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Carbon metabolism in transgenic roots with altered levels of hexokinase and triosephosphate isomerase and growing under different nitrogen status

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Ce mémoire intitule :

Carbon metabolism in transgenic roots with altered levels of hexokinase and triosephosphate isomerase and growing under different nitrogen status

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David Morse, président-rapporteur
Jean Rivoal, directeur de recherche
Mario Jolicoeur, membre du jury

Résumé

Ce projet a pour but d'évaluer la capacité de la voie des pentoses phosphates (VPP) dans les racines transgéniques de pomme de terre (*Solanum tuberosum*) modifiées pour exprimer différents niveaux de l'hexokinase (HK) et de la triosephosphate isomérase cytosolique (cTPI). Dans les racines, la VPP alimente la voie de l'assimilation de l'azote en equivalents réducteurs et permet donc la biosynthèse des acides aminés. Le glucose-6-phosphate produit par l'HK est consommé par la partie oxydative de la VPP catalysée par la glucose-6-phosphate déshydrogénase (G6PDH) et la 6-phosphogluconate déshydrogénase (6PGDH). Les changements dans l'expression de HK et cTPI peuvent affecter le fonctionnement de la VPP et les mécanismes qui sont liés à l'utilisation des équivalents réducteurs produits par la VPP, comme l'assimilation de l'azote et la synthèse des acides aminés. Afin d'évaluer l'effet des manipulations génétiques de l'HK et de la cTPI sur l'assimilation de l'azote, nous avons cultivé les racines transgéniques sur des milieux contenant des concentrations élevées (7 mM) ou basses (0,7 mM) de nitrate d'ammonium comme source d'azote. Les résultats montrent que la culture sur un milieu riche en azote induit les activités G6PDH et 6PGDH. Les données montrent que la capacité de la VPP est plus grande avec des niveaux élevés en HK ou en cTPI. Nous avons aussi pu démontrer une plus grande activité spécifique de l'HK dans les conditions pauvres en azote. Ces données ont été complémentées par des mesures des pools d'acides aminés dans les racines transgéniques cultivées sur différents niveaux d'azote. Aucune tendance notable des pools d'acides aminés n'a été remarquée dans les racines modifiées pour leur contenu en HK suggèrant que la manipulation de HK n'affecte pas l'assimilation de l'azote. Dans les racines transgéniques modifiées pour la cTPI, les ratios Gln/Glu et Asn/Asp sont plus élevés chez les clones antisens, indiquant une assimilation de l'azote plus élevée. Ces résultats ont démontré l'activation de l'assimilation de l'azote chez les clones antisens cTPI dans les conditions élevées et basses d'azote alors que la manipulation de l'HK n'affecte pas l'assimilation de l'azote.

Mots-clés: hexokinase, triosephosphate isomérase, nutrition azotée, voie des pentoses phosphates, acides aminés

Abstract

This study investigates the capacity of the oxidative pentose phosphate pathway (oxPPP) and nitrogen metabolism in transgenic potato (*Solanum tuberosum*) roots modified to express different levels of hexokinase (HK) or cytosolic triosephosphate isomerase (cTPI) growing under different nitrogen regimes. The flux of carbon through the oxPPP in cTPI antisense roots is higher than control roots growing under high supply of N. On the other hand, the conversion of Glucose (Glc) to Glucose-6-phosphate (G6P) is higher in roots overexpressing HK than in antisense HK roots growing at a high level of N. Therefore, overexpression of HK or down regulation of cTPI activities in transgenic roots might be compensated by increased C catabolism through the oxPPP. In order to see the affect of HK and cTPI manipulation on N assimilation, the transgenic roots were grown on media with low or high concentration of ammonium nitrate as the N source. The specific activity of the oxPPP enzymes glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) were both increased by an increased N supply in HK and cTPI transgenic roots. This is consistent with the provision of reducing equivalents for N assimilation. The data also show that the capacity of the oxPPP is higher in roots with high HK or cTPI activity. We were able to detect higher HK specific activity in N deficient conditions. These data were complemented with measurements of amino acid pools in transgenic roots. No trend in amino acid pools was found in roots modified for HK activity. However, down regulation of cTPI led to higher Gln, Gln/Glu and Asn/Asp ratios, indicating higher assimilation of N. These results demonstrated the activation of N assimilation in cTPI antisense clones while the manipulation of HK is unlikely to affect the N assimilation.

Keywords: Hexokinase, Triosephosphate isomerase, nitrogen assimilation, oxidative pentose phosphate pathway, amino acid content

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Abbreviations

ALD: Aldolase

Asn: Asparagine

Asp: Aspartate

ATP-PFK: ATP-dependent phosphofructokinase

α-KG: Alphaketoglutarate

DHAP: Dihydroxyacetone phosphate

DTT: Dithiothreitol

Fru: D-fructose

Man: D-manose

Gal: D-galactose

FNR: ferredoxin-NADP oxidoreductase

FK: fructokinase

F1, 6BF: Fructose 1,6-bisphosphate

F6P: Fructose-6-phosphate

GK: glucokinase

G1P: Glucose-1-phosphate

G6P: Glucose-6-phosphate

GPT: Glc-6-P/phosphate translocator

G3P: Glyceraldehyde-3-phosphate

G6PDH: Glucose-6-phosphate 1-dehydrogenase

GDH: glutamate dehydrogenase

Gln: Glutamine

Glu: Glutamate

Gs: Glutamine synthetase

GOGAT: Glutamate synthase

GDH: Glutamate dehydrogenase

HK: Hexokinase

miETC: Mitochondrial electron transport chain

MA: NAD-dependent malic enzyme

NR: Nitrate reductase

MDH: Malate dehydrogenase

ME: Malic enzyme

NiR: Nitrite reductase

NO: Nitric oxid

TCA: Mitochondrial tricarboxylic acid

OAA: Oxaloacetate

oxPPP: Oxidative section of the PPP

PEP: Phosphoenol pyruvate

PFK: Phosphofructokinase

PFP: Pyrophosphate-dependent phosphofructokinase

PEPC: PEP carboxylase

PEPase: PEP phosphatase

PGI: phosphoglucose isomerase

PK: Pyruvate kinase

PPT: Phosphoenolpyruvate/phosphate translocator

PDC: Programmed cell death

ROS: Reactive oxygen species

Ru5P: Ribulose-5-phosphate

RPI: Ribose-5-phophate isomerase

RPE: Ribulose-5-phosphate 3-epimerase

TK: Transketolase

TA: Transaldolase

Triose-P: Triosephosphate

TPI: Triosephosphate isomerase

Xlu-5-P: Xylulose 5-phosphate

XPT: xylulose 5- phosphate/phosphate translocator

1,3-DPGA: 1,3-diphosphoglycerate

2-OG: 2-oxoglutarate

2-PGA: 2-phosphoglycerate

3-PGA: 3-phosphoglyceric acid

6PG: 6-phosphogluconolactone

6PGL: 6-phosphogluconolactonase

6PGDH: 6-phosphogluconate dehydrogenase

1. Introduction and literature review

1.1. Glycolysis and plant respiration

In the photosynthetic process, plants use light energy for the oxidation of water into O_2 and the simultaneous reduction of CO_2 into carbohydrates, commonly stored as the insoluble polysaccharide starch or the soluble disaccharide sucrose. Conversely, respiration oxidizes these reduced carbohydrates via the glycolytic pathway, the pentose phosphate pathway and the mitochondrial tricarboxylic acid (TCA) cycle, thereby producing CO_2 and reducing equivalents (NAD(P)H and $FADH_2$). In aerobic respiration, the reducing equivalents produced during carbohydrate oxidation transfer their electrons to O_2 via the mitochondrial electron transport chain (miETC), resulting in respiratory O_2 consumption and ATP production (Plaxton 1996, Siedow & Day 2000). Respiration is required for growth, development and the production of C skeletons for biosynthetic purposes in all photosynthetic organisms (Kromer 1995). Respiration also serves for ATP production by the ATP synthase complex in the inner mitochondrial membrane. This process uses the proton electrochemical gradient generated by the transport of respiratory electrons in the miETC.

Plant respiration produces the C skeletons for the biosynthesis of several intermediates including secondary metabolites, isoprenoids, fatty acid and amino acids (Moraes & Plaxton 2000, Plaxton 1996, Plaxton & Podestà 2006). The interaction between carbon and N metabolism is pivotal in plants because the inorganic N, absorbed in the form of nitrate or ammonium, must be assimilated into amino acids (Plaxton & Podestà 2006). Although plant respiration can alleviate oxidative stress by NAD(P)H production which is required for scavenging reactive oxygen species (ROS), respiration is also a source of ROS production in the miETC (Tiwari et al 2002). Therefore it could participate in the oxidative burst leading to ATP depletion and programmed cell death (PCD) (Tiwari et al 2002, Xie & Chen 1999). Increased ROS generation by respiration has been suggested to integrate the plant responses to several biotic and abiotic stresses (McDonald & Vanlerberghe 2005, Møller 2001).

1.1.1. Steps of Glycolysis

Glycolysis serves to oxidize stored sucrose or starch concomitantly with the production of NADH, ATP and metabolite precursors. In contrast to the animal cells that start glycolysis with glucose, in most higher plant cells, glycolysis starts with sucrose and ends with pyruvate (Sung et al 1988).

Sucrose can be cleaved by an invertase to generate glucose and fructose (Figure 1). The resulting hexoses will be converted into hexose phosphates by hexokinase (HK, EC 2.7.1.1) via the consumption of ATP in the cytosol.

$$\text{Hexose} + \text{Mg ATP}^{2-} = \text{HexosePO}_3^{2-} + \text{Mg ADP}^{-} + \text{H}^{+}$$

The reaction catalyzed by HK will be explained in more detail in the text below. Sucrose can also be cleaved by sucrose synthase (EC 2.4.1.13) to form UDP–glucose and fructose. Following phosphorylation, the resulting glucose-6-phosphate (G6P) and/or fructose-6-phosphate (F6P) can be further metabolized through glycolysis (Pego & Smeekens 2000). In addition, glucose phosphate isomerase catalyzes the reversible isomeration of G6P to F6P. The next glycolytic reaction is the conversion of F6P to fructose 1,6 bisphosphate (F1,6bisP), catalyzed by an irreversible ATP-dependent phosphofructokinase and therefore coupled to ATP hydrolysis (PFK, EC 2.7.1.11). However, this reaction can also be catalyzed by a reversible pyrophosphate-dependent phosphofructokinase (PFP, EC 2.7.1.90) in higher plants. Aldolase (ALD, EC 4.1.2.5) can split the ketose ring in F1,6bisP into the two triosephosphates dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P). Triosephosphate isomerase (TPI, EC 5.3.1.1) then catalyzes the reversible conversion of these two products. However due to the consumption of G3P by the bottom part of glycolysis in the subsequent reaction, the net flux goes towards the production of G3P. The steps between G3P and pyruvate are called the payoff phase of glycolysis, which yields energy in the form of ATP and NADH (Plaxton 1996).

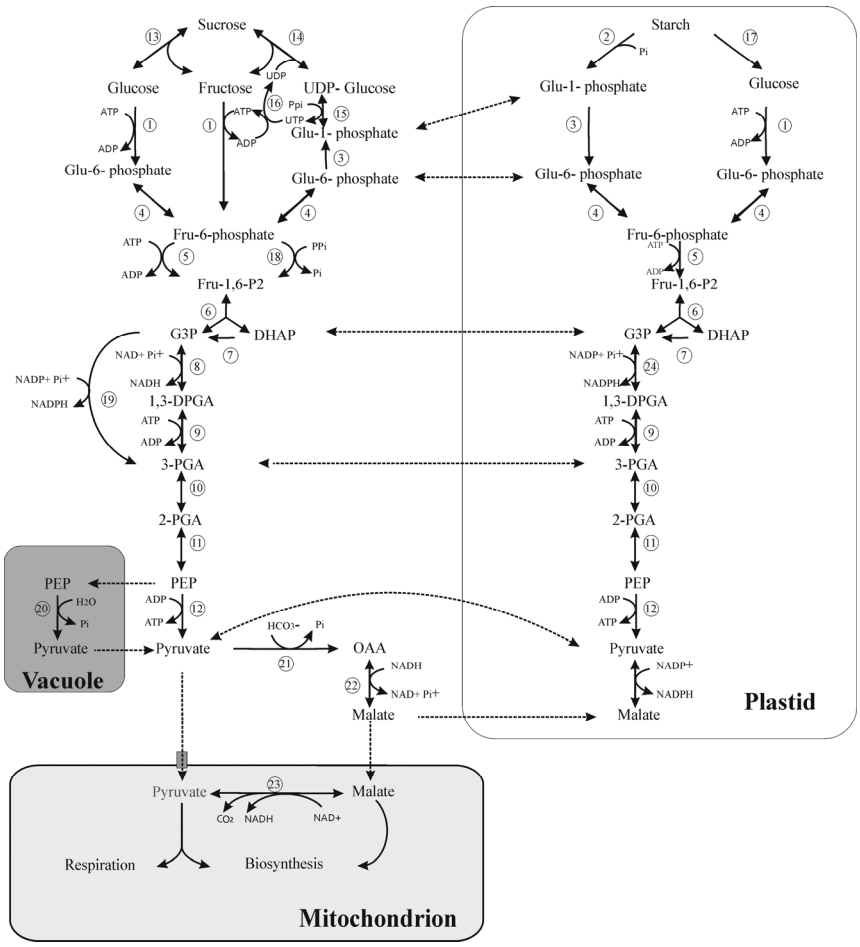

Figure 1. The organization of plant glycolysis. The numbers appearing in the figure correspond to the following enzymes: 1, HK; 2, Starch phosporylase (EC 2.4.1.1); 3 phosphoglucomutase (EC 5.4.2.2); 4, phosphoglucose isomerase (PGI, EC 5.3.1.9); 5, PFK; 6, ALD; 7, TPI; 8, NAD-dependent GAPDH (EC 1.2.1.12) (phosphorylating); 9, 3-PGA kinase (EC 2.7.2.3); 10, phosphoglyceromutase (EC 5.4.2.1); 11, enolase (EC 4.2.1.11); 12, pyruvate kinase (PK 2.7.1.40); 13, invertase (EC 3.2.1.26); 14, sucrose synthase (EC 2.4.1.13); 15, UDP-glucose pyrophosphorylase (EC 2.7.7.9); 16, nucleoside diphosphate kinase (EC 2.7.4.6); 17, α- and β-amylase (EC 3.2.1.1 and EC 3.2.1.2); 18, PFP; 19, NADP-dependent (non-phosphorylating) GAPDH (EC. 1.2.1.13); 20, PEP phosphatase (PEPase, EC 3.1.3.60); 21, PEP carboxylase (PEPC, EC 4.1.1.31); 22, malate dehydrogenase (MDH, EC 1.1.1.37); 23, malic enzyme (ME, EC1.1.1.40), 24, NADP-dependent GAPDH (EC. 1.2.1.13). Note that due to the formation of two molecules of G3P from one molecule of hexose, the number of substrate and product molecules in all reactions from G3P to pyruvate should be doubled (Plaxton 1996).

In the later steps of glycolysis, G3P is oxidized to 1,3 diphosphoglyceric acid via an NAD-dependent G3P dehydrogenase (EC 1.1.1.8) in the cytosol. An NAD-linked in the cystosol oxidizes G3P to 3-phosphoglyceric acid (3-PGA) while an NADP-linked enzyme (EC 1.2.1.9.) in plastids catalyzes the same reaction. The 3-PGA produced is converted to 2-phosphoglycerate (2-PGA) by phosphoglyceromutase (EC 2.7.5.3). Then, the product of this reaction is converted to phospho*enol*pyruvate (PEP) by the action of enolase. In the cytosol, PEP is a substrate for two enzymes named PK and PEPC. PK generates pyruvate, concomitant with energy in the form of ATP, that can directly enter the mitochondria to feed the TCA cycle. The second enzyme, PEPC, forms oxaloacetate (OAA) that can also enter the mitochondria (Kromer et al 1996). OAA may be reduced to malate by malate dehydrogenase (EC 1.1.1.37) in the cytosol and the produced malate transported to the mitochondria. The conversion of PEP to pyruvate can also be catalyzed in vacuole by a PEPase that can bypass the ADP-dependent PK reaction during Pi starvation (Theodorou & Plaxton 1993, Ukaji & Ashihara 1987).

The summary reaction of glycolysis:

$$\text{Glucose} + 2\ \text{ADP} + 2\ \text{Pi} + 2\ \text{NAD}^{+} \leftrightarrow 2\ \text{Pyruvate} + 2\ \text{ATP} + 2\ \text{NADH}$$

In aerobic conditions, pyruvate produced by glycolysis is consumed in the TCA cycle to generate both ATP and reducing equivalents, which then transfer their electrons to the electron transport chain to produce more ATP. In anaerobic conditions, pyruvate can be converted to lactate or ethanol by fermentative pathways with the consumption of NADH as reducing power.

