Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase is post-translationally phosphorylated in heterotrophic cells of wheat (*Triticum aestivum*)

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Abstract In wheat, non-phosphorylating, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPN) was found to be encoded by one gene giving rise to a single protein. However, Western blots revealed two different subunits of about 58 and 60 kDa in endosperm and shoots. The latter was attributed to in vivo phosphorylation of shoot GAPN. No modification occurred in leaves, where the enzyme is composed by a single 58 kDa polypeptide. GAPN partially purified from shoots and endosperm was dephosphorylated in vitro with alkaline phosphatase. Phosphorylated GAPN exhibited similar affinity for substrates but a lower V_{max} compared to the non-phosphorylated enzyme. Results suggest that reversible phosphorylation of GAPN could regulate NADPH production in the cytosol of heterotrophic plant cells.

Key words: Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; Post-translational modification; Enzyme phosphorylation; *Triticum aestivum*

1. Introduction

Non-phosphorylating D-glyceraldehyde-3-phosphate dehydrogenase (GAPN; EC 1.2.1.9) is a cytosolic enzyme catalyzing the irreversible oxidation of glyceraldehyde-3-phosphate (Ga3P) into 3-phosphoglycerate coupled to $NADP^+$ reduction to NADPH [1,2]. GAPN was early described in photosynthetic cells [3] and then purified and characterized from different plants [4–9], green algae [10], and eubacteria [11]. The enzyme from archaebacteria is NAD-dependent [12]. Stereospecificity for $NADP^+$ reduction and sequence comparison between proteins have demonstrated that GAPN is related to the aldehyde dehydrogenase (ALDH) superfamily [13,14].

GAPN plays a cardinal role in a shuttle system for the transport of photosynthetically generated NADPH from the chloroplast to the cytosol [15]. This role for GAPN is strictly related to photosynthetic metabolism within plant cells, in agreement with the finding that the enzyme is a specific feature of organisms having chloroplasts or cyanelles [5,8]. However, GAPN was also detected in plant non-photosynthetic tissues such as endosperm, cotyledons, and roots [7,8]. In non-green cells GAPN would couple NADPH production needed for anabolic reactions with glycolysis [1].

The occurrence of GAPN in the cytosol of plant cells establishes an alternative for glycolysis [1,2,16]. In fact, Ga3P can be metabolized to 3-phosphoglycerate either by the couple of phosphorylating NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPC; EC 1.2.1.12) and phosphoglycerate kinase (EC 2.7.2.3) or by GAPN [16]. In the first case, NADH and ATP are produced in the pathway; whereas the second route renders NADPH but not ATP [2]. This branch point in glycolysis is expected to be regulated in order to effectively modulate the production of energetic and reductive power within cells [16]. Despite the relevance of GAPN for plant metabolism, cross-regulation of the phosphorylating and non-phosphorylating enzymes is poorly understood and the occurrence of specific mechanisms affecting the activity for these dehydrogenases have not been reported.

In the present work we show that GAPN from non-photosynthetic tissues of wheat undergoes phosphorylation. To the best of our knowledge, this is the first time that one enzyme from the ALDH superfamily is found to be post-translationally modified. The consequences of GAPN modification for regulation of carbohydrate metabolism within the cytosol are discussed.

2. Materials and methods

2.1. Plant material and reagents

Wheat (*Triticum aestivum*) plants were grown in a greenhouse at 18 ± 2°C. Green material utilized was the third and fourth leaves from plants grown in Hoagland's medium under a 14 h photoperiod. Shoots were developed for 2 days in the dark, in 5 mM P_i (pH 7.0) buffered medium. Endosperm was from developing seeds (20 days post-anthesis) of plants grown in the field. Leaves and shoots were harvested and used immediately; seeds were detached and stored at −80°C until use.

Radiochemicals were from DuPont NEN. Prestained kit for molecular mass determination (27–180 kDa) was from Sigma Chemical Co. All reagents were of the highest quality available.

2.2. Protein measurement and enzyme assay

Total protein was determined after Bradford [17], using bovine serum albumin as the standard.

