons (Figure 5, Control). The amount of NADP+-IDH polypeptide was higher in 0.5-cm cotyledons, whereas GS polypeptides were more abundant in 1.5-cm cotyledons. Application of PPT differentially affected the amounts of NADP+-IDH and GS polypeptides, depending on seedling developmental stage. The 0.5-cm cotyledons responded to PPT with a 5-fold increase in the amount of GS polypeptide but PPT had no effect on the amount of NADP+-IDH polypeptide. In contrast, the 1.5-cm cotyledons responded to the herbicide treatment with a 2-fold increase in the amount of NADP+-IDH, but there was no significant effect of PPT on the amount of GS polypeptide (Figure 5).

Discussion

In higher plants, NADP+-IDH isoenzymes are encoded by a small gene family. In most plants studied so far, only one or two genes seem to encode each NADP+-IDH isoenzyme. For example, in *A. thaliana* unique genes encode the cytosolic and peroxisomal forms, whereas the chloroplastic and mitochondria isoenzymes are apparently encoded by the same gene (Hodges 2002). Similarly, EST or gene annotations in poplar, rice, papaya and grape genomes indicate that one or two genes encode the cytosolic NADP+-IDH protein (http://supfam.cs.

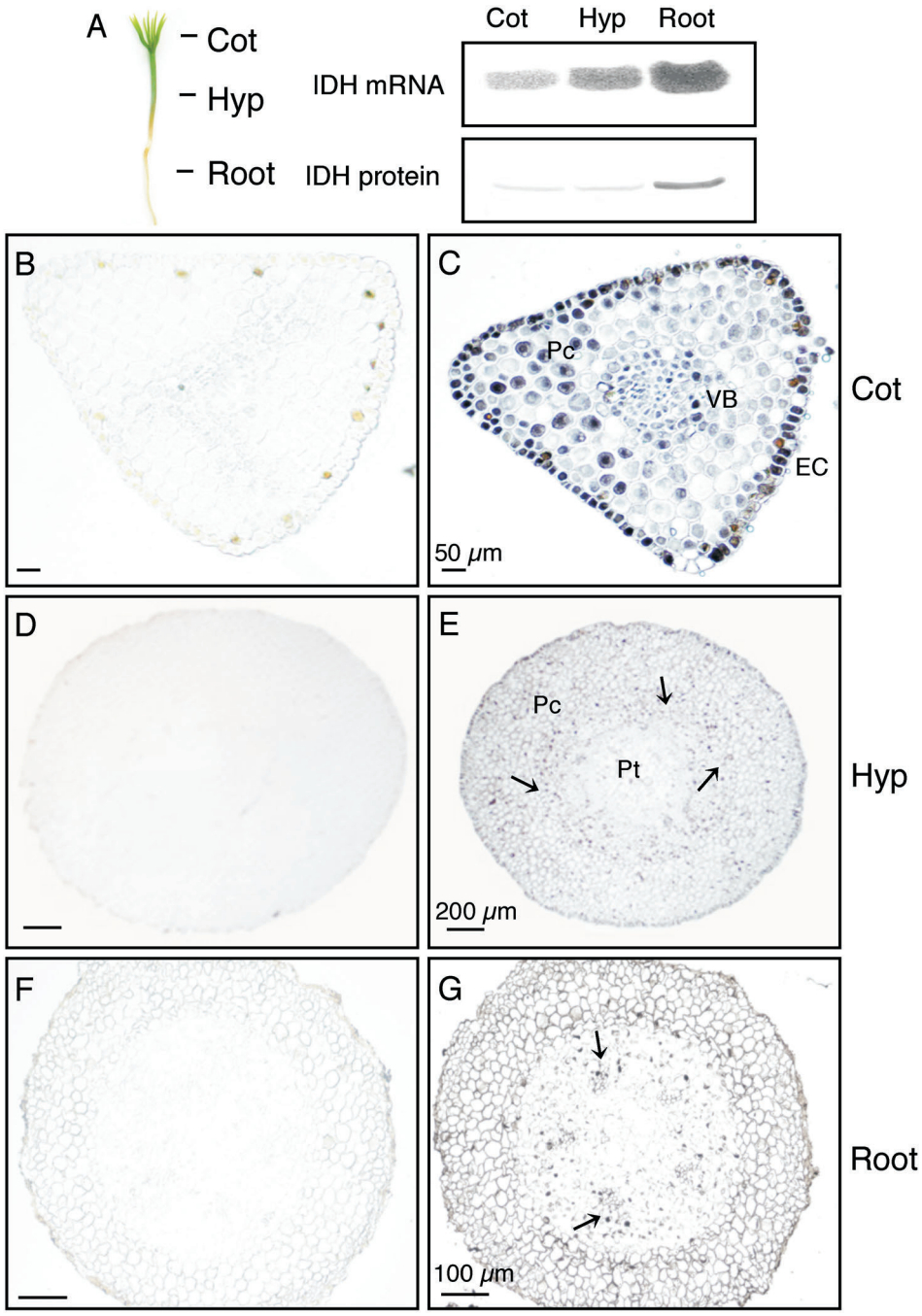

Figure 3. Expression analysis of NADP$^+$-IDH in developing *Pinus pinaster* seedlings. (A) Expression analysis of NADP$^+$-IDH transcripts and polypeptides in cotyledons (Cot), hypocotyls (Hyp) and roots of pine seedlings. (B–G) Sections (10 μm thick) of cotyledon (B and C), hypocotyl (D and E) and root (F and G) were probed with purified polyclonal NADP$^+$-IDH IgGs (right panels) or with a pre-immune antiserum