1.1.1.1. Hexokinase

HK is a key enzyme in glycolysis (Figure 2) and has also been suggested to act as a sugar sensor in lower and higher eukaryotes (Frommer et al 2003, Rolland et al 2001). HK is thus able to mediate hexose responses at the level of gene expression (Jang et al 1997).

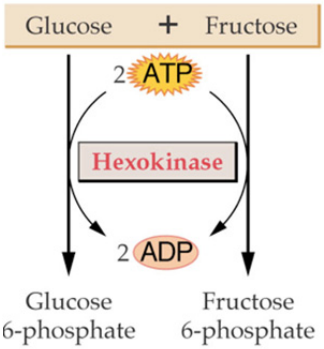

Figure 2. The conversion of hexose to hexose-6-phosphate by HK (Dennis & Blakeley 2002). This material is copyrighted by the American society of plant biologists and is reprinted with permission.

HK is a part of glycolysis in all living organisms. This enzyme can phosphorylate several substrates, such as D-glucose (Glc), D-fructose (Fru), D-mannose (Man) and D-galactose (Gal) (Claeyssen & Rivoal 2007). In most organisms, HK have a high affinity for Glc and Man but a significantly lower affinity for Fru (Claeyssen et al 2006). In plants several enzymes are able to phosphorylate hexoses. These enzymes are classified as Hexokinases, Fructokinases and Glucokinases according to their substrate specificity. Fructokinases and Glucokinases should not be confused with HK since they are highly specific to glucose and fructose, respectively. The specificity of HK for each hexose gives the enzyme the capacity to act as a gateway to glycolysis. Glc and Fru do not limit HK activity since they are generally quite abundant in plant tissues (Cardenas et al 1998, Claeyssen & Rivoal 2007, Troncoso-Ponce et al 2011). The product of glucose phosphorylation feeds glycolysis and can also be a precursor for oxidative section of pentose phosphate pathway (oxPPP) (Claeyssen & Rivoal 2007, Neuhaus & Emes 2000).

Plant cells contain several HK isoforms in different compartments. In potato tubers, at least four isoforms have been identified and one of them has been purified to homogeneity (Moisan & Rivoal 2011). HK either is soluble and targeted to the plastid stroma or has a hydrophobic N-terminal domain and is found bound to the mitochondrial membrane. Most isoforms of this enzyme are controlled by the ATP/ADP ratio and are sensitive to acidic pH (Moisan & Rivoal 2011).

1.1.1.2. Triosephosphate isomerase

TPI has a central role in carbon metabolism since its substrates DHAP and G3P are precursors for several other metabolic processes like synthesis of sucrose and of secondary metabolites. DHAP is also used in the synthesis of glycerolipids (Dorion et al 2012, Rohmer 1999, Schwender et al 1996, Tambasco-Studart et al 2005).

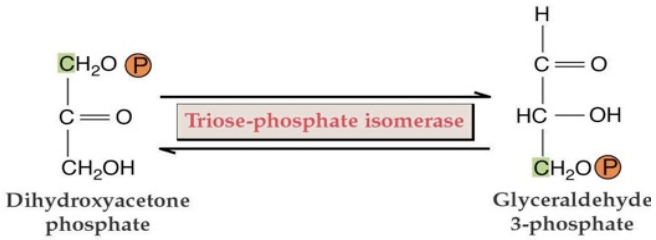

Figure 3. The isomerization of triosephosphates by TPI (Dennis et al 1991). This material is copyrighted by the American Society of Plant Biologists and is reprinted with permission.

TPI is an example of a perfect catalyst because its reaction rate is controlled only by the rate of substrate diffusion (Knowles & Albery 1977). Due to this fact, it is generally not considered as an important enzyme in the regulation of glycolysis (Fell 1997). The isoform enzyme is present in the cytosol plays a role in glycolysis and gluconeogenesis, whereas the plastidic isoform functions in the Calvin cycle (Pichersky & Gottlieb 1984). It has been suggested that cytosolic TPI is regulated by glutathionylation, which means that under oxidizing conditions glutathione would suppress TPI activity (Ito et al 2003). Protein glutathionylation is the formation of an intermolecular disulfide bridge between a cysteine residue of a target protein with glutathione. Glutathionylation was initially thought to protect the irreversible oxidation of reactive protein-bound Cys (Dixon et al 2005, Fratelli et al 2004). However, it can also cause changes in protein function that might regulate the activity of the protein. Therefore, glutathionylation can act as a redox-driven regulator of metabolic pathways (Cotgreave & Gerdes 1998, Ito et al 2003).

1.1.2. Compartimentation of glycolysis

In plants, glycolysis can occur independently in both the cytosol and the plastid (Figure 1). The intermediates of cytosolic and plastidic glycolysis can be exchanged between these two compartments through the action of highly selective transporters, present in the inner plastid envelope such as G6P, triosephosphates, 3-PGA, malate/glutamate and PEP transporters (Figure 4) (Dennis & Blakeley 2002, Plaxton 1996). Except for the malate/glutamate transporter, these proteins reversibly exchange phosphorylated intermediates with inorganic P_i, and the direction of transport at the inner plastid membrane is dependent on the concentrations of Pi and metabolites on each side (Dennis & Blakeley 2002). In addition to the oxidation of carbohydrates to gain reductant and ATP for anabolic pathways in the cytosol, in non-photosynthetic plastids or in the chloroplast in the dark, glycolysis also provides carbon skeletons for these pathways (Blakeley & Dennis 1993, Dennis et al 1991, Neuhaus & Emes 2000). Cytosolic and plastidial glycolysis share several metabolites with the pentose phosphate pathway, which can therefore participate in the exchange of intermediates between the two compartments (Neuhaus & Emes 2000).

In the cytosolic glycolytic network, several parallel enzymatic reactions exist at the level of sucrose, F6P, G3P and PEP metabolism (Figure 1). The various alternative reactions in the cytosol and the existence of parallel glycolytic reactions in the cytosol and plastid, give glycolysis the flexibilities to acclimate to different biotic and abiotic stresses (Mertens 1991, Plaxton 1996, Theodorou & Plaxton 1993). A good example of this is the alternative conversions of F6P to F1,6P2 which can be done using ATP-PFK or PFP since PPi serves as a phosphoryl donor of the plant cytosol, specially under stresses like anoxia and Pi starvation (Plaxton 1996).

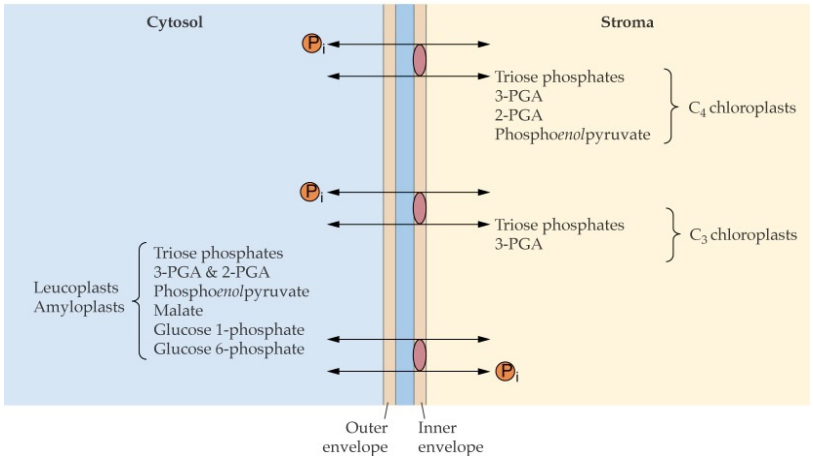

Figure 4. Transporters of glycolytic intermediates in the inner plastid membrane (Dennis & Blakeley 2002). This material is copyrighted by the American Society of Plant Biologists and is reprinted with permission.

1.2. Pentose phosphate pathway

The pentose phosphate pathway (PPP) is a central metabolic pathway that oxidizes a hexose phosphate and leads to the production of pentose phosphates and other phosphate esters together with the release of CO_2 and provision of NADPH for biosynthesis processes. This pathway has two distinct sections, namely the oxidative (Figure 5) and non-oxidative sections (Dennis & Blakeley 2002, Xiong 2009) (Figure 6). In non-photosynthetic cells, the irreversible oxidative reactions of the pathway are a major source of the reducing equivalent NADPH for various biosynthetic processes, such as fatty-acid synthesis, assimilation of inorganic forms of nitrogen and sulfur, as well as for and maintaining the redox potential necessary to protect against oxidative stress. The reversible non-oxidative section of the pathway is a source of carbon skeletons for the synthesis of nucleotides, aromatic amino acids, phenylpropanoids and their derivatives.

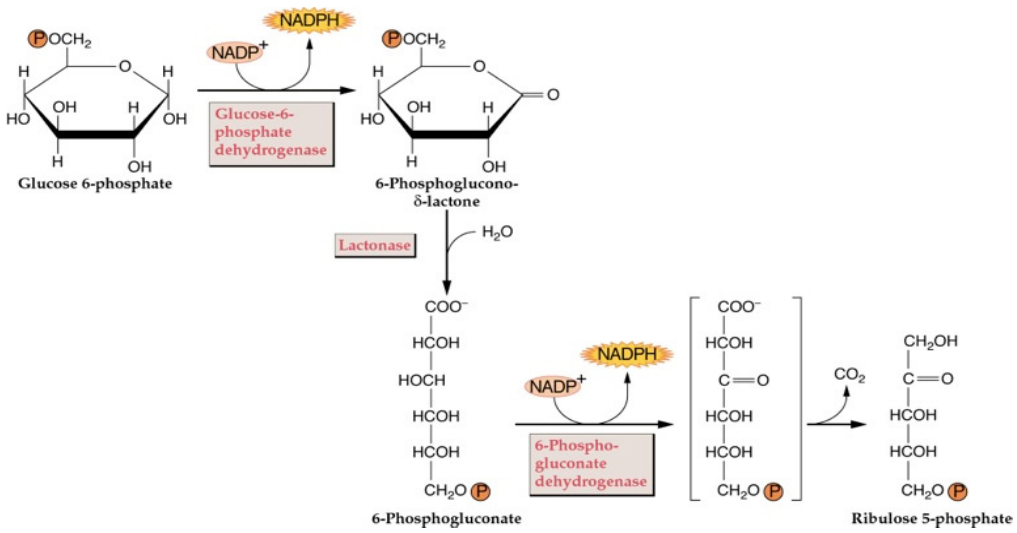

Figure 5. The oxidative section of the PPP (Dennis & Blakeley 2002). G6P is oxidized to Ru5P concomitantly with the release of CO_2 and NADPH production. This material is copyrighted by the American Society of Plant Biologists and is reprinted with permission.

The PPP contains a series of reactions catalyzed by seven different enzymes: glucose-6-phosphate 1-dehydrogenase (G6PDH, EC 1.1.1.49), 6-phosphogluconolactonase (6PGL, EC 1.1.1.44), 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44), ribose-5-phophate isomerase (RPI, EC 5.3.1.6), ribulose-5-phosphate 3-epimerase (RPE, EC5.1.3.1), transketolase (TK, EC 2.2.1.1) and transaldolase (TA, EC 2.2.1.2) (Xiong 2009) (Figure 6). It is generally believed that the enzymes catalyzing the oxidative section exist in both the cytosol and plastids, whereas those catalyzing the non-oxidative section are restricted to plastids (Debnam & Emes 1999, Schnarrenberger et al 1995) (Figure 7).

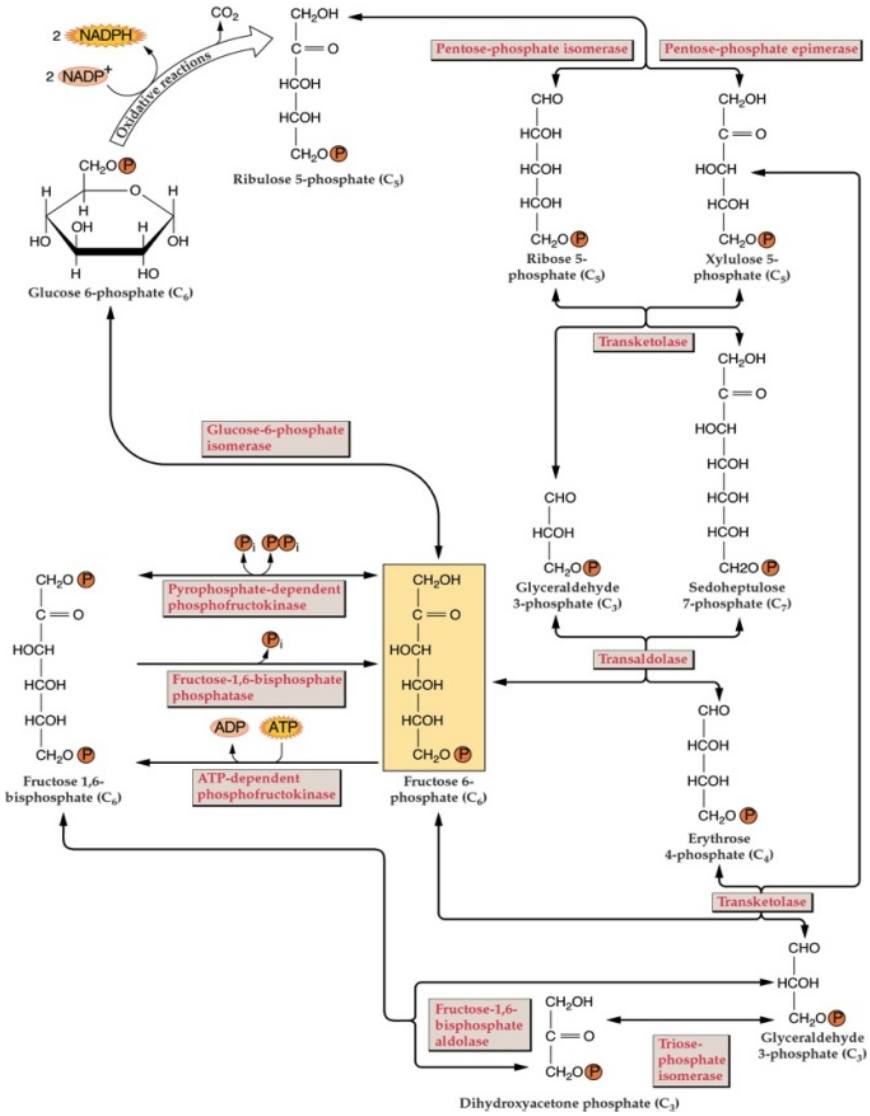

Figure 6. Pentose phosphate pathway. The details of the non-oxidative section (Dennis & Blakeley 2002). This material is copyrighted by the American Society of Plant Biologists and is reprinted with permission.

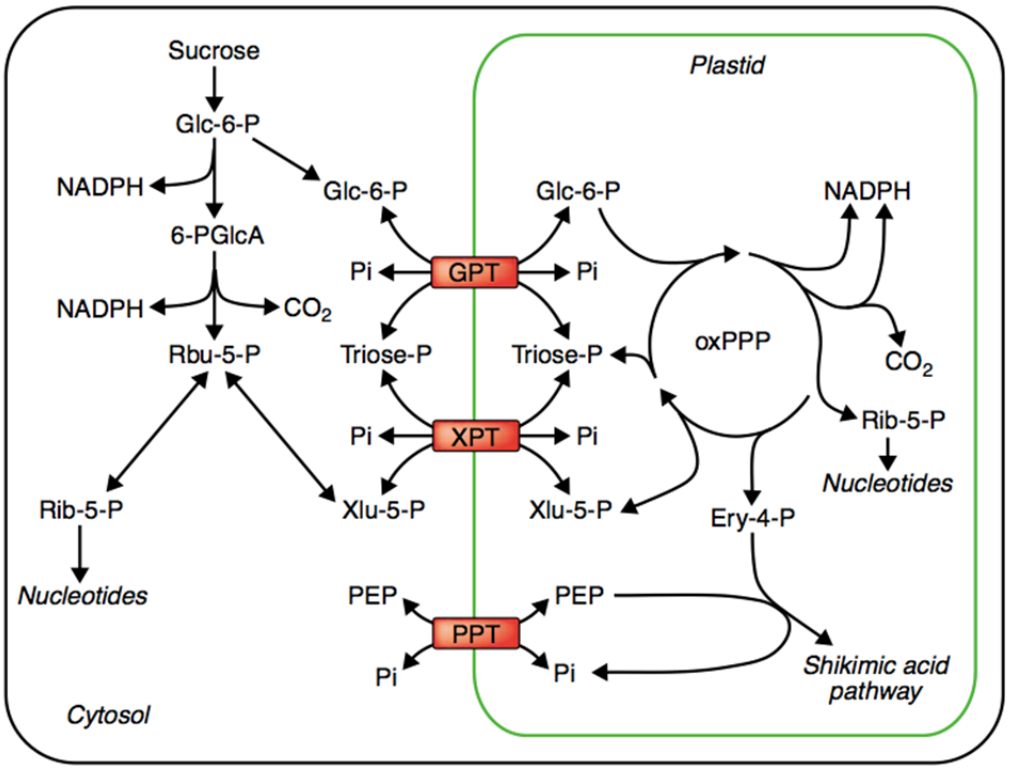

Figure 7. Exchange of metabolites between cytosol and plastid in PPP. The intermediates transfer by Glc-6-P/phosphate translocator (GPT), xylulose 5- phosphate/phosphate translocator (XPT) and the phospho*enol*pyruvate/phosphate translocator (PPT) (Kruger & von Schaewen 2003). This material is reprinted by permission of Elsevier provided by copyright clearance center.