GAPN activity was assayed spectrophotometrically at 30°C by

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Abbreviations: ALDH, aldehyde dehydrogenase; Ga3P, glyceraldehyde-3-phosphate; GAPC, NAD-dependent glyceraldehyde-3-phosphate dehydrogenase, phosphorylating; GAPN, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; RACE, rapid amplification of cDNA ends

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monitoring NADPH generation at 340 nm as previously described [4]. One unit (U) is the amount of enzyme catalyzing the formation of 1 μmol NADPH per min. Values of K_m and Hill coefficients (n_H) for $NADP^+$ and D-Ga3P were determined using saturating concentrations of the cosubstrate. The experimental data were fitted to the general Hill equation by a non-linear least-square regression kinetics computer program [18].

2.3. Partial purification and molecular mass determination of native GAPN

GAPN from wheat leaf and endosperm was partially purified by ammonium sulfate fractionation (35–60%) and fast flow DEAE–Sepharose chromatography as previously described [4]. The enzyme from both sources was purified near 25-fold by this procedure to reach specific activities of about 0.35 U/mg (leaf) and 0.12 U/mg (endosperm).

The molecular mass of native GAPN was determined by using a Superose 12 HR10/30 column (FPLC-Pharmacia) calibrated with standard protein markers as specified previously [4]. Running buffer contained 50 mM Mops–NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 100 NaCl.

2.4. PCR cloning of GAPN cDNA

Total RNA was extracted from approximately 200 mg mature wheat leaves or 500 mg endosperm using guanidine thiocyanate (Promega). First-strand cDNA synthesis was performed using M-MLV RNAse H-point mutant (Promega). One degenerated oligonucleotide primer was designed according to the highest conserved region in GAPN sequences as follows: primer T2, 'sense'-primer, 5′-CCK GTC TAG AGG ATC GCA TGG G-3′, corresponding to the region starting at base 1322 in maize *gapN* [14]. PCR was performed using 1 μl of the 25 μl reverse transcriptase reaction, 20 pmol T2 primer, 40 pmol oligo(dT) primer, and 2.5 U Taq DNA polymerase (Promega), according to the manufacturer's instructions. After purification with GFX kit (Pharmacia), the PCR products were cloned by pGEM-T Easy Vector System (Promega). 5′-RACE (rapid amplification of cDNA ends) was performed by terminal deoxynucleotide transferase according to [19], using the degenerated oligonucleotide primer T3: 5′-DGT TCA AGC TTC CAT BGC ATC AC-3′, starting from the base 1474 in maize *gapN* [14]. All clones were sequenced (at least twice) on both strands. Sequence alignment and comparison were performed with Clustal W and BLAST search of GenBank sequence databases. Sequences for GAPN from wheat endosperm and leaf were deposited in the GenBank database with accession numbers AF521190 and AF521191, respectively.

2.5. Southern analysis

Wheat genomic DNA was purified from 10 g mature wheat leaves, digested with *Eco*RI, *Bam*HI, and *Hin*dIII and separated on a 0.8% (w/v) agarose gel. DNA was capillary transferred (overnight) to nylon membranes (Hybond N^+, Amersham) in 10×SSC and fixed at 80°C for 1.5 h. PCR was used to ^{32}P-label a 1000 bp fragment of maize *gapN*. Membranes were prehybridized 2 h at 42°C in buffer PIPES×2, 0.1% (w/v) SDS and 50% (v/v) formamide. Hybridization was performed overnight under the same conditions with addition of the ^{32}P-labeled probe. Following hybridization, membranes were rinsed twice in 6×SSC, 0.1% (w/v) SDS, at 56°C. Two additional 20 min washes were performed prior image capture, as described for the cloning of plant *gapN* [20].

2.6. In vivo phosphorylation and in vitro dephosphorylation of GAPN

GAPN was phosphorylated in vivo by incubating about 2 g of plant material (wheat leaf circles or shoots) in 20 ml of 100 mM Tris–HCl buffer (pH 7.5) containing 10 μCi [^{32}P]P_i in the dark or under illumination (200 μmol m^{-2} s^{-1}). After 8 h (leaves) or 2 days (shoots) incubation at room temperature, plant material was immediately processed for the respective analysis.