as control (left panels). The NADP$^+$-IDH signal is broadly distributed in pine seedling. The arrows indicate the regions with a strong signal in hypotocyl and root. Abbreviations: EC, epidermal cells; VB, vascular bundle; Pc, parenchyma; and Pt, pith.

bris.ac.uk/SUPERFAMILY/). Analysis and full sequencing of *P. pinaster* ESTs allowed us to identify a full-length cDNA with higher homology to cytosolic NADP$^+$-IDH than to peroxisomal, chloroplastic or mitochondrial NADP$^+$-IDH from angiosperms. Genomic analysis indicated that pine NADP$^+$-IDH was encoded by one gene or by two genes with a high degree of homology (Figure 1). The cDNA sequence described in our study is the first complete sequence for a cytosolic NADP$^+$-IDH from a gymnosperm to be deposited in GenBank (Accession no. AY344584). The alignment and phylogenic tree based on amino acid sequences of 19 species indicated that cytosolic NADP$^+$-IDH has been highly conserved during higher plant evolution. The phylogenetic tree provided no evidence that the pine sequence belongs to a separate branch from the angiosperms (Figure 1). These features—high conservation among species and a low number of gene copies—are shared with other cytosolic enzymes in angiosperms that are considered housekeeping proteins such as NAD$^+$-malate dehydrogenase, aconitase, triosephosphate isomerase and aspartate aminotransferase, which also exist as isoenzymes in different subcellular compartments. Phylogenetic studies of these housekeeping enzymes indicate that gene duplications, prior to isoenzyme evolution, are ancient, and occurred before separation of the main kingdoms (Schultz and