Analysis of the *Arabidopsis* genes encoding the enzymes of the oxPPP has shown that TK and TA, which catalyze part of the non-oxidative phase of the pathway, may be present only in plastids (Batz et al 1998, Kruger & von Schaewen 2003).

1.2.1. The regulation of the enzymes of oxPPP

Whereas both G6PDH and 6PGDH catalyzed reactions are irreversible, these enzymes are believed to be rate limiting enzymes for the oxPPP (Huang et al 2003). Their role in resistance to different stresses has been investigated in various organisms. All eukaryotic G6PDHs studied are feedback inhibited by NADPH, hence they are presumed to act as cellular redox sensors. In addition to the enzyme inhibition by the redox balance of the NADPH/ $NADP^+$ pool, plastidial G6PDH is regulated by the ferredoxin/thioredoxin system

(Buchanan 1991, Knight et al 2001, Kruger & von Schaewen 2003, Wakao et al 2008). Therefore, plastidial G6PDH is inactivated under reducing conditions, and this is presumed to prevent unnecessary oxidation of carbon when NADPH generation by photosynthesis is sufficient (Wakao et al 2008). *In vitro*, dithiothreitol (DTT) can inactivate plastidic G6PDH (Scheibe et al 1989, Wright et al 1997). The mechanism that is responsible for the regulation of the cytosolic isoform is different. The presence of several SURE elements, which confer sugar responsiveness, in the promoter region of the potato (*Solanum tuberosum*) *g6pdh* genes suggest the regulation of cytosolic G6PDH by sugar availability at the gene expression level (Hauschild & von Schaewen 2003). The high amount of sugar in the cytosol causes elevated transcription of the cytosolic *G6PDH* gene via sugar-mediated signaling to the nucleus. Greater mRNA expression increases the enzyme levels and G6PDH activity (Hauschild & von Schaewen 2003).

1.2.2. G6PDH

G6PDH, a homotetramer with subunits of 50–60 kDa, is the first NADPH-generating enzyme of the oxPPP (Figure 5). The cytosolic isoform is very stable *in vitro*, whereas the plastidic isoform is unstable and is generally lost during the purification procedure (Krepinsky et al 2001). Recently, an *Arabidopsis* genome-wide analysis identified two cytosolic G6PDH and four plastidial G6PDH isoforms, the latter divided into P1-G6PDH and P2-G6PDH groups (Wakao & Benning 2005). The biochemical characteristics and the specific gene expression patterns were different for these two plastidial isoforms in potato (Wendt et al 2000). Although in most of the higher plants all these G6PDH isoforms have been described, the regulatory properties of each isoform are still not well characterized (Esposito et al 2001, Esposito et al 2005, Hauschild & von Schaewen 2003, Huang et al 2002, Nemoto & Sasakuma 2000, Wendt et al 2000). *P1-G6PDH* transcripts are expressed in most of the photosynthetic and non-photosynthetic tissues under light conditions (Wendt et al 2000) and are absent from roots, whereas *P2-G6PDH* transcripts are found throughout the plant especially in stems and roots (Knight et al 2001).

The main role of G6PDH is to produce reducing equivalents for reductive biosynthesis (Kruger & von Schaewen 2003, Wakao et al 2008, Wenderoth et al 1997). The cytosolic and

plastidial isoforms have different roles in plant metabolism. For example, the cytosolic enzyme is involved in the production of precursors for nucleic acid biosynthesis (Anderson et al 1974, Dennis et al 1997, Esposito et al 2001, Schnarrenberger et al 1973). Recent studies with knockout mutants of the two cytosolic G6PDH suggested a crucial role of cytosolic isoforms in providing carbon substrates for biosynthesis of storage components in developing seeds of *Arabidopsis thaliana* (Wakao et al 2008). The cytosolic isoform is also required to generate the NADPH necessary for defense responses (Scharte et al 2009) such as the induction of G6PDH as an antioxidant in response to salt stress and the production of ROS in olive plants (Valderrama et al 2006). The plastidial G6PDH plays a major role in the production of reducing power for nitrite reduction (Bowsher et al 1989, Emes & Fowler 1983, Oji et al 1985) and fatty acid biosynthesis (Dennis et al 1997). The P2-G6PDH protein is induced in roots by N (Bowsher et al 2007). In addition, the P1-G6PDH protein increases in leaves in response to N supply (Esposito et al 2005). Also plastidic G6PDH might be the major source of reducing power for glutamate synthase (EC 1.4.1.13) in non photosynthetic tissues (Bowsher et al 1989).

1.2.3. 6PGDH

6PGDH, the second enzyme of oxPPP, catalyzes the conversion of 6PG to Ru5Pconcomitant with the production of NADPH and the release of one molecule of CO_2 (Figure 5). The cytosolic isoform is stable but, like G6PDH, the plastidial isoform is extremely unstable which has limited all the efforts for its purification. In addition to the plastid and cytosol, Both G6PDH and 6PGDH enzyme activity have been detected in peroxisomes purified from pea leaves, so it has been proposed that oxPPP may also exist in peroxisomes (Kruger & von Schaewen 2003). The cytosolic 6PGDH cDNA has been isolated from alfalfa (*Medicago sativa L.*) (Fahrendorf et al 1995), maize (*Zea mays L.*) (Redinbaugh & Campbell 1998), spinach (*Spinasia oleracea L.*) (Krepinsky et al 2001)and rice (*Oryza sativa L.*) (Hou et al 2007), while the plastidial gene has been identified from spinach (Krepinsky et al 2001) and *Arabidopsis*(Kruger & von Schaewen 2003b). The lack of cytosolic 6PGDH in maize seedling roots decreased the capacity of oxPPP to promote carbon flux when the demand of NADPH is increased by nitrite treatment (Averill et al 1998).

1.3. TCA cycle and mitochondrial transporter chain

Following glycolysis, the respiratory process continues with the mitochondrial reactions of the TCA cycle. PEP, which is synthesized by glycolysis, is converted to pyruvate and malate in the cytosol. The presence of a NAD-malic enzyme (NAD-ME, EC 1.1.1.39) offers the possibility to oxidize malate in the TCA cycle, in addition to oxidation of pyruvate delivered by glycolysis. Therefore, pyruvate and malate should be imported into the mitochondria matrix by transporters in order to feed the TCA cycle. The stepwise oxidation of pyruvate to CO_2 generates NADPH and $FADH_2$ as reducing power and ultimately yields ATP (Fernie et al 2004) (Figure 8). In addition to the production of energy, the TCA cycle produces several intermediates for other plant biosynthetic pathways. For example, acetyl-CoA can feed fatty acid biosynthesis (Ohlrogge & Browse 1995), nucleic acids are generated from alpha-ketoglutarate (α-KG), OAA provides carbon skeletons for amino acid biosynthesis through the reaction of glutamate dehydrogenase (GLDH EC 1.4.1.2.) or transamination reactions and succinyl-CoA serves in the synthesis of porphyrin. Therefore, the TCA cycle functions in both catabolism and anabolism (Siedow & Day 2000).

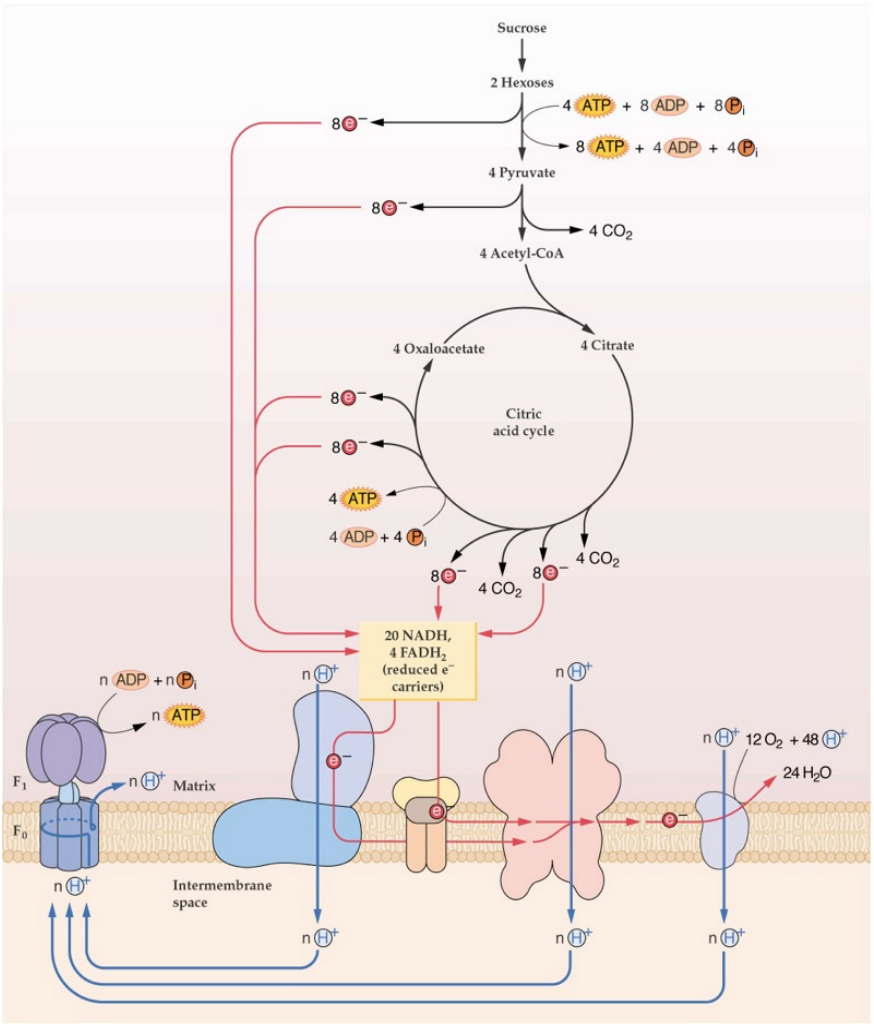

Figure 8. TCA cycle and mitochondrial electron transporter chain (Siedow & Day 2000). This material is copyrighted by the American Society of Plant Biologists and is reprinted with permission.

The reducing equivalents produced during glycolysis and TCA cycle are subsequently oxidized by the electron transport chain located in the inner mitochondrial membrane. The free energy that is released by the miETC is coupled to the translocation of protons in the inner membrane from the mitochondrial matrix to the intermembrane space. The electrochemical gradient generated across the inner membrane is then used by the ATP synthase complex to phosphorylate ADP and generate ATP as a source of chemical energy for metabolism (Siedow & Day 2000) (Figure 8).

1.4. N assimilation in non-photosynthetic tissues

The process by which mineral N is taken up by plants for the synthesis of organic forms of N is called N assimilation. Nitrate and ammonia are forms of soil inorganic N that can be readily absorbed by plants. The initial conversion of nitrate to nitrite occurs in the cytosol and is catalyzed by nitrate reductase (NR, EC 1.6.6.2) (NO_3^- + NADH+ $H^+ \rightarrow NO_2^-$ + NAD^+ + H_2O). This is followed by a reduction to ammonia by nitrite reductase (NIR, EC 1.7.7.1) in the plastids.

The electrons needed for nitrite reduction are provided by NAD(P)H (Figure 9). In roots, NADPH acts as the initial reductant to reduce ferredoxin through a root-specific ferredoxin-NADP oxidoreductase (FNR, EC, 1.18.12). Subsequently, ammonium is assimilated into organic molecules. Nitrite is toxic and must be rapidly reduced to ammonium, which is then used for the synthesis of amino acids like glutamine (Gln) and glutamate (Glu), mainly by the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle (Figure 9), although assimilation by glutamate dehydrogenase (GDH, EC 1.4.1.2) is also possible. The activity of the GS/GOGAT cycle is pivotal for plants because the products of this cycle, Gln and Glu, are the primary donors of the organic N for all compounds which require N in their structure, including amino acids/proteins, chlorophylls, nucleic acids, polyamines, and several important secondary metabolites like alkaloids (Plaxton & Podestà 2006). The reduced ferrodoxin substrate is required for the reaction of both NIR and GOGAT, which are regarded as control points that regulate N assimilation (Bowsher et al 2007). After assimilation of ammonia, Glu and Gln transfer their amino groups to Aspartate (Asp) and Asparagine (Asn) as well as other amino acids. Asn biosynthesis includes the transfer of an amino group from glutamine to aspartate, which is catalyzed by asparagine synthase (EC 6.3.5.4) in an ATP-dependent reaction (Ta et al 1986). The amino group can be transferred to Asp directly from ammonium by an Aspartate-ammonia ligase (EC 6.3.1.4) (Coruzzi & Last 2000). However the first reaction is more preferable. Glu, Gln, Asp and Asn believed to play an important role in primary N assimilation (Lam et al 1996).

The dependence of G6PDH activities on nitrate assimilation has been investigated in non-photosynthetic tissues (Bowsher et al 1992, Emes & Fowler 1983, Oji et al 1985,

Redinbaugh & Campbell 1998). It has long been proposed that the reducing power required for nitrate metabolism in the roots is produced by the oxPPP.

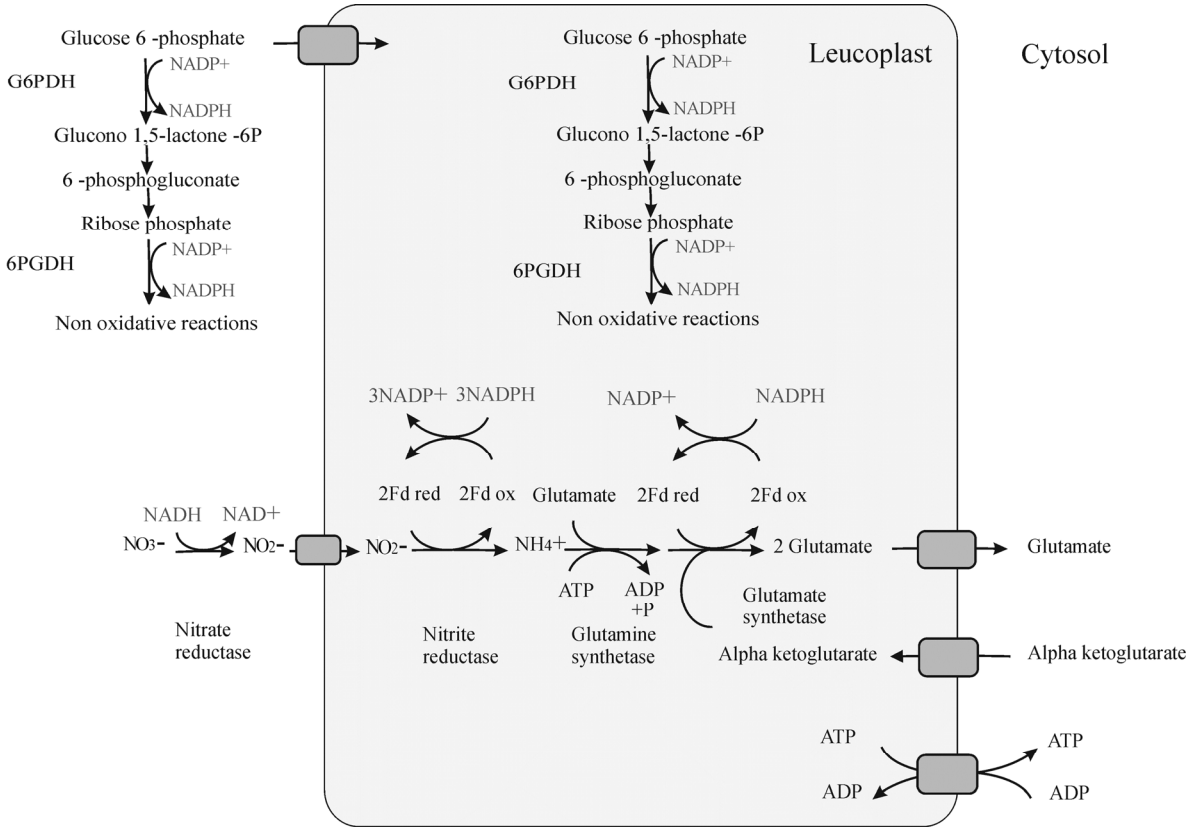

Figure 9. N assimilation in non-photosynthetic tissues. This figure shows the production of reducing equivalent for N assimilation by oxPPP in plastid and cytosol. Gray squares indicate transporters across the plastid membrane (Heldt & Piechulla 2004).