Dephosphorylation of GAPN from wheat leaves, shoots, or endosperm was performed by incubation of the respective partially purified enzyme with 4 U of calf intestine phosphatase (Promega) in 1 ml of 50 mM Tris–HCl (pH 8.5), 1 mM EDTA, 10 mM $MgCl_2$, 1.2 mM $CaCl_2$, 20 mM 2-mercaptoethanol, 1 mM PMSF [21]. After 10 min at 37°C, the reaction was stopped with 1 mM NaF and samples were utilized for activity assays or SDS–PAGE analysis.

2.7. Immunoprecipitation, protein electrophoresis and immunoblotting

Plant material was homogenized using a mortar and pestle in a buffer (about 2.5 ml/g) containing 100 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% (v/v) Nonidet P40, 1 mM EDTA, 20 mM 2-mercaptoethanol, and 1 mM PMSF. After centrifugation at 20 000×g for 15 min, the clear extracts were incubated at 4°C for 1 h with 2 μl of rabbit anti-celery leaves GAPN antiserum [4], followed by a 1 h incubation with 0.5 ml of streptavidin-coated paramagnetic beads and biotin-labelled anti-rabbit IgGs (Streptavidin Magnesphere from Promega; anti-rabbit, biotin-labelled IgG from Gibco BRL). Beads were washed twice with 1 ml of the above extraction buffer, then once with extraction buffer plus 1 M NaCl. Anti-GAPN and GAPN were released from the beads with 100 mM glycine (pH 2.2), and the samples then boiled in Laemmli buffer for 5 min and subjected to SDS–PAGE (see below). Gels were stained with Coommasie brilliant blue R-250, dried, and exposed for autoradiography at −80°C.

Electrophoresis under denaturating conditions (SDS–PAGE) was performed in 10% running gels [22]. Following electrophoresis, gels were either stained for protein or electroblotted. Nitrocellulose membranes were treated with rabbit anti-celery leaves GAPN antiserum and the antigen–antibody complex was visualized with horseradish-linked anti-rabbit IgG followed by chemiluminescent staining (ECL kit, Pharmacia).

3. Results and discussion

GAPN was found in both photosynthetic and heterotrophic tissues of higher plants [1,8]. In the different cells, the enzyme is involved in different functions dictated by the specific metabolic scenario [1]. To investigate the possible existence of GAPN isoenzymes in different plant cells, we utilized three main approaches: (i) to search for the occurrence of one or more genes encoding the enzyme in plant tissues, (ii) to perform the molecular cloning of the gene(s) encoding the enzyme in wheat leaf and endosperm, and (iii) to analyze the kinetic and structural properties of the enzyme present in different plant cells.

3.1. GAPN is encoded by one gene in plant tissues

Fig. 1 shows genomic southern analysis in wheat, performed with a maize *gapN* cDNA fragment as a probe. As shown, a single signal was observed with different digestion treatments. The non-stringent hybridization and washing conditions (56°C) used in this procedure assure that genes and pseudogenes with sequence similarities of about 75% should be detected [20]. Thus, results in Fig. 1 indicate in wheat that GAPN is encoded by a single gene.

To evaluate possible post-transcriptional modifications, the gene encoding GAPN was cloned from cDNAs of a photosynthetic (leaf) and a non-photosynthetic (endosperm) wheat tissue. Using degenerated primers derived from highly conserved regions in *gapN* from different sources, one 400 bp DNA band was amplified by PCR from the first-strand cDNA from both wheat tissues. Each 400 bp clone showed high homology to maize, pea, and tobacco *gapN*; and was utilized to design nested gene specific primer to obtain another first-strand cDNA. The products were purified and subjected to terminal deoxynucleotide transferase reactions and 5′-RACE with gene-specific and anchor primers as previously described [19]. Sequences of genes thus cloned from endosperm and leaves (GenBank database accession numbers AF521190 and AF521191, respectively) were identical; both containing an ORF encoding a 497 amino acids polypeptide (predicted M_r of 53 322 Da), which agrees with the size for GAPN from different sources [1].