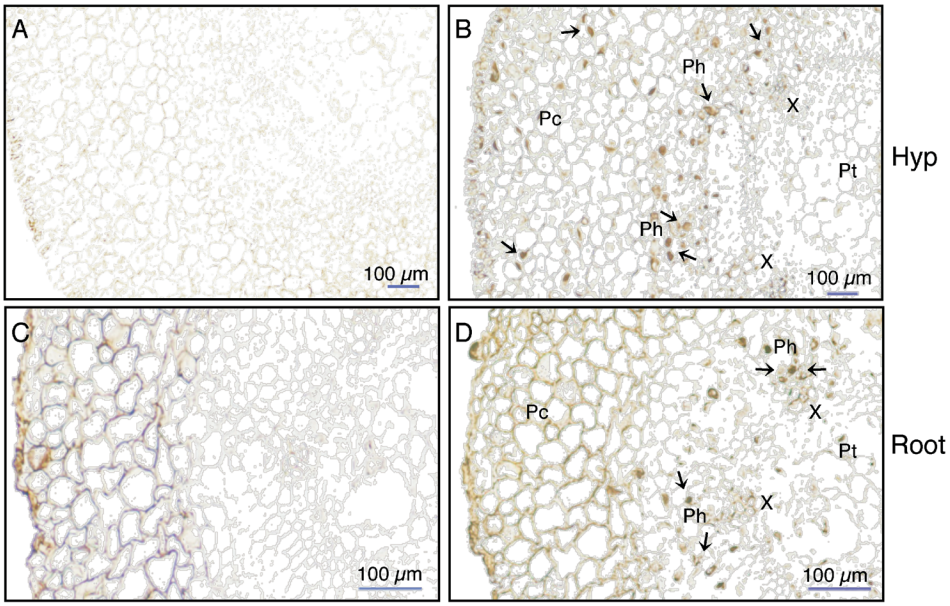

Figure 4. Magnifications of (A, B) hypocotyl and (C, D) root sections shown in Figure 3. The signal due to NADP+-IDH protein is broadly distributed. Controls correspond to left panels (A, C). Arrows indicate phloem cells (Ph) and parenchyma cells (Pc) with stronger signals. Abbreviations:X, xylem; and Pt, pith.

Coruzzi 1995, Canback et al. 2002, Schnarrenberger and Martin 2002, Ding and Ma 2004). In contrast, the evolution of NADP+-IDH isoenzymes seems to have arisen by independent gene duplications in animals, fungi (Nekrutenko et al. 1998), and higher plants (Schnarrenberger et al. 2002, Hodges et al. 2003), suggesting that NADP+-IDH isoenzymes play different roles in different organisms.

Another characteristic indicative of the evolutionary stage of enzymes is their catalytic efficiency (Albery and Knowles 1976, Whitford 2005). The catalytic efficiency of cytosolic pine NADP+-IDH (10^5 M^{-1} s^{-1}, Palomo et al. 1998) is one order higher than the values estimated for other enzymes involved in the GS/GOGAT pathway (Palomo et al. 1998); however, it is still far from the maximum attainable efficiency (i.e., 10^8–10^9 M^{-1} s^{-1}, Whitford 2005). This finding and the data derived from the phylogenetic studies may indicate that NADP+-IDH isoenzymes are still evolving in the different kingdoms.

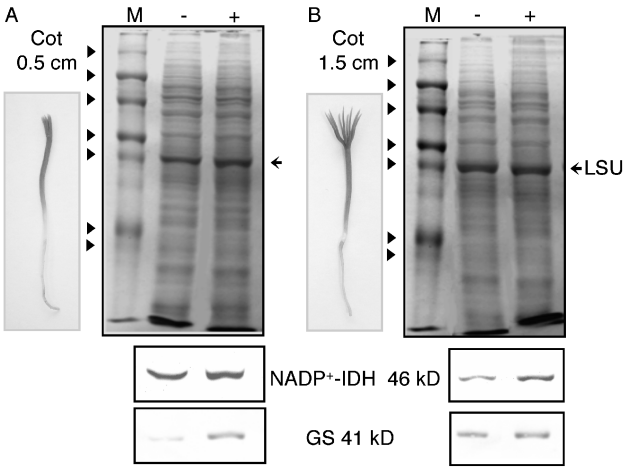

Figure 5. Analyses of NADP+-IDH and GS in developing *Pinus pinaster* seedlings treated with phosphinothricin (PPT). Pine seedlings having cotyledons (Cot) 0.5 (A) or 1.5 cm (B) in length were sprayed with 25 mM PPT (+) or water as control (-), and protein extracts were prepared 8 h after treatment. Protein markers (M) corresponding to 180, 116, 84, 58, 48.5, 36.5 and 26.6 kD are indicated at left. Results represent three independent experiments.