The effect of nitrate supply on the specific activity of G6PDH and 6PGDH was investigated in maize, and increases of 27- and 12- fold, respectively, were measured in plastids (Redinbaugh & Campbell 1998). The cytosolic isoform of G6PDH is insensitive to regulation by reductant (Scheibe et al 1989) compared to the P2 G6PDH, which is linked to N assimilation (Esposito et al 2002). The expression of the P2 form of G6PDH was increased by nitrate treatment in *Nicotiana tabacum* roots (Knight et al 2001), while the activity of G6PDH was increased after ammonium/glutamate feeding in barley roots (Esposito et al 2002). Indeed,

the activity of G6PDH in pea root plastids was increased by external nitrate supply. The maximum catalytic activity of G6PDH was determined to be more than sufficient to support the reductant requirements of both NiR and GOGAT (Bowsher et al 2007). Taken together, the details above suggest that oxPPP supplies the reductant for N assimilation in the dark and in non-photosynthetic tissues.

The organic N originally generated can be distributed to other amino acids by transamination reactions, which transfer an amino group from an amino acid to an α-ketoacid via enzymes belonging to the aminotransferase class (Wilson et al 1954).

In order for metabolism to provide the necessary carbon skeletons (α-ketoacids) for amino acid synthesis, as well as ATP and reductant for the GS/GOGAT cycle, N assimilation must have a close interaction with carbon metabolism. This interaction involves three compartments in plants: mitochondria, plastid and cytosol (Brito & Kronzucker 2005, Huppe & Turpin 1994, Lancien et al 2000) (Figure 10). GS requires energy in the form of ATP, while GOGAT requires reductant such as reduced ferredoxin or NADH and a carbon skeleton in the form of α-KG, which is produced by the TCA cycle. For the generation and export of TCA intermediates, the only net source of carbon skeletons for the GS/GOGAT cycle, the substrates required for generating acetyl-CoA and OAA should have been imported to the mitochondria. In addition, PK and PEPC in the cytosol control the glycolytic flux and PEP provision during NH^+_4 assimilation to feed the TCA cycle or provide the necessary OAA for N-compounds synthesis (Brito & Kronzucker 2005, Moraes & Plaxton 2000, Plaxton 1996, Plaxton & Podestà 2006).

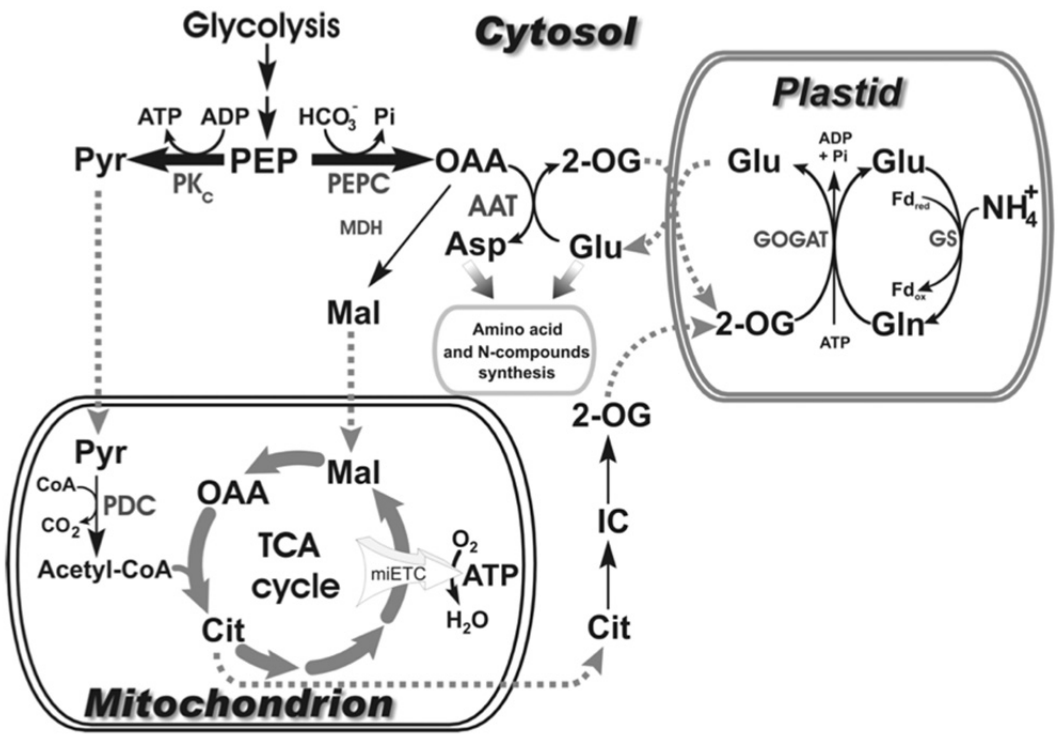

Figure 10. The connection between carbon and N metabolisms in plants between cytosol, plastid and mitochondria (Plaxton & Podestà 2006). Dash arrows indicate the transfer of intermediates between different compartments. This figure is reprinted by permission of Taylor & Francis (http://www.tandfonline.com).

1.5. Previous results from the laboratory in connection to the function of the OPPP and glycolysis

Our laboratory investigates the function and regulation of primary metabolism in non-photosynthetic plant cells. In particular, several current projects are aimed at understanding the relationships between the activity level of individual enzymes in glycolysis and the metabolic flux that passes through the glycolytic pathway. Recently, our laboratory has focused on the enzymes HK and TPI. Using transgenic approaches, the activity of these two enzymes has been modified in potato roots. These experiments have led to several important results in connection with the function of the PPP and my own project.

1.5.1. Transgenic roots containing modified levels of HK

In order to study the role of HK in root growth and glycolytic flux, potato (*Solanum tuberosum*) root clones with different levels of HK activity were generated using both sense and antisense strategies. The*ScHK2* gene from *Solanum chacoense* was used, and since it shared 82-98% overall sequence identity with known potato HK sequences (Claeyssen et al 2006), it was expected that it would be efficient in both strategies. The sense and antisense constructs of *ScHK2* cDNA were inserted in the pGA643 vector under the control of the CaMV35S promoter in order to transform potato using *Agrobacterium rhizogenes* as the transformation vector. A population of 22 transformants, including one antisense (As213), two control (CTL2, CTL3) which were transformed by an empty vector and two sense (S111, S101) clones were selected for in-depth metabolic characterization (Claeyssen et al 2012). For the measurement of HK control over the phosphorylation of Glc, the Glc analogue (U^{14}-C)DOG was used as a tracer. The tracer is phosphorylated toDOG6P by HK enzyme in potato roots. Since DOG6P is not normally metabolized, it accumulates in plant cells. The measurement of (U^{14}-C)DOG6P accumulation as well as the specific radioactivity of Glc allows examination of carbon flux at the level of the HK reaction and the level of HK control over the phosphorylation of Glc (Claeyssen et al 2012). The capacity of the roots to generate G6P was found to be tightly correlated with the activity of HK (Figure 11). However, (Claeyssen et al 2012) found no proportional increase in the rate of glycolysis measured as the flux from sucrose to CO_2, indicating that other processes consumed most of G6P formed by HK. It was hypothesized that futile recycling of G6P to glucose could occur. In support of this hypothesis, a hexose-P phosphatase previously described in plants was detected in transgenic potato roots and tracer evidence of the recycling of the glucose moiety of G6P to the carbohydrate pool (Claeyssen et al 2012). However, the possible catabolism of G6P by oxPPP remains to be investigated in this context.

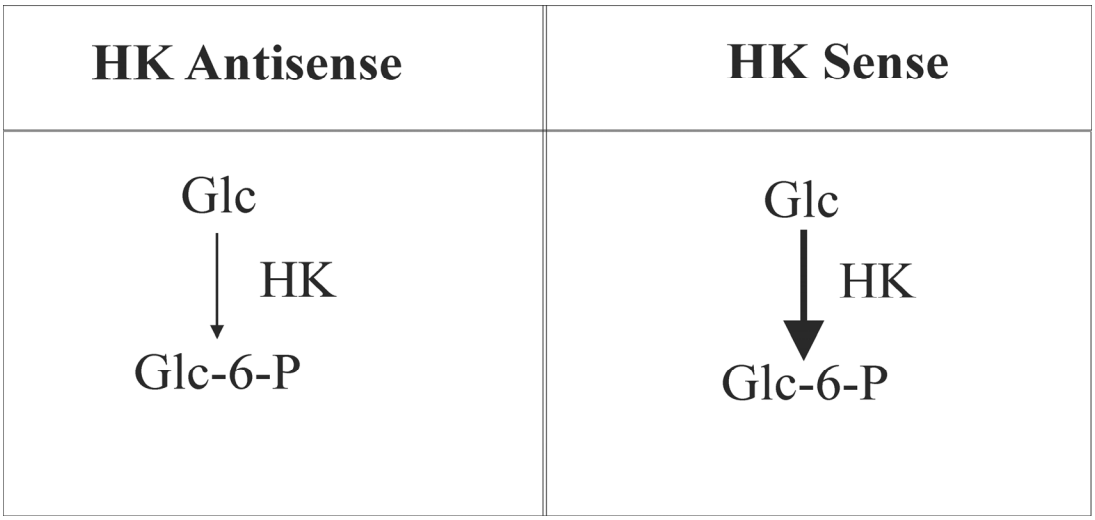

Figure 11. The phosphorylation of Glc by HK in sense and antisense HK potato roots (Claeyssen et al 2012). The higher thickness of the arrow in HK sense indicates a higher conversion rate of Glc to G6P in these clones.

1.5.2. Transgenic TPI roots

To examine the metabolic role of the cytosolic TPI enzyme, an antisense cTPI construct was generated. Using a cTPI cDNA from *S. chacoense* with 98.8% identity to *S. tuberosum* cTPI. The antisense construct was under the control of the CaMV35S promoter in the pGA643 plasmid. The transformation was done by *Agrobacterium rhizogenes*. An empty pGA643 plasmid was also transformed to potato in order to generate control clones. Among a population of 97 transgenic clones, three control (A1.5, A1.6 and A2.5) and two antisense (G1.8 and G3.10) clones were selected for in-depth metabolic characterization. Clones G1.8 and G3.10 had the lowest TPI activity in enzyme assays, approximately one third that of control clones, yet did not display any visual phenotypic difference from the control roots. TPI enzyme activity assays represent the sum of activities of plastidial and cytosolic isoforms, so to determine the contribution of the cytosolic isoform, anion exchange chromatography and immunoblot analyses with an antiserum specific for cTPI were performed. It was shown that the plastidial isoform was not affected by the antisense strategy while the cytosolic isoform was decreased by up to 90% in theG1.8 and G3.10 antisense clones.

To observe the effects of allowed cTPI activity on carbon distribution in transgenic roots, O_2 uptake as well as the content of soluble carbohydrate and starch pools, major organic acids, glycolytic intermediates, and free amino acids were measured. The decrease in cTPI in transgenic potato roots did not change the activity of other enzymes in the glycolysis and respiratory pathways. However, some glycolytic intermediates such as G6P and pyruvate, as well as fatty acids, and some amino acids that are produced from intermediates of the bottom part of glycolysis and TCA cycle accumulated in those transgenic roots with the greatest reduction in TPI activity. Total amino acid content, the total amount of N that is present as amino acids, and the Gln/Glu ratio were not changed in these roots while the amount of Glu and (Glu+Gln) were decreased (Figure 12).

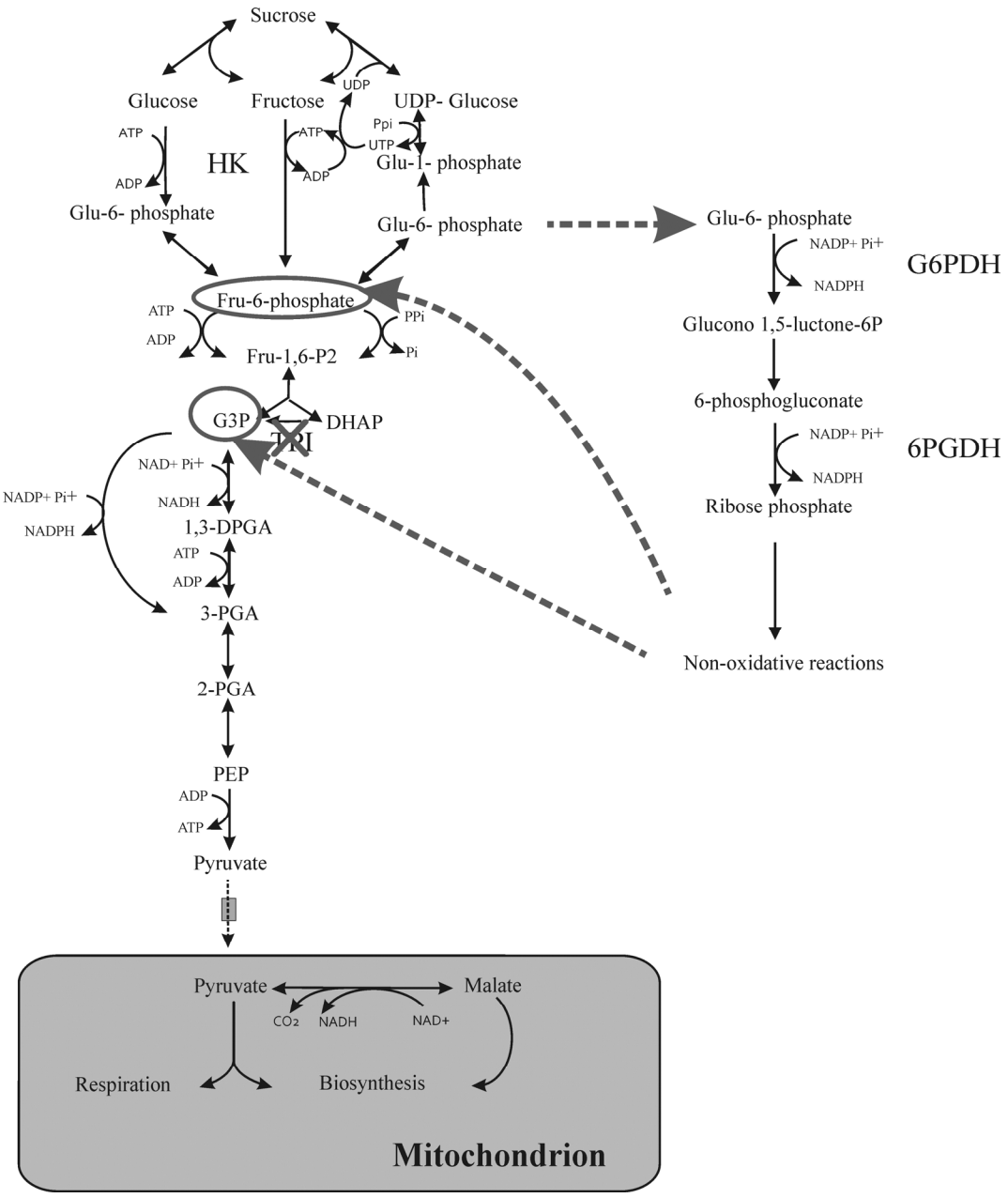

Figure 12. The bypass for C catabolism through PPP in transgenic roots with low cTPI. Scheme of metabolic events occurring in roots containing low levels of cTPI, according to results from (Dorion et al 2012). Dashed arrows represent the provision of G6P for oxPPP and consequently the provision of carbon skeleton for G3P and F6P by non–oxidative section of PPP in cTPI antisense roots.

Radioactive Glc labeling experiments indicated that the carbon flux through G6PDH and 6PGDH was increased in TPI antisense roots. The higher flow of C toward the oxPPP in

cTPI antisense clone as further supported using a metabolic modelization approach and a kinetic-metabolic model (Valancin et al 2012). It was concluded that GAP generated by the PPP might feed the latter stages of glycolysis and consequently the TCA cycle. It was hypothesized that the reducing power generated in oxPPP might be directly consumed via the NAD(P)H dehydrogenase in the inner membrane of mitochondria, increasing O_2 uptake rates (Dorion et al 2012, Valancin et al 2012). Therefore in cTPI antisense roots, increased flux through the PPP might provide the reducing power necessary for fatty acid synthesis and N assimilation that is used in amino acid production (Figure 12).