Sequence analysis of the 5′- and 3′-untranslated region re-

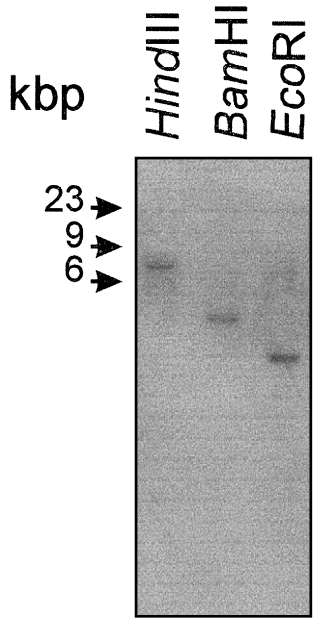

Fig. 1. Southern blot of genomic DNA from *T. aestivum* hybridized with maize *gapN* fragment. Restriction digests with the specified enzymes were performed with 10 μg DNA each and blotted onto a Hybond N^+ membrane (Amersham). Hybridization conditions are described under Section 2. Molecular size markers correspond to λ*Hin*dIII.

vealed that clones obtained from wheat leaf and endosperm derive from a single gene, containing the complete coding region. The sequences are highly conserved with respect to other GAPNs, with an amino acid identity of 95.5% between wheat and maize. Fig. 2 shows that wheat GAPN contains an ALDH domain between amino acid residues 25 and 495. The three most conserved regions (I, III, and IV in Fig. 2) common to all ALDHs [5] are also present in the wheat enzyme. Region I is involved in binding of $NAD(P)^+$ [5,23], region III is an interdomain for nucleotide binding and catalysis [24]; whereas no function was assigned to region IV (Fig. 2). The region containing one essential cysteine absolutely conserved among the ALDH superfamily [23,25] is also present in wheat GAPN (region II in Fig. 2).

3.2. GAPN has more than one species in non-photosynthetic plant tissues

Despite the identity of genes encoding GAPN from photosynthetic and heterotrophic cells of wheat, Western blots revealed that the enzymes found in leaf or endosperm extracts are different. As shown in Fig. 3, antiserum raised against celery leaf GAPN cross-reacted with a single polypeptide of molecular mass about 58 kDa in leaves (Fig. 3A), but two protein bands of about 58 and 60 kDa were immunoreactive in endosperm (Fig. 3A). Identical results were obtained when all the tissues were extracted under denaturing conditions [26], thus discarding that partial in vitro proteolysis is modifying the structure of GAPN (data not shown). To determine if differences can be attributed to the auto- or heterotrophic characteristic of the tissue, we analyzed extracts from shoots. Immunodetection of GAPN in the latter tissue gave identical results than those found in endosperm (data not shown), suggesting that the presence of two protein bands for GAPN is a characteristic of non-photosynthetic cells. These results, together with the above data showing only one gene encoding GAPN, suggest that in certain tissues the enzyme undergoes post-translational modification rendering one protein with different mobility in SDS–PAGE.

Interestingly, amino acids sequence analysis using programs NetPhos (http://www.cbs.dtu.dk/services/NetPhos/) and PPSEARCH, from EMBL, predict the existence of putative phosphorylation sites in plant GAPN. As possible target residues, Tyr222, Tyr224 and Ser253 are hidden in the protein core according to the Swiss PDB viewer; thus being not accessible to protein kinases. Conversely, Ser29 and Ser404 are somewhat exposed to the solvent, with phosphorylation probabilities of 0.963 and 0.995, respectively. As shown in Fig. 2, the sequence surrounding Ser29 (region V) is somewhat conserved between plant but not bacterial GAPNs. Ser404 is located in one of most conserved regions in ALDHs (IV in Fig. 2) [5]. Region IV also exhibits a higher homology to plant rather than bacterial GAPNs and thus constitutes a likely target for phosphorylation in the enzyme from eukaryotes.

Combining the above experimental and theoretical data we determined whether phosphorylation of GAPN occurs in photosynthetic or heterotrophic plant tissues or not. In vivo phosphorylation, followed by immunoprecipitation analysis re-