Because seed germination is associated with the mobilization of nitrogen reserves accumulated during seed maturation in both the megagametophyte and the embryo itself, the early seedling development model for gymnosperms has proved useful in understanding the roles of enzymes involved in nitrogen metabolism during these early developmental phases (García-Gutiérrez et al. 1998, Suárez et al. 2002, Cañas et al. 2006, 2007, Cánovas et al. 2007). Several studies have shown up-regulation of enzymes such as GS and asparagine synthetase during seed germination, indicating roles for these enzymes in mobilization of nitrogen reserves to support early development of pine seedlings (Suárez et al. 2002, Cañas et al. 2006). We found that expression of cytosolic NADP+-IDH transcripts and polypeptides increased following germination of pine embryos (Figure 2), suggesting a role of the enzyme during active cell proliferation and development of seedling organs and structures.

Up-regulation of NADP+-IDH during germination in the parenchyma of embryonic cotyledons (Figure 2), and the strong RNA and polypeptide signals in cotyledons of seedlings (Figure 3) may be associated with the synthesis of nitrogen compounds required for acquisition of photosynthetic competence. The NADP+-IDH protein is up-regulated by PPT in pine cotyledons (Figure 5). The PPT herbicide impairs amino acid metabolism by inhibiting the GS holoenzyme, resulting in depletion of glutamate and other amino acids and leading to the inhibition of photosynthesis and ultimately plant death (Lea and Ridley 1989, Hoerlein 1994). The herbicide also affects GS expression, and the up-regulation of GS and cytosolic NADP+-IDH followed a pattern similar to that previously observed in PPT-treated poplar plants (Pascual et al. 2008).

Thus, increased amounts of GS were observed after PPT treatment in organs with low photosynthetic competence, whereas increased amounts of NADP$^+$-IDH was observed in organs with high amounts of LSU and GS, that is, with high photosynthetic competence (Figure 5; Pascual et al. 2008). These findings suggest that inhibition of amino acid synthesis by PPT generates a signal that up-regulates the synthesis of NADP$^+$-IDH to meet the demand for 2-oxoglutarate/glutamate in tissues with high photosynthetic competence (Pascual et al. 2008).

In both germinating embryos and seedling cotyledons, NADP$^+$-IDH expression showed a broad distribution (Figures 2 and 3). Although the concomitant increases in cytosolic NADP$^+$-IDH and GS during germination and chloroplast development initially suggested a coordinated expression of both enzymes (Figure 2; Palomo et al. 1998), the broad localization of NADP$^+$-IDH signals in the embryo and pine cotyledon did not match with the specific localization of either the cytosolic GS1a isoenzyme, which is expressed in photosynthetic cells in green cotyledons, or the GS1b isoenzyme, which is restricted to the procambium in the embryo (Pérez-Rodríguez et al. 2006) and to the vascular cylinder in green cotyledons (Suárez et al. 2002). The NADP$^+$-IDH polypeptide signal was detected not only in the same cells as those in which GS1a or GS1b was detected, but also in the epidermis of green cotyledons (Figure 3) where no GS expression has previously been reported (Suárez et al. 2002). Accordingly, we suggest that NADP$^+$-IDH facilitates plant metabolism in different ways, depending on the features of the particular tissue and cell type. For example, cytosolic NADP$^+$-IDH could catalyze the production of 2-oxoglutarate required for glutamate synthesis through the GS/GOGAT cycle during early seedling development (cf. Palomo et al. 1998), or via ornithine aminotransferase, an enzyme implicated in arginine metabolism, during germination (Figure 6; Slocum 2005, Cañas 2006, Cánovas et al. 2007). In addition, cytosolic NADP$^+$-IDH may be involved in amide synthesis for transport because GS and asparagine synthetase are up-regulated during embryo elongation and early development in pine seedlings (Figure 6; Cañas et al. 2006, Cánovas et al. 2007).