1.6. Hypotheses and goals of the present research project

As outlined above, changes in carbon metabolism occur in HK and TPI transgenic roots. The possibility thatG6P might be consumed by the PPP led us to investigate the function of the bypass through the PPP in both HK and TPI transgenic roots. This investigation was also expected to reveal if changes in the levels of HK and TPI could affect the capacity of the PPP and the mechanisms that are related to the function of oxPPP in roots. As the oxPPP is known to provide reducing power for N assimilation, we decided to investigate our transgenic roots growing under two nutritional N conditions. In order to test directly whether N provision affects the maximum catalytic capacity of the oxPPP, we opted to measure the specific activity of G6PDH isoforms and 6PGDH in roots grown in N sufficient and N deficient conditions. We predicted differences in root metabolism under these two N conditions that will be explained in more detail in the following section.

1.6.1. Working hypotheses for HK transgenic roots

As mentioned above, the overexpression of HK in HK sense clones caused an increase in the conversion of Glc to G6P in high N conditions, yet this increase did not impact on the rest of the glycolytic pathway. Both enzymatic and tracer evidence suggested that G6P formed by HK could be recycled to the carbohydrate pool (Claeyssen et al 2012), but the fate of G6P in the PPP was not investigated. The activity of the PPP could potentially consume G6P in roots containing higher amount of HK enzyme. Thus sense clones, if metabolism of G6P

occurs through this bypass, are predicted to have a higher flux of oxPPP even if the changes in HK enzyme do not affect the capacity of oxPPP (Table 1). In turn, by increasing the oxPPP flux, we should expect a greater capacity to generate the NADPH necessary for N assimilation (Table 1). Under high N conditions, the total amount of organic N (as amino acids) was unaffected in sense clones (Claeyssen et al 2012) (Table1). A possible explanation for this could be that the required carbon provision to support amino acid biosynthesis was not available in these clones because of a lack of increased C flux through the glycolytic pathway. We will compare and contrast cultures growing in high (7 mM) and low N (0.7 mM) conditions. These concentrations are more physiologically relevant than the concentration of total N in MS medium (100 mM) that was used in the previous study (Claeyssen et al 2012). In sense clones growing on low N, if the higher rate of Glc to G6P conversion is maintained, we expect more G6P will be supplied to the PPP than in control and HK antisense roots. In that case, HK overexpressors growing in low N condition may have a higher capacity to generate reducing power (Table 1) than control and antisense clones. It is also possible that the limitation of anaplerotic C supply for N assimilation is not as important in N limited compared to N sufficient conditions. Therefore, this hypothesis predicts that we should observe higher amounts of total N present as amino acids in sense clones compare to the controls in roots growing under N limitation (Table 1).

Table 1. The capacity of PPP and the amount of total N as amino acids in engineered HK roots.

HK	Results & Hypotheses	Hypotheses
	High N	**Low N**
Glucose→G6P	* As < S	* As < S
oxPPP capacity	? As = S	? As = S
oxPPP flux	* As < S	* As < S
Total N as amino acids	? As = S	? As < S

Note: The asterisk (*) indicates a postulate made to elaborate the hypothesis and the question mark (?) indicates a hypothesis that will be tested in this study.

1.6.2. Working hypotheses for TPI transgenic roots

In transgenic roots expressing low levels of cTPI growing in high N condition, the flux through the oxPPP was higher than in control clones where the capacity of oxPPP was unchanged (Dorion et al 2012) (Table 2). Therefore, we predict to observe the same capacity of oxPPP in antisense and control TPI clones under N deficient condition, although the manipulation of TPI might affect the flux of oxPPP in N deficient as well as N sufficient (Table 2). In these clones, more reducing equivalents were produced to sustain anabolism. However it was hypothesized that the provision of some intermediates, which are required for amino acid biosynthesis (anaplerotic C flux) and are generated below the TPI reaction, could have been limited in antisense clones. This situation could explain the observation that the same amount of total N as amino acids was present in control and antisense roots under high N conditions (Table 2). We postulate that under low N condition (0.7 mM), the anaplerotic C

flux is not limiting the assimilation of N. We therefore hypothesize that if the higher activity of the oxPPP is maintained in cTPI antisense growing under low N, we should observe a higher amount of total organic N in AS TPI in a situation where N is limiting but C is sufficient (Table 2).

Table 2. The capacity of PPP and the amount of total N as amino acids in engineered TPI roots.

TPI	**Results**	**Hypothesis**
	High N	**Low N**
oxPPP capacity	? As = Control	? As = Control
oxPPP flux	* As > Control	* As > Control
Total N as amino acids	? As = Control	? As > Control

Note: The asterisk (*) indicates a postulate made to elaborate the hypothesis and the question mark (?) indicates hypothesis that will be tested in this study.

2. Materials and methods

2.1. Chemicals and plant materials

All chemicals, buffers and commercial coupling enzymes were purchased from Fisher Scientific (Nepean, ON, Canada) or Sigma chemical Co. (St.-Louis, MO). GAP used in TPI assays was obtained by synthesis and was a generous gift from Sonia Dorion. Transgenic potato roots (*S. tuberosum*), previously established in the laboratory, were maintained as described (Claeyssen et al 2012, Dorion et al 2012). Potato cells were maintained in MS medium containing 30 g/L sucrose and 1 mg/L 2,4D on a gyratory shaker (145 rpm) at room temperature. Potato cells were subcultured every two weeks and harvested at 7 d by filtration over a Büchner Funnel using a 9 cm filter paper. Following filtration, cells were weighted, frozen in liquid nitrogen and stored at -80°C (Dorion & Rivoal 2003).

Young potato roots used for maintenance and amplification prior to liquid culture, were laid in the center of Petri plates containing 15 mL of solid MS medium, and left to grow in the dark. After 14 d, root clones were transferred in 250 mL Erlenmeyer flasks containing 30 mL of liquid MS medium with (0.7 mM) or (7 mM) of NH_4NO_3 as a source of N respectively for N deficient and sufficient treatments. The flasks were agitated continuously at 145 rpm on a gyratory shaker, at room temperature. Roots were harvested, precisely weighted in aliquots of 500 to 600 mg, frozen in liquid nitrogen and stored at -80°C until used.

2.2. Protein extraction from plant tissues, enzyme activity and protein assays

All steps were done at 4 °C. Potato roots were ground with a pestle and mortar using a ratio of 3:1 (mL extraction buffer/g fresh weight) in a buffer containing 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM ε-CA, 1 mM benzamidine, 5% (w/v) insoluble PVPP, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mg/ml leupeptin and 1 mM PMSF. Potato cell cultures were homogenized in the same buffer using a polytron as described previously

(Dorion & Rivoal 2003). Following extraction, the homogenates were centrifuged for 15 min at 16,500 x*g*. The clear supernatants were desalted on PD10 columns equilibrated with 50 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 1 mM EDTA. The desalted extract was used for all enzyme activity and protein measurements on a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). One unit (U) of enzyme activity corresponds to the disappearance of 1 µmol of substrate or the appearance of 1 µmol of product per minute. Enzyme activities were measured using direct or coupled enzyme assays based on the consumption or the production of NAD(P)H. The rate of absorbance change at 340 nm was followed by spectrophotometer for the initial 5 min at 30°C. Blank reaction rates were also measured in assay mixtures without substrate, and these were deducted from rates obtained in the presence of substrates. For all enzymes, the reactions were performed in 96-well microplates in a final volume of 200 µL. Protein concentration was measured according to (Bradford 1976), using BSA as standard and the Bio-Rad protein assay reagent (Bio-Rad, Mississauga, ON, Canada). The specific activity of the enzyme (the activity of an enzyme per mg of total protein) is reported hereafter in U mg^{-1} protein.

2.2.1. G6PDH assay

The G6PDH was measured as described by (Hauschild & von Schaewen 2003) in an assay buffer containing 100 mM Tris–HCl, pH 8.0, 2 mM glucose 6-phosphate, and 0.2 mM NADP. The G6PDH assay was measured based on the reduction of NADP to NADPH determined spectroscopically. In order to distinguish the contribution of the cytosolic and the plastidial isoenzymes, the activity was measured with or without a prior 10 min incubation under reducing conditions (62.5 mM DTT) for 10 min. The measurements of G6PDH activity were based on the calculation of G6PDH activity in the absence (total activity) and presence of DTT (cytosolic activity). The activity of plastidial isoform was total minus cytosolic (Hauschild & von Schaewen 2003). The specific activity of each isoform is the individual activity divided by the mass of total protein.

2.2.2. 6PGDH assay

The 6PGD assay was measured as described by (Simcox & Dennis 1978). A 20 µl aliquot of the desalted extract was added to 180 µl of assay buffer (50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 0.25 mM 6-phosphogluconate and 0.25 mM NADP). Blank reaction rates were measured without 6PG. The assay was measured based on NADPH production.

2.2.3. TPI assay

TPI was measured in the GAP to DHAP direction in a coupled enzyme assay with α-glycerophosphate dehydrogenase (α-GPDH, EC 1.1.1.8) as described (Dorion 2005). The assay mixture contained 0.1 M Tris–Cl pH 7.8, 1 mM GAP, 1 mM EDTA, 2.5 U/mL α-GPDH and 0.3 mM NADH. The disappearance of NADH was followed over time.

2.2.4. HK assay

HK activity assays were conducted as described (Claeyssen et al 2012) using Glc and Fru as substrates to assay respectively glucokinase (GK) and fructokinase (FK) activities. The HK reaction was coupled to the G6PDH reaction. For GK activity, the 200 µl reaction mixture contained 50 mM Tris–HCl, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 0.3 mM NAD^+, 1 mM ATP, 1.4 U/mL commercial G6PDH and 5 mM Glc. For FK, the 200 µl reaction mixture contained 50 mM Tris–HCl, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 0.3 mM NAD^+, 1 mM ATP, 1.4 U/mL commercial G6PDH, 5 mM Fru and 3.5 U/mL phosphoglucose isomerase (PGI, EC 5.3.1.9).

2.3. Extraction and analysis of amino acid pools

For HPLC analysis, aliquots of 500 to 600 mg FW transgenic roots material grown for 7 days in the presence of low or high N were harvested and frozen in liquid nitrogen. Frozen roots were extracted in 10 mL of 80% (v/v) ethanol at 75°C using a Büchi Rotovap. Extracts were dried and then resolubilized in 3 mL H_2O. Solubilized extracts were fractionated by ion exchange on 3 ml columns of Dowex AG 50-X8 (H^+) and Dowex AG 1-X8 (formate)

arranged in tandem (Claeyssen et al 2012, Rivoal & Hanson 1993). Sugars, organic acids and amino acids were eluted respectively by 20 mL water (neutral fraction), 15 mL8 N formic acid (anionic fraction) and 15 mL1 M NH_4OH (cationic fraction). Recovered fractions were stored at -20°C until analyzed. One ml aliquot of the basic fractions (containing the amino acids) was evaporated overnight in a speed vac centrifugal concentrator for HPLC analysis of free amino acids. Samples were derivatized with the AccQ reagent and separated on an AccQ·Tag column according to the manufacturer's indications. HPLC samples were analyzed using the AccQ reagent (Waters) according to the manufacturer's instructions. The HPLC system was controlled by the Empower Pro software and was equipped with a 600 controller, a 717 plus refrigerated automatic sample injector and a 2996 Diode Array Detector. The quantification was done by use of standard curves, which were generated using commercial amino acid standards. Recovery of external amino acid standards was 99.9%. Results were corrected for recovery.

2.4. Statistical analyses

All data presented are based on the analysis of 3 to 10 biological replicates and each biological replicate routinely represents three technical replicates. To evaluate significant differences between treatments, we used the Student *t* test tool available in the Sigma Plot 8.0 package. Correlation studies between series of experimental data were made by calculating the Pearson correlation coefficient with Microsoft Excel.

3. Results

3.1. Stability of G6PDH and 6PGDH after extraction

In order to investigate the maximal capacity oxPPP for providing reducing equivalents and the possible subsequent effects on nitrogen assimilation, we needed to measure the specific activities of G6PDH and 6PGDH by spectrophotometry. The extraction and assay conditions were first optimized using cell cultures, a material that can easily be generated in large quantities and similar in metabolism to roots since it is non photosynthetic and grows in MS medium. Since the assay of G6PDH involves dilution in a medium in the presence and absence of DTT, we determined the influence of time after extraction and/or dilution on enzymatic stability/activity. The stability of the enzymes was assessed by activity measurement immediately after extraction and 2 and 4 hours after extraction using a new dilution for each time point (Figure 13). The cytosolic G6PDH and 6PGDH specific activities were stable for up to 4 hours after extraction but the activity of the plastidial isoform declined rapidly after extraction (Figure 13). Following extraction, the activity of enzymes was also measured after 0, 30 and 60 min of dilution (Figure 14). Again cytosolic G6PDH and 6PGDH, were stable up to 60 min after dilution. (Figure 14 A, C). The activity of the plastidial G6PDH isoform, however, decreased after dilution (Figure 14 B). These experiments served as guidelines for experiments carried out on root extracts. Assays for G6PDH and 6PGDH were done within 5 min after recovery of supernatant in order to avoid any loss of activity.

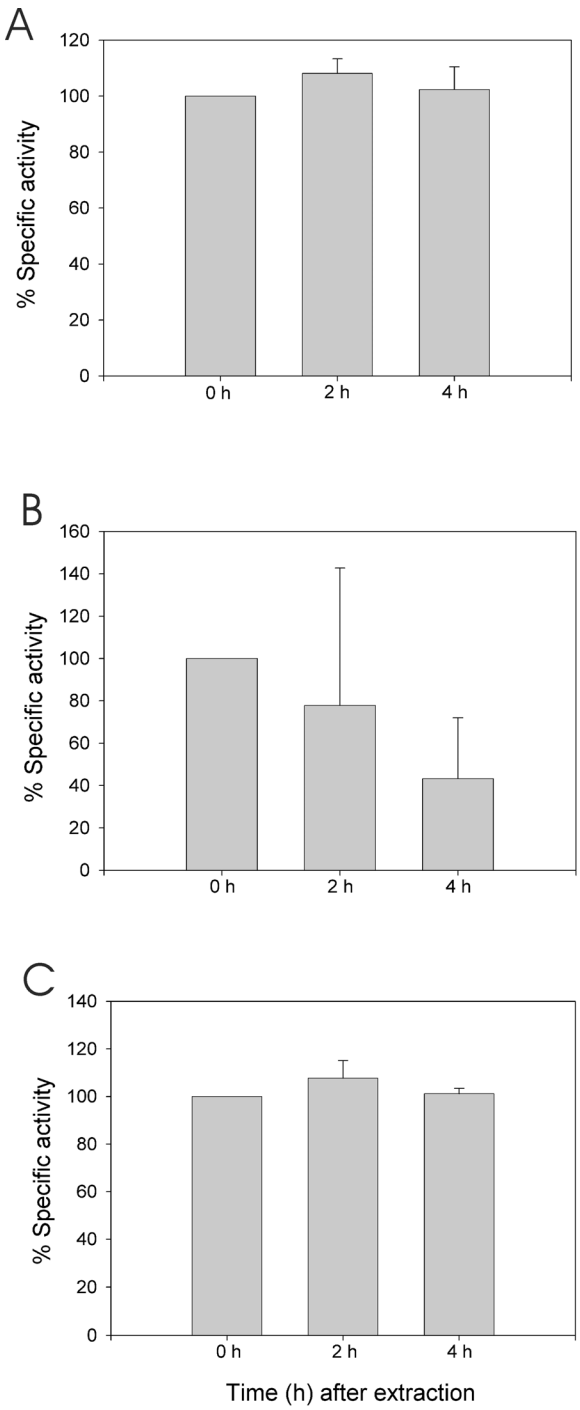

Figure 13. The stability of G6PDH and 6PGDH activities after extraction. The specific activity of the enzymes was measured after 0, 2 and 4 h of extraction and immediately after dilution. A, Cytosolic G6PDH, B, Plastidial G6PDH; C, 6PGDH assayed after 0, 2 and 4 h of extraction.

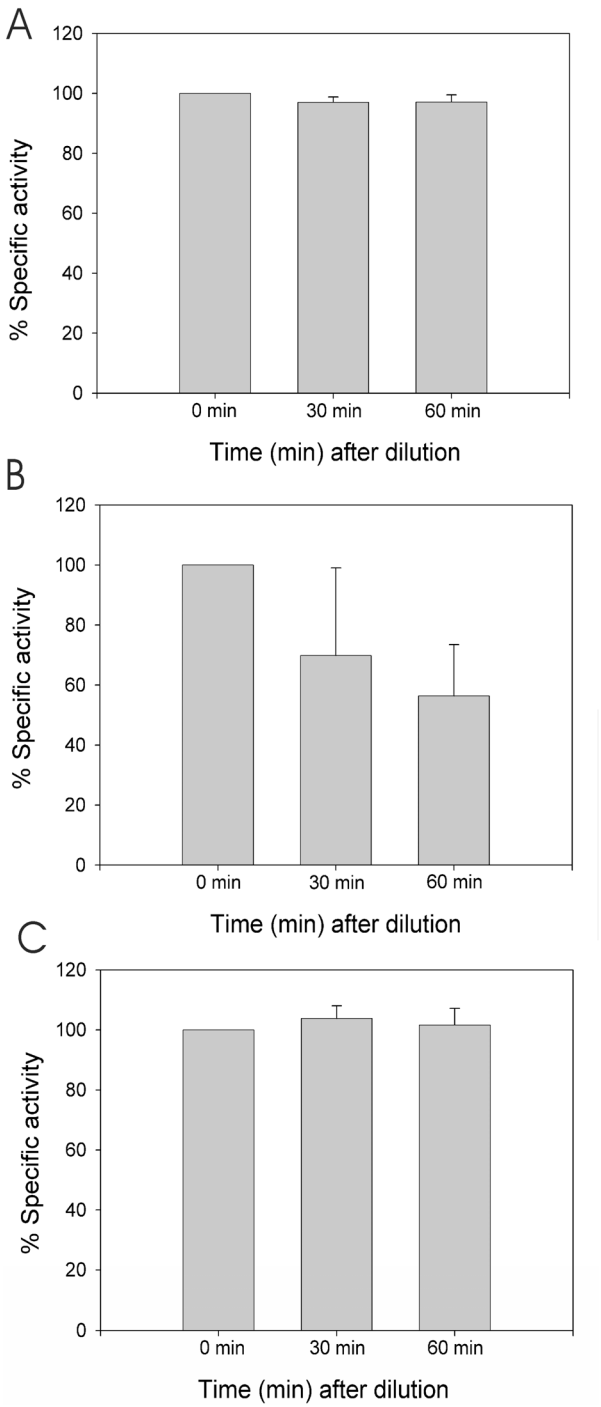

Figure 14. The stability of G6PDH and 6PGDH activities upon dilution. After extraction, the specific activity of the enzyme was measured after 0, 30 and 60 min of dilution. A, Cytosolic G6PDH assay; B, Plastidial G6PDH; C, 6PGDH specific activity after 0, 30 and 60 min of dilution.

3.2. Nitrogen treatment

It is known that the oxPPP is induced by N availability in different systems (Redinbaugh & Campbell 1998). Although the first step of nitrogen assimilation, nitrate reduction to ammonium, takes place in the cytosol, the subsequent reactions occur in plastids. The reducing power required for plastidial nitrogen assimilation is generated within the same compartment where it will be used. The plastid envelopes are usually considered as impermeable to NAD and NADP. The inner membrane restricts the passage of molecules between the cytosol and the interior of the organelle. The transfer of the reducing equivalents may be mediated in directly through a carrier in non-photosynthetic tissues, it is thought that nitrogen assimilation is dependent on the oxPPP for reducing power. Since the MS medium used in our experiments is very rich in N and because data on oxPPP regulation in this medium are scarce, we performed preliminary tests to determine the conditions under which nitrogen provision in MS medium would affect the activity of the enzymes oxPPP. We measured the specific activities of G6PDH isoforms and 6PGDH in cells grown in MS medium adjusted to different N concentrations. These preliminary experiments were designed to evaluate the appropriate and physiologically relevant concentrations for N sufficient and N deficient conditions on *in vitro* grown cultures. The activity of G6PDH and 6PGDH were therefore evaluated in cells growing on media containing 0.7 mM, 7.7 mM, 101 mM and 107 mM of ammonium dihydrogen phosphate $(NH_4^+)H_2PO_4$ and potassium dihydrogen phosphate $(K^+)H_2PO_4$ as a source of N in cell medium (Figure 15). We used the mixture of $(NH_4^+)H_2PO_4$ and $(K^+)H_2PO_4$ to vary the amount of N with the same amount of P. The activity of the plastidial G6PDH isoform was higher in 101 and 107 mM compared to N concentrations of 0.7 and 7.7 mM (Figure 15 B) while the cytosolic and 6PGDH were not affected (Figure 15 A, C). Plastidial isoform was not detectable in 0.7 mM N due to a lack of sizable activity. None of the enzymes had significantly different activities between 101 mM and 107 mM concentration of N (Figure 15). N concentrations in the 100 mM range are representative of what is normally found in typical MS medium. However, such a concentration range is not physiologically relevant for plant N nutrition since generally plant growth will fall under a N supply above 10 mM (Sánchez et al 2004). The activity of both G6PDH isoforms was significantly higher in 7.7 mM concentration of N compared to the concentration of 0.7 mM

(Figure 15). Hence, the concentration of 0.7 mM of N for N deficient and 7 mM for N sufficient condition were used for cell and root cultures.

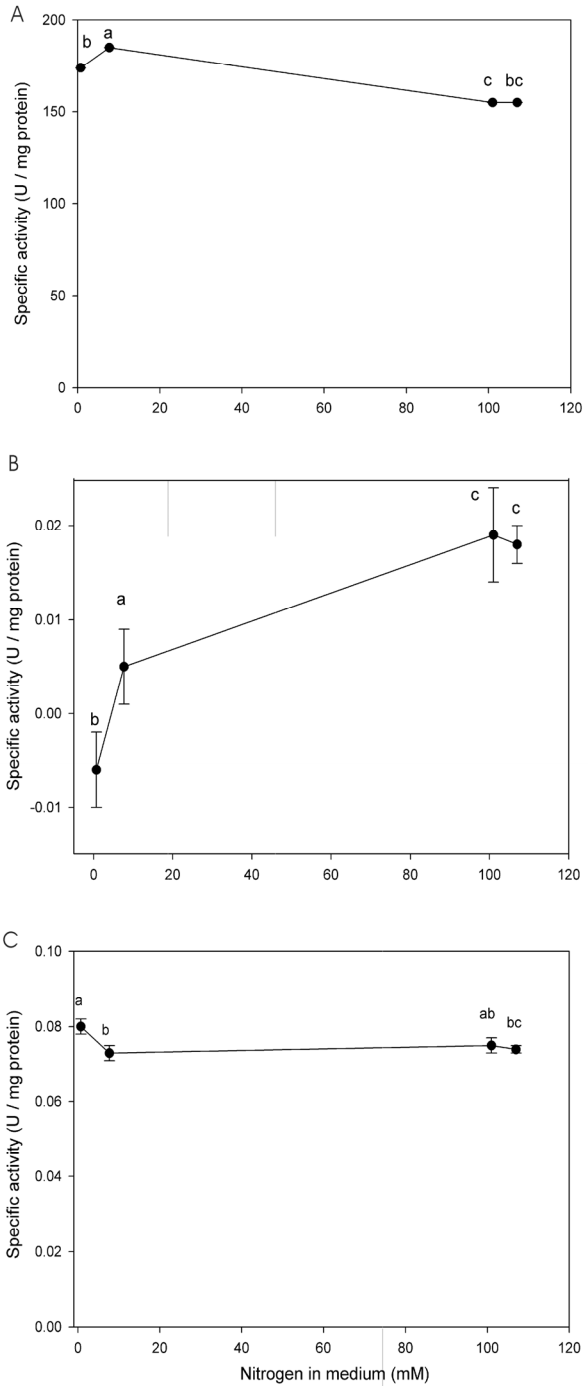

Figure 15. Specific activities of the enzymes in 7 d old cells grown in different N concentrations. A, Cytosolic G6PDH; B, Plastidial G6PDH; C, 6PGDH. Data points marked with different letters (a, b and c) are statistically different (Student *t* test, $P>0.05$).

3.3. Enzyme activities in HK transgenic roots

3.3.1. G6PGD and 6PGDH

The activity of the oxPPP enzymes in HK transgenic roots was surveyed in one antisense clone (As213), two control clones (CTL2, CTL3) and two antisense clones (S111, S101). As seen below, the activity of cytosolic G6PDH and 6PGDH were significantly higher in N sufficient compared to N-limited conditions (Figure 16 and 17). The activity of the plastidial isoform was not detectable (not significantly different from 0). We did not observe any significant difference between antisense, control and sense HK clones in either N sufficient or in N deficient conditions.

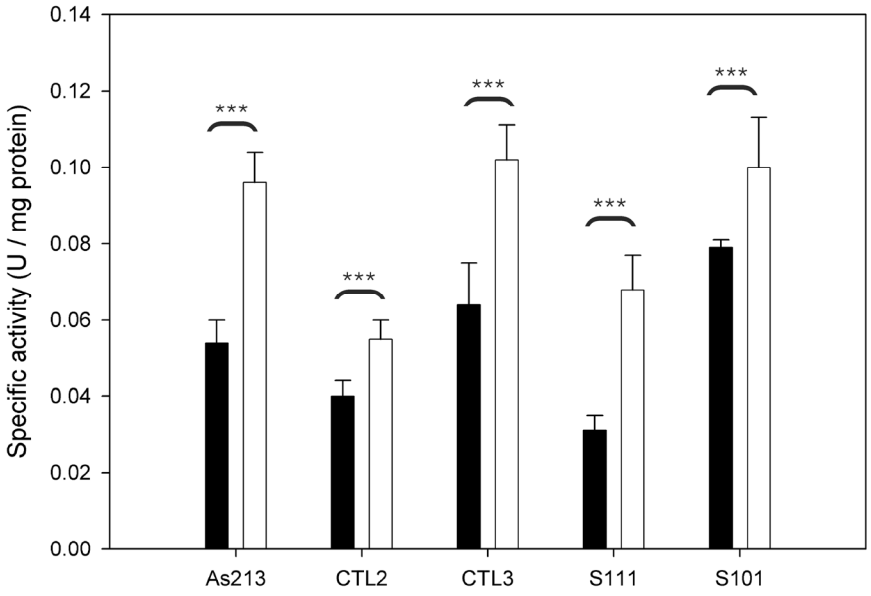

Figure 16. Specific activity of cytosolic G6PDH in HK transgenic roots. Seven days old antisense (As213), control (CTL2, CTL3) and sense (S111, S101) HK transgenic roots grown in different N conditions. Each bar represents 7 to 9 biological replicates. Black bars represent low N and white bars represent high N conditions. A bracket with three stars indicates that the difference between the two treatments is significant (Student *t* test, $P<0.05$).

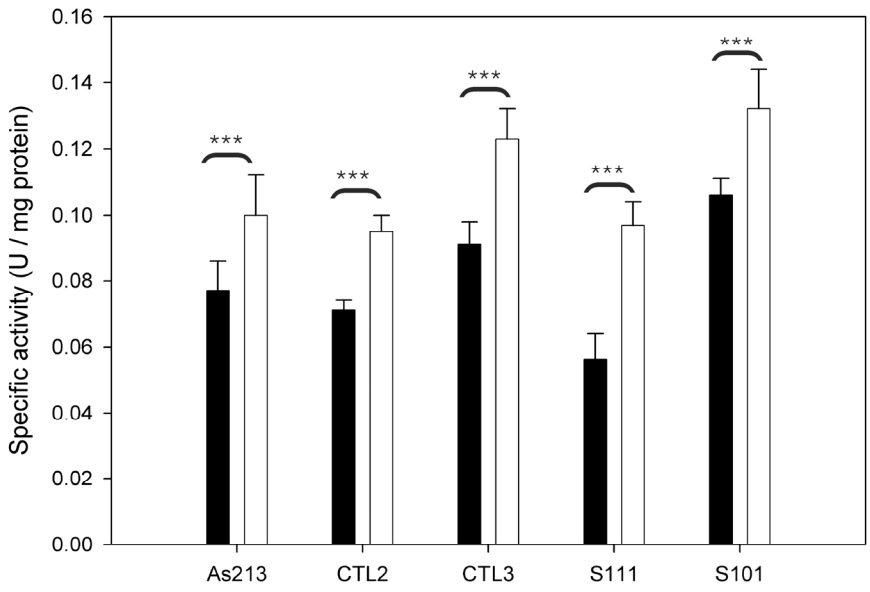

Figure 17. Specific activity of 6PGDH in HK transgenic roots. Seven days old antisense (As213), control (CTL2, CTL3) and sense (S111, S101) HK transgenic roots grown in different N conditions. Each bar represents 7 to 9 biological replicates. Black bars represent low N and white bars represent high N conditions. A bracket with three stars indicates that the difference between the two treatments is significant (Student *t* test, $P<0.05$).

3.3.2. HK

The activity of HK was assayed with two different substrates, Glc and Fru in HK transgenic roots (Figure 18). As expected, in HK sense clones, the activity of HK was significantly higher than in control and antisense roots. Interestingly, the higher activity of GK and FK enzymes were found in sense clones in N deficient condition (Figure 18). As the measured FK activities include the activity of HK with fructose substrate and also the activity of FK enzyme families, the levels of FK activity were higher than GK in all clones (Figure 18).

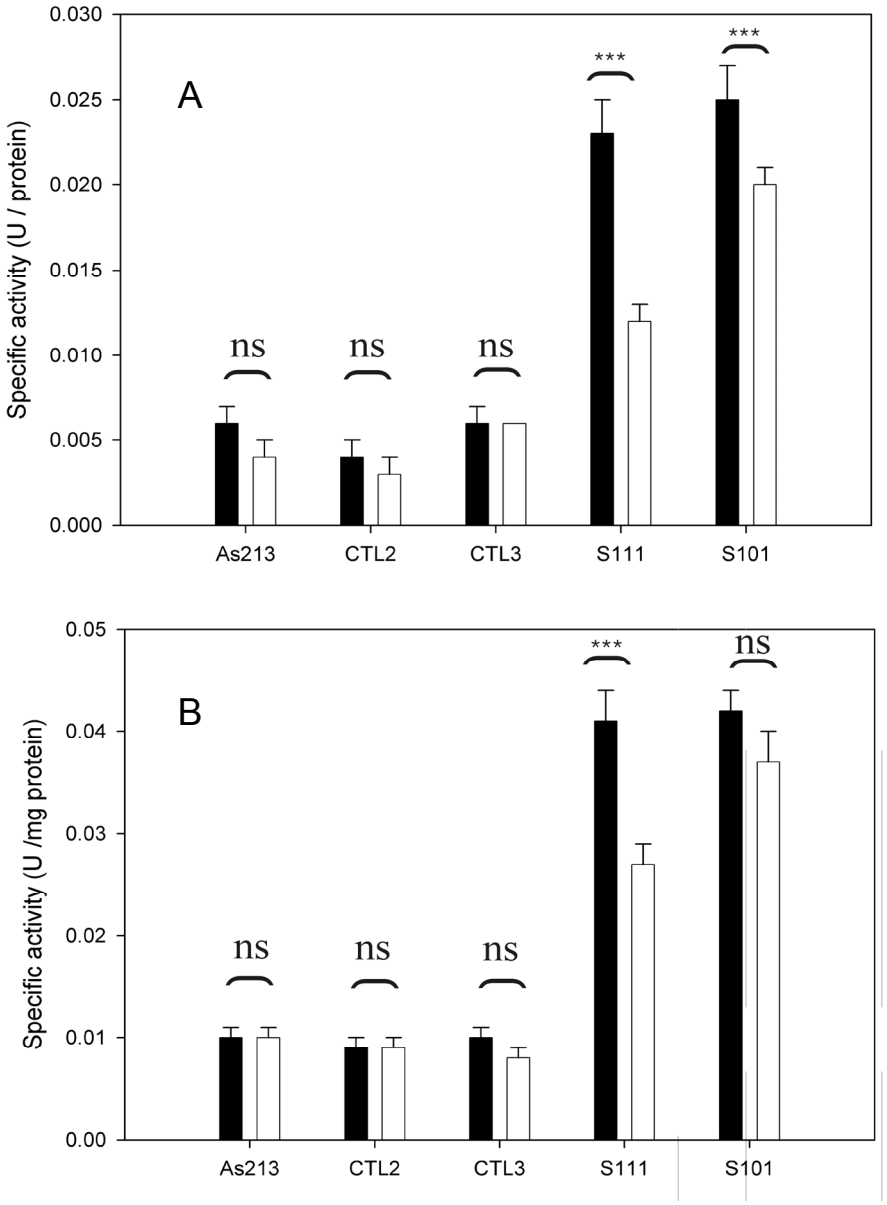

Figure 18. Specific activities of GK (A) and FK (B) in HK transgenic roots. Seven days old antisense (As213), control (CTL2, CTL3) and sense (S111, S101) HK transgenic roots grown in different N conditions. Each bar represents 7 to 9 biological replicates. Black bars represent low N and white bars represent high N conditions. A bracket with three stars indicates that the difference between the two treatments is significant (Student *t* test, $P<0.05$). A bracket with "ns" indicates that the difference between the two treatments is not significant (Student *t* test, $P>0.05$).

3.4. Enzyme activities in cTPI transgenic roots

3.4.1. G6PDH and 6PGDH enzymes

The activity of 6PGDH and G6PDH isoforms was measured in two antisense (G1.8 and G3.10) and three control (A1.5, A2.5 and A1.6) root clones (Figures 19 and 20). The activity of cytosolic G6PDH was significantly different between N deficient and N sufficient conditions in all clones. The specific activity of cytosolic G6PDH was higher in the presence of high N (Figure 19). The plastidial activity was not detectable. The 6PGDH activity was also significantly higher in N deficient condition in all cTPI transgenic clones (Figure 20).