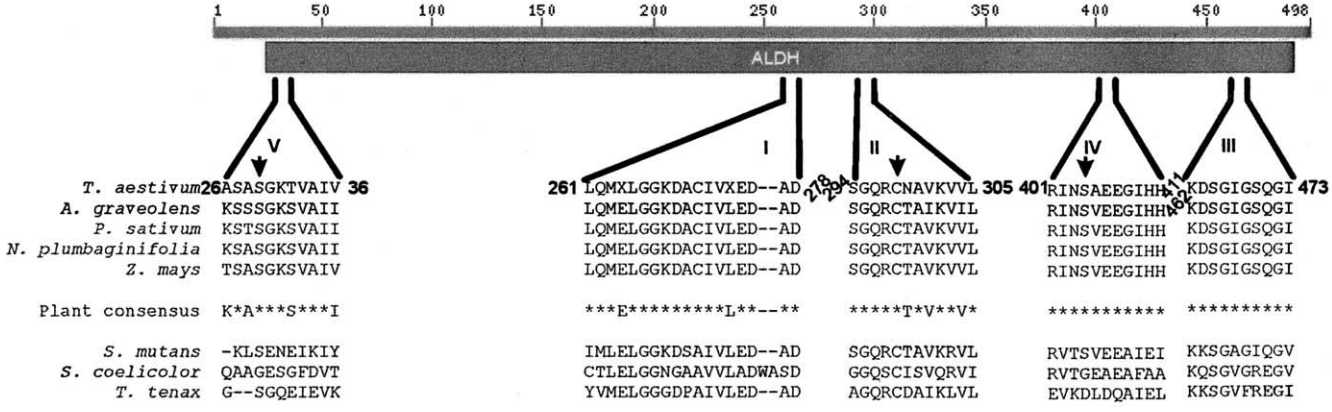

Fig. 2. Schematic representation of the primary structure corresponding to wheat GAPN related to ALDH superfamily proteins. Roman numbers indicate regions highly conserved (I–IV) and/or containing Ser residues exposed to the environment (arrows in IV and V) constituting probable phosphorylation sites. Identical amino acids are replaced by asterisks in the plant consensus sequence. Gaps in the sequences were replaced by dashes. The conserved Cys residue in region II is marked in bold.

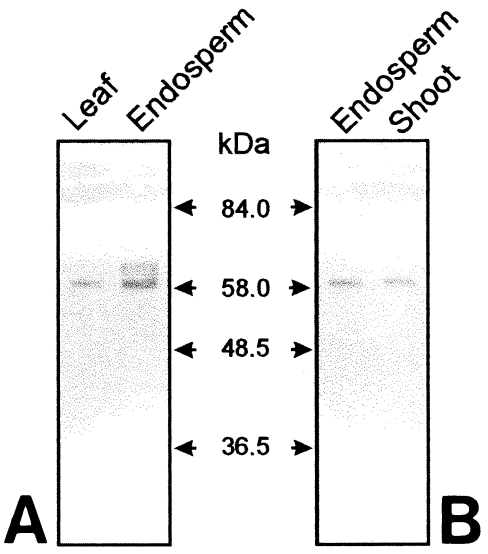

Fig. 3. Western blot analysis of GAPN from wheat leaf and endosperm. A: Samples from leaf or endosperm extracts were loaded in the respective lane. B: Samples of the enzyme partially purified from endosperm or shoot were treated with alkaline phosphatase and then loaded as indicated. SDS–PAGE was performed in 10% polyacrylamide gels, electroblotted, and immunodetected using anti-celery leaves GAPN immune serum.

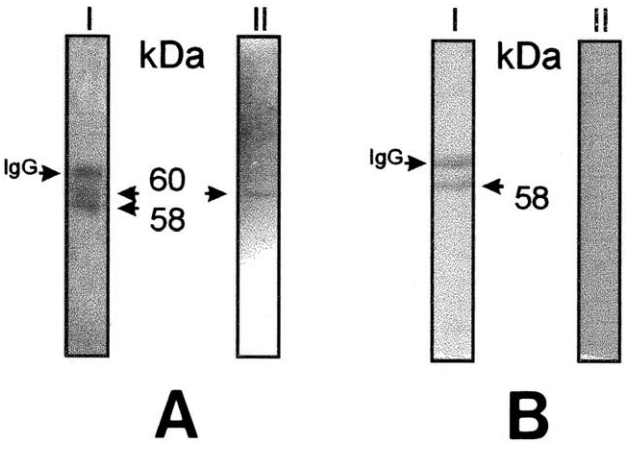

Fig. 4. Immunoprecipitation and autoradiography of GAPN from wheat extracts. After in vivo phosphorylation assays, extracts from shoots (A) or leaves (B) were immunoprecipitated with anti-GAPN and then resolved by SDS–PAGE. Gels were stained for protein (I) followed by drying and exposure for autoradiography (II). Heavy chain IgG was identified in control immunoblots of solubilized immunoprecipitate probed only with secondary antibody.