We observed cytosolic NADP$^+$-IDH expression in vascular tissues in various organs of pine seedlings (Figures 3 and 4), and its expression has also been reported in vascular tissues of *Eucalyptus* roots (Boiffin et al. 1998) and leaf petioles in potato (Fiew et al. 1995). In addition, cytosolic NADP$^+$-IDH ESTs have been identified in libraries prepared from the cambial zone in pine and poplar (Cantón et al. 2004, Sterky et al. 2004). The role of cytosolic NADP$^+$-IDH in vascular tissues could be associated with the recovery of ammonia released by lignifying cells (Gallardo et al. 2003), or with the catabolism of asparagine that is reported to occur during lignification of pine hypocotyls (Cañas et al. 2006; cf. Figure 6). In addition, NADP$^+$-IDH activity in vascular tissues could be associated with signaling because recent evidence indicates that 2-oxoglutarate interacts with protein PII (Hodges 2002), a regulator of C/N status in bacteria and higher plants (Moorhead et al. 2007) that has been detected in pine xylem (Cánovas, unpublished results).

In conclusion, cytosolic NADP$^+$-IDH expression in different cell types and tissues in pine seedlings, and its high homology to their angiosperm counterparts indicates a basic role for NADP$^+$-IDH in conifer seedling development. Genomic analyses of pine and angiosperms suggest that no gene specialization has been required for cytosolic NADP$^+$-IDH during the evolution of vascular plants. This lack of gene specialization could be associated with our poor understanding of the different and essential roles of this housekeeping protein in metabolism and differentiation. Experimental modification of gene expression may provide new information about the functions of this isoenzyme. Down-regulation of cytosolic NADP$^+$-IDH expression by antisense cDNA expression has been detected in potato (Kruse et al. 1998) and tobacco (Hodges 2002) without noticeable effects on plant development, perhaps because of redundancy in reactions producing 2-oxoglutarate. Additional studies, including the overexpression of cytosolic NADP$^+$-IDH in transgenic plants, are necessary to establish the role of this housekeeping enzyme in plant development.

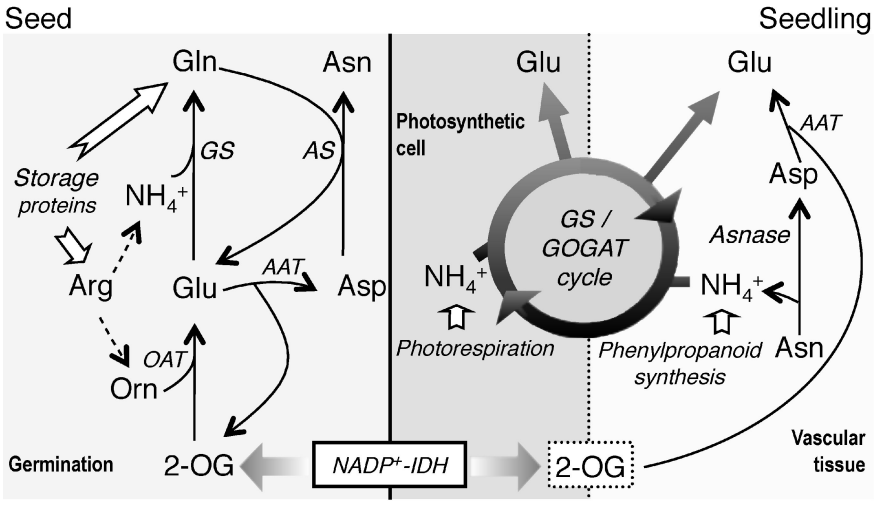

Figure 6. Possible pathways of 2-oxoglutarate metabolism during primary development in *Pinus pinaster*. Abbreviations: AAT, aspartate aminotransferase; Asnase, asparaginase; GS, glutamine synthetase; GOGAT, glutamate synthase; NADP$^+$-IDH, cytosolic NADP$^+$-dependent isocitrate dehydrogenase; and OAT, ornithine aminotransferase.

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