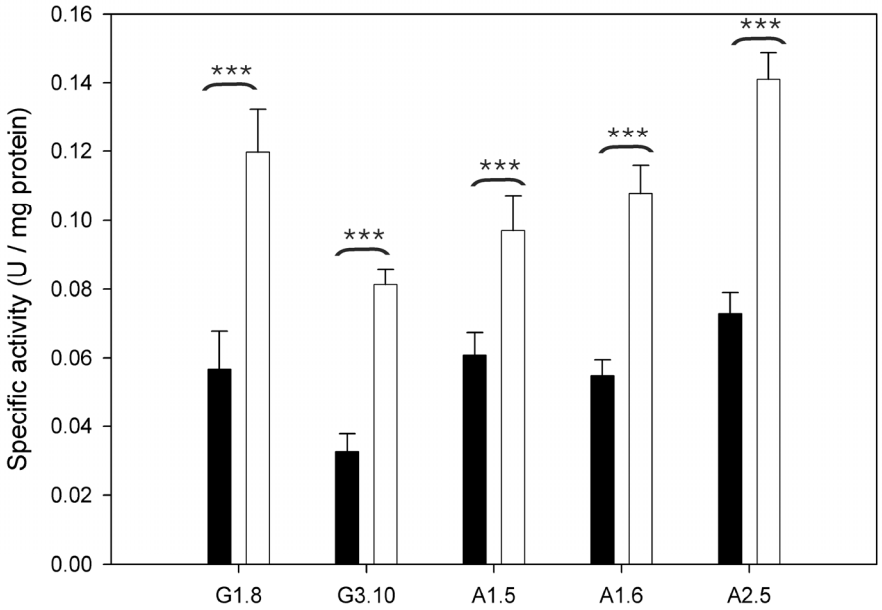

Figure 19. Specific activities of the cytosolic G6PDH in TPI transgenic roots. Seven days old antisense (G1.8, G3.10) and control (A1.5, A2.5, A1.6) TPI transgenic roots grown in different N conditions. Each bar represents 7 to 9 biological replicates. Black bars represent low N and white bars represent high N conditions. A bracket with three stars indicates that the difference between the two treatments is significant (Student *t* test, $P<0.05$).

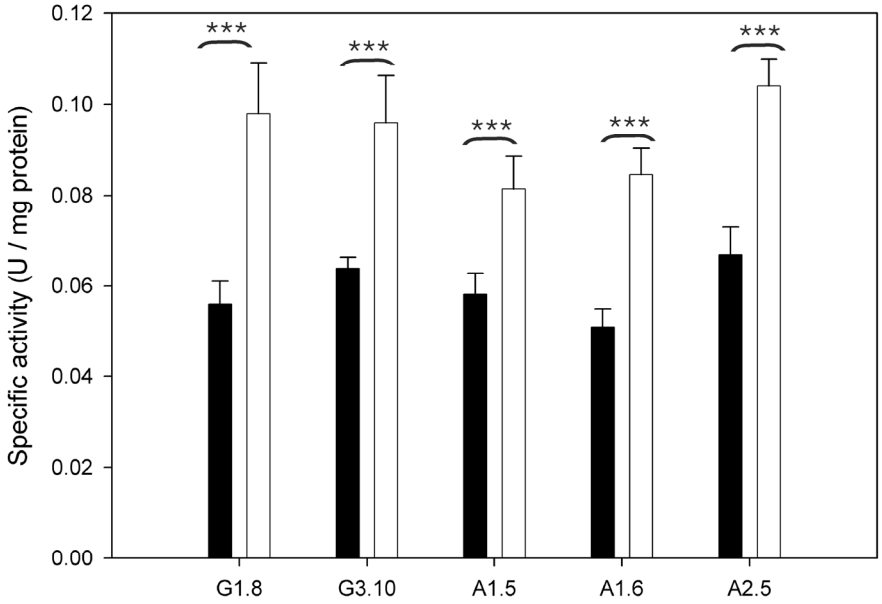

Figure 20. Specific activities of 6PGDH in TPI transgenic roots. Seven days old antisense (G1.8, G3.10) and control (A1.5, A2.5, A1.6) TPI transgenic roots grown in different N conditions. Each bar represents 7 to 9 biological replicates. Black bars represent low N and white bars represent high N conditions. A bracket with three stars indicates that the difference between the two treatments is significant (Student *t* test, $P<0.05$).

3.4.2. TPI enzyme

The activity of TPI enzyme in cTPI transgenic roots was slightly increased in N sufficient condition but except for A1.6 clone, the difference between N sufficient and N deficient was not significant (Figure 21).

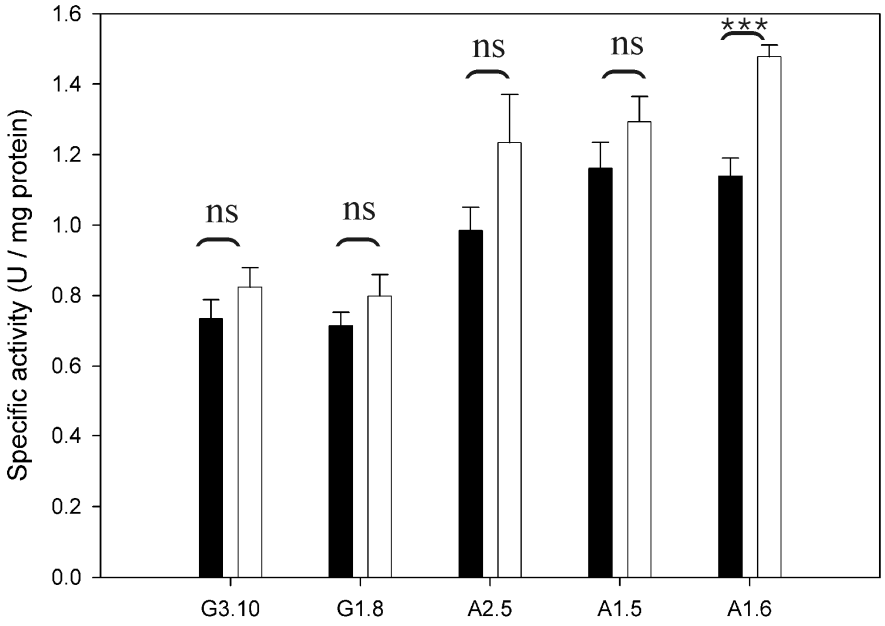

Figure 21. Specific activities of TPI enzyme in cTPI transgenic roots. Seven days old antisense (G1.8, G3.10) and control (A1.5, A2.5, A1.6) TPI transgenic roots grown in different N conditions. Each bar represents 7 to 9 biological replicates. Black bars represent low N and white bars represent high N conditions. A bracket with three stars indicates that the difference between the two treatments is significant (Student *t* test, $P<0.05$). A bracket with "ns" indicates that the difference between the two treatments is not significant (Student *t* test, $P>0.05$).

3.5. Correlations between the activity of the oxPPP enzymes and the levels of TPI or GK in transgenic roots

Using the above results, the activities of the oxPPP enzymes are plotted against that of TPI and GK in the transgenic clones (figure 22). We also performed an analysis of the Pearson correlation coefficient to evaluate the correlation between GK or TPI expression level and G6PDH or 6PGDH activity (Table III). As HK activities measured with fructose as substrate include the activity of HK and also the activity of the true FK enzyme family, GK is a better representative of HK activity. The specific activities of cytosolic G6PDH and 6PGDH were positively correlated with that of GK but that correlation was not strong for 6PGDH (Table III). In high N conditions, a positive correlation was found between the specific activities of

oxPPP enzymes and GK. The specific activity of oxPPP enzymes was highly correlated with TPI enzyme ($R^2 > 0.7$) in both N sufficient and N deficient conditions (Table III).

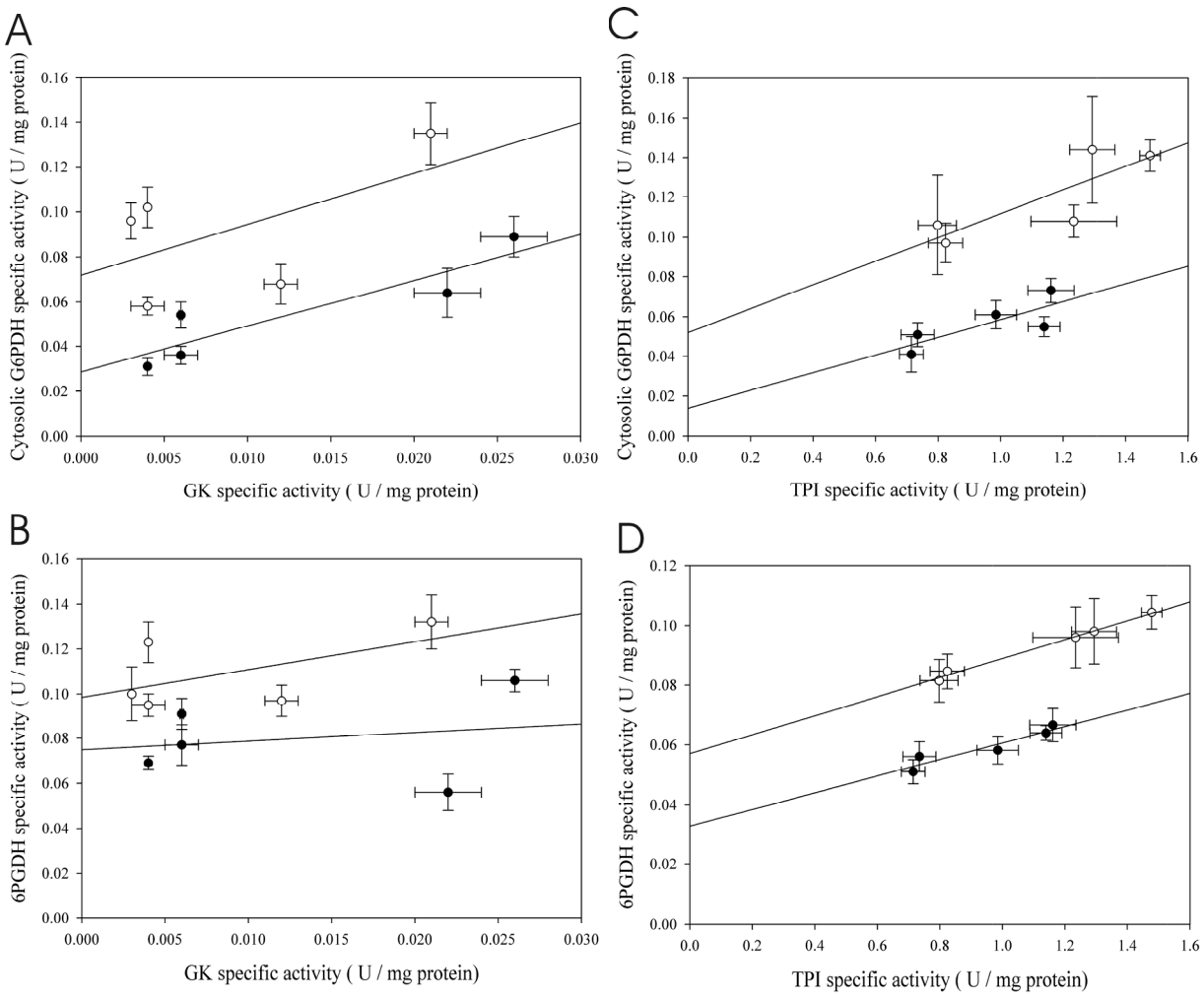

Figure 22. Correlation between oxPPP and GK or TPI specific activity. A, cytosolic G6PDH and GK specific activity; B, 6PGDH and GK; C, cytosolic G6PDH and TPI, D; 6PGDH and TPI specific activity. Each dot represents the mean of enzyme specific activities in a clone. Black symbols represent low N and white symbols represent high N conditions.

Table 3. Summary of the observed correlations between OxPPP enzymes and GK or TPI specific activities. Correlation between these parameters was assessed by calculating the Pearson correlation coefficient.

	Low N		High N	
Enzymes	G6PDH	6PGDH	G6PDH	6PGDH
GK	0.910	0.205	0.614	0.562
TPI	0.804	0.943	0.828	0.996

3.6. Analysis of free amino acid pools in HK transgenic roots

Glycolysis and respiratory pathways provides carbon skeletons and reducing equivalents for nitrogen assimilation and amino acid biosynthesis. The influence of HK manipulation and N provision on nitrogen assimilation was investigated. The level of free amino acid pools was measured in HK transgenic clones in both N deficient (Figure 23) and N sufficient (Figure 24) conditions.

In N deficient conditions, the variation of free amino acids between antisense, control and sense clones was unremarkable. Only Glu, 4-aminobutyrate and leucine correlated slightly and negatively with HK activity (Figure 23 a, d, h). Proline increased slightly with HK activity (Figure 23 a). In N sufficient conditions, the levels of most of the free amino acids increased compared to N deficient conditions (Figure 24). In particular, the Gln content increased approximately eight times in N sufficient than N deficient condition (figure 24, b). However, the different amino acid pools were not different between HK clones, similar to what was observed for N deficient conditions.

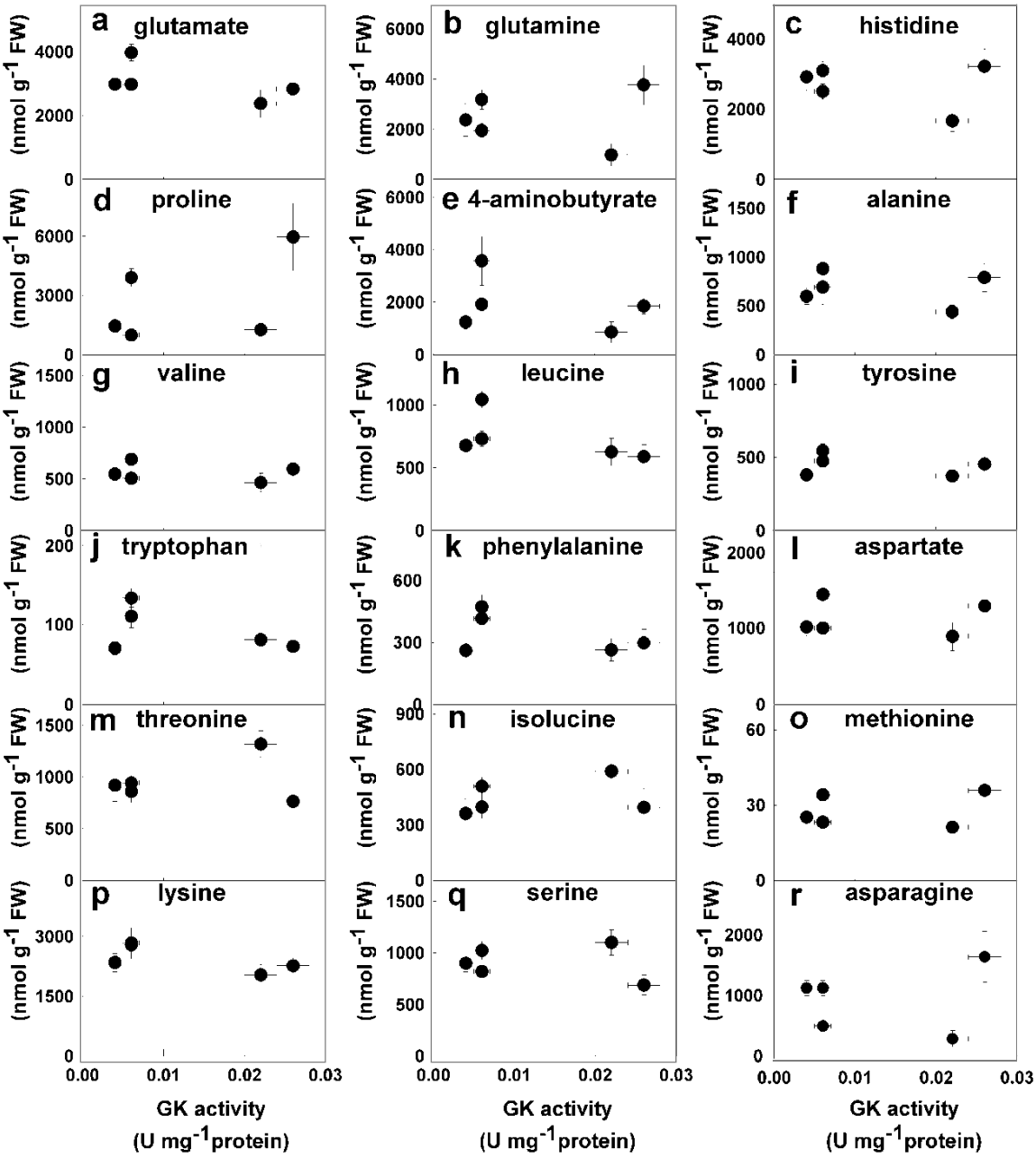

Figure 23. Levels of free amino acids in HK transgenic roots in N deficient condition. Metabolites were assayed by HPLC. The amino acid contents are plotted as a function of HK activity. Each dot represents the mean of 5 biological replicates.

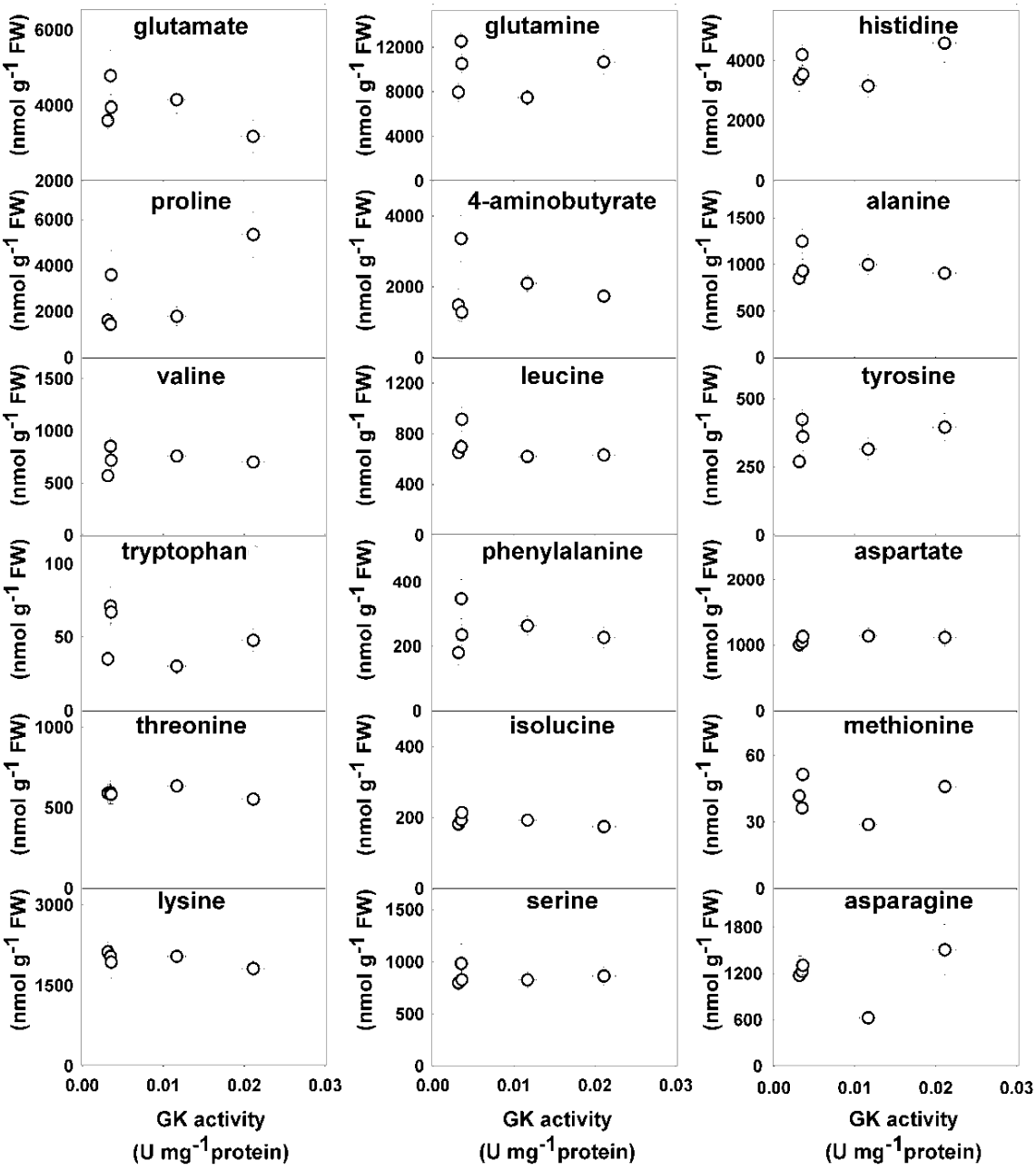

Figure 24. Levels of free amino acids in HK transgenic roots in N sufficient condition. Metabolites were assayed by HPLC. The amino acid contents are plotted as a function of HK activity. Each dot represents the mean of 5 biological replicates.

To have a better understanding of amino acid contents, the data represented in figures 23 and 24 were used for several other analyses. The total free amino acids and also the total N present as free amino acids were calculated in both N treatment (Figures 25, 26).

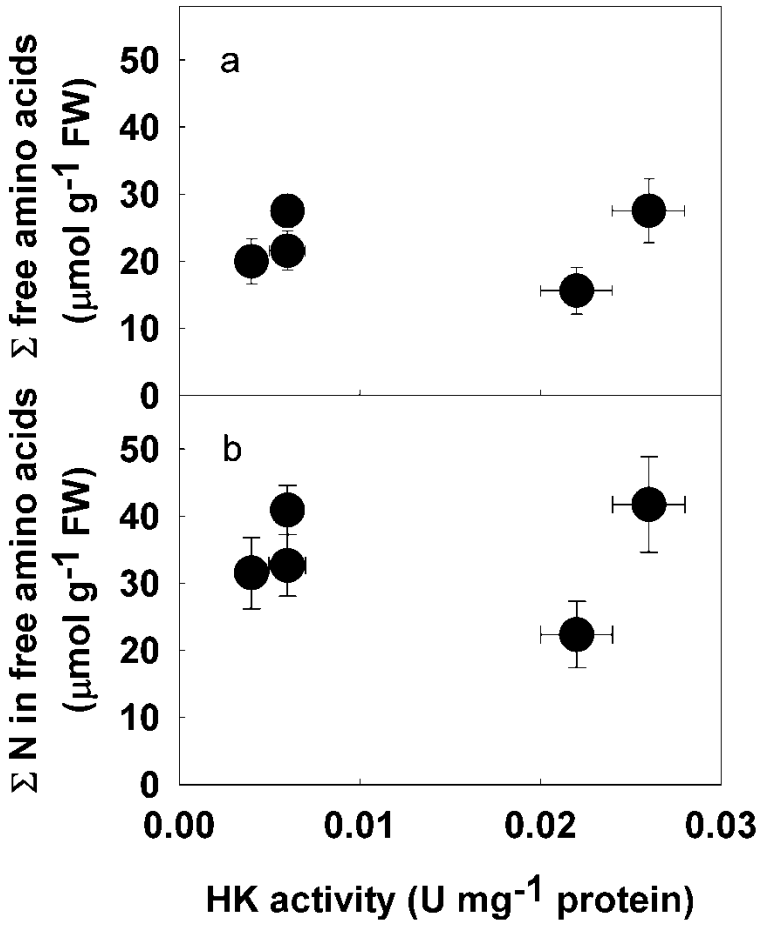

Figure 25. Sum of all free amino acids (a) and N present in amino acids (b) in HK transgenic roots in N deficient conditions. Metabolites were assayed by HPLC. The amino acid contents were calculated from data present in figure 23. Each dot represents mean ± SE (n = 5).

The total free amino acids as well as total N as free amino acids were stable among the different HK clones (Figure 25). Similar results were obtained for N sufficient condition (Figure 26).

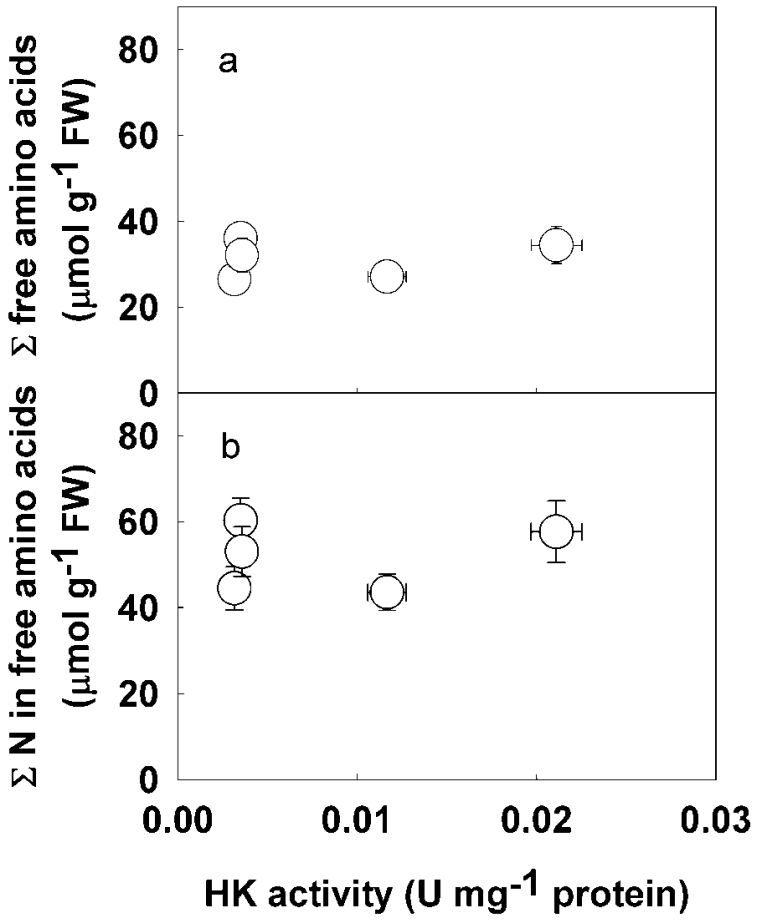

Figure 26. Sum of all free amino acids (a) and N present in amino acids (b) in HK transgenic roots in N sufficient condition. Metabolites were assayed by HPLC. The amino acid contents were calculated from data present in figure 24. Each dot represents mean ± SE (n = 5).

The Gln/Glu ratio as well as Asn/Asp ratio did not vary between antisense, control and sense clones in either N deficient (Figure 27, 28 a) or in N sufficient conditions (Figure 27, 28 b).

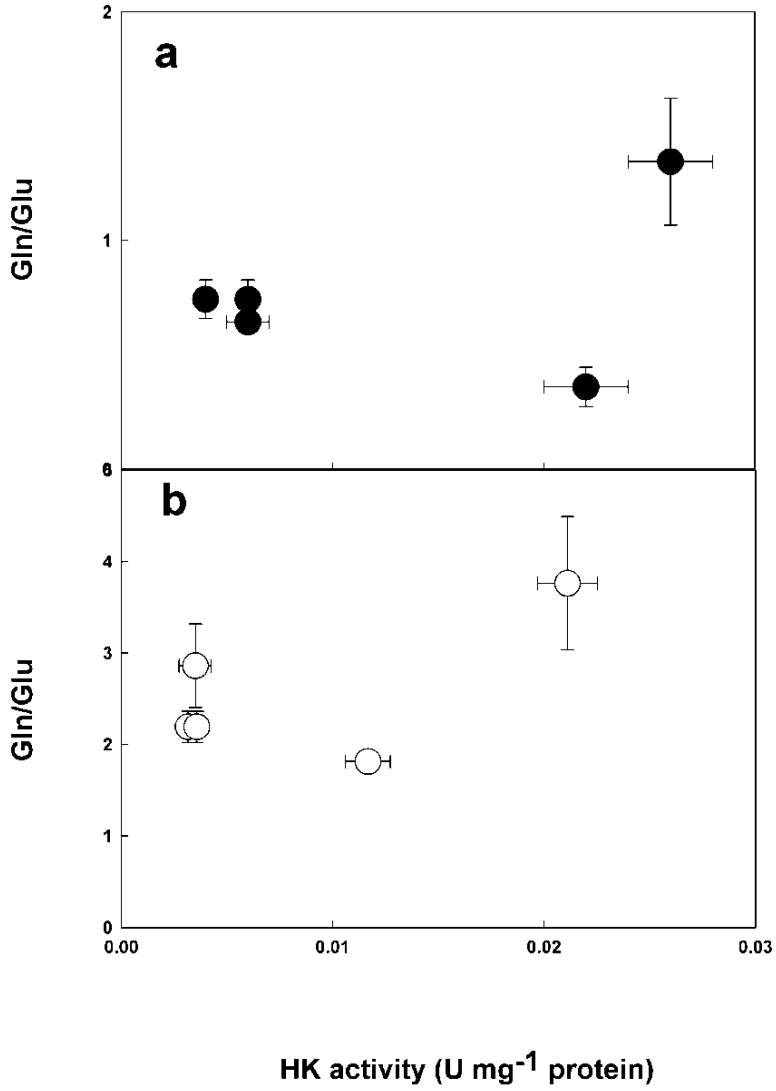

Figure 27. Interaction between the pools of Glu and Gln in transgenic HK roots. Data for Glu/Gln in N deficient (a) and in N sufficient (b) were calculated from data present in figure 23 and 24. Each dot represents mean ± SE (n = 5). Symbols are used: white circles; high N condition, black circles; low N condition.

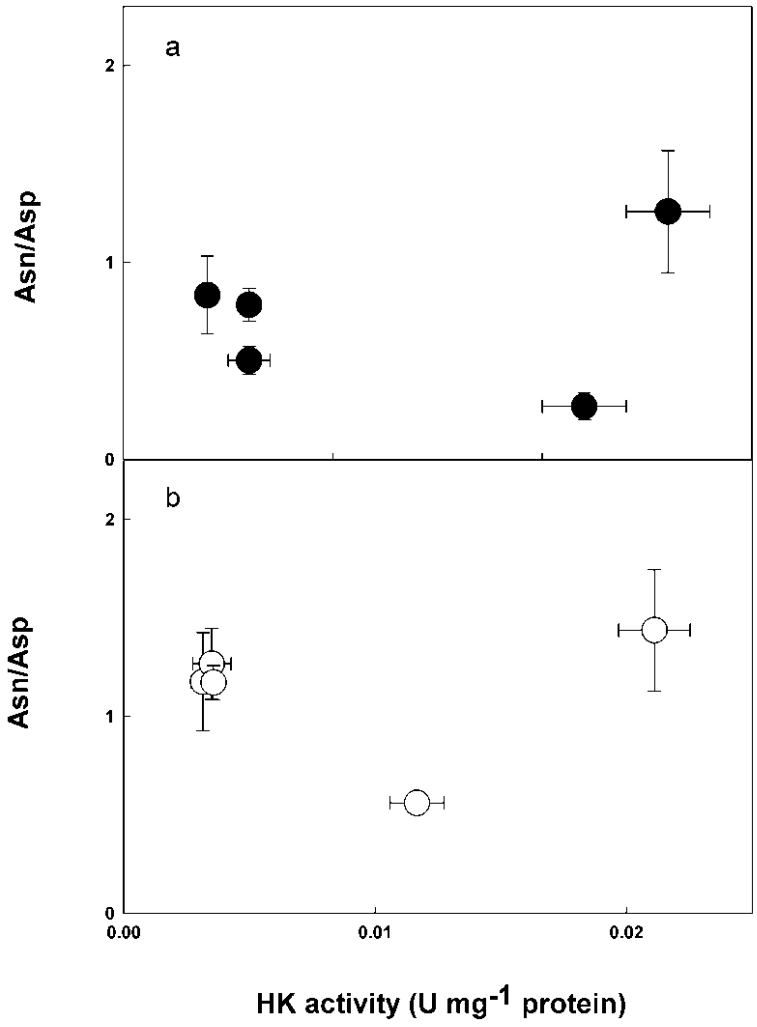

Figure 28. Interaction between the pools of Asn and Asp in transgenic HK roots. Data for Asn/Asp in N deficient (a) and in N sufficient (b) were calculated from data present in figure 23 and 24. Each dot represents mean ± SE (n = 5). Symbols are used: white circles; high N condition, black circles; low N condition.

3.7. Analysis of free amino acid pools in TPI transgenic roots

The same analyses were done for TPI transgenic roots in N sufficient and N deficient treatments. First the free amino acid pools were quantified by HPLC to see the affect of TPI manipulation on the distribution of amino acids in TPI antisense and control clones.

In the N deficient treatment (Figure 29), the levels of Gln, 4-aminobutyrate and alanine were slightly negatively correlated with TPI activity. Asn negatively correlated with TPI activity more strongly. Glu, Asp and proline, and to a much lesser extent, valine, tryptophan, lysine, phenylalanine, and isoleucine seem to be positively correlated with TPI activity. For serine, methionine, leucine, histidine and threonine no significant trend was detected.

In the N sufficient condition, the amount of most amino acids was higher than under N deficient condition across the TPI population (Figure 30). As with HK transgenic roots, the amount of Gln was more than eight times elevated in high N condition. Glu and 4-aminobutyrate levels were stable regardless of TPI activity. The trend of other amino acids was almost similar to what we observed in N deficient condition.