vealed that GAPN is effectively phosphorylated in a tissue-dependent manner (Fig. 4). Radioactive P_i was incorporated into the 60 kDa protein band corresponding to one of the subunits immunorecognized as GAPN in shoots (Fig. 4A). Fig. 4 also shows that the immunoprecipitated 58 kDa polypeptide (corresponding to a second band in shoots or the single band for leaf GAPN) exhibited no phosphorylation. Identical results were obtained independently of the incubation conditions (i.e. dark versus light, data not shown), suggesting the occurrence of a tissue- rather than light-dependent phosphorylation/dephosphorylation mechanism.

To further analyze the phosphorylation state of GAPN in different wheat tissues we partially purified the enzymes from leaves, shoots, and endosperm to characterize their kinetic properties before and after incubation with alkaline phosphatase. Dephosphorylation eliminates the 60 kDa band in the enzyme from endosperm and shoots (Fig. 3B), reinforcing the idea that the higher molecular mass protein species results from a post-translational modification. Estimation of the molecular mass of the enzyme by size exclusion chromatography showed that native GAPN is a protein of 240 ± 30 kDa (data not shown) independently of its phosphorylation state. These results agree with the tetrameric structure of the enzyme from different sources [1,4,6,9,10], and they suggest that phosphorylated GAPN is also a tetramer composed by a mixture of modified and non-modified subunits.

Table 1 shows similar K_m values for substrates of wheat leaf and endosperm GAPNs, independently of the phosphorylation state. However, dephosphorylation caused a three-fold increase in the maximal activity of the endosperm (Table 1) and shoot enzymes (not shown); whereas the activity of leaf GAPN was not affected (Table 1). These data strongly suggest that GAPN is phosphorylated in plant heterotrophic tissues to produce an enzyme with similar affinity for substrates but significant lower V_{max}.

3.3. Concluding remarks

Triose phosphates are key intermediates in plant cells metabolism; representing a central branch point for routes involving glycolysis, gluconeogenesis as well as photosynthetic carbon assimilation and partitioning [2,16]. A main event in the partition of photoassimilates in the cytosol is the conversion of Ga3P to 3-phosphoglycerate which may occur by alternative pathways involving GAPC/P-glycerate kinase or GAPN, and rendering NADH and ATP, or NADPH, respectively [2,16]. How these enzymes are involved in the control of energy metabolism and production of reducing equivalents is poorly understood. At present, only NAD-dependent GAPN from the hyperthermophilic archaeon *Thermoproteus tenax* has been found to play a main regulatory role in glycolysis [12]. The enzyme from *T. tenax* is allosterically regulated by a

Table 1
Kinetics parameters of GAPN partially purified from wheat endosperm and leaf before and after treatment with alkaline phosphatase

Parameter[a]	Endosperm		Leaf	
	before	after	before	after
K_m NADP (μM)	40	60	34	40
K_m D-Ga3P (μM)	118	118	101	101
Relative velocity[b] (%)	100	300	100	100

[a]Values are means of duplicates determined from three independent preparations.
[b]Velocities were determined at saturating substrate concentrations: 0.4 mM $NADP^+$ and 1.2 mM Ga3P. 100% of activity corresponds to values of 0.12 U/mg and 0.40 U/mg, for the enzyme from wheat endosperm and leaf, respectively.

variety of intermediates of carbohydrate and energy metabolism; which was reported as a new feature exemplifying structural and functional plasticity within the ALDH superfamily [1,12,14].

The main contribution of the present work is to show that GAPN is subjected to post-translational modification in heterotrophic plant cells, which is a first evidence for the occurrence of a distinctive regulation of this enzyme with respect to its counterpart from photosynthetic cells. Phosphorylated GAPN occurring in non-photosynthetic tissues exhibited lower activity and this could be a regulatory strategy to control the partitioning of glycolytic intermediates towards synthesis of NADPH in the cytosol. A more detailed study of the kinetic and regulatory properties of this post-translationally modified enzyme will contribute to a better understanding of the physiological regulation of GAPN and carbon metabolism in plant cells. Such studies are currently in progress.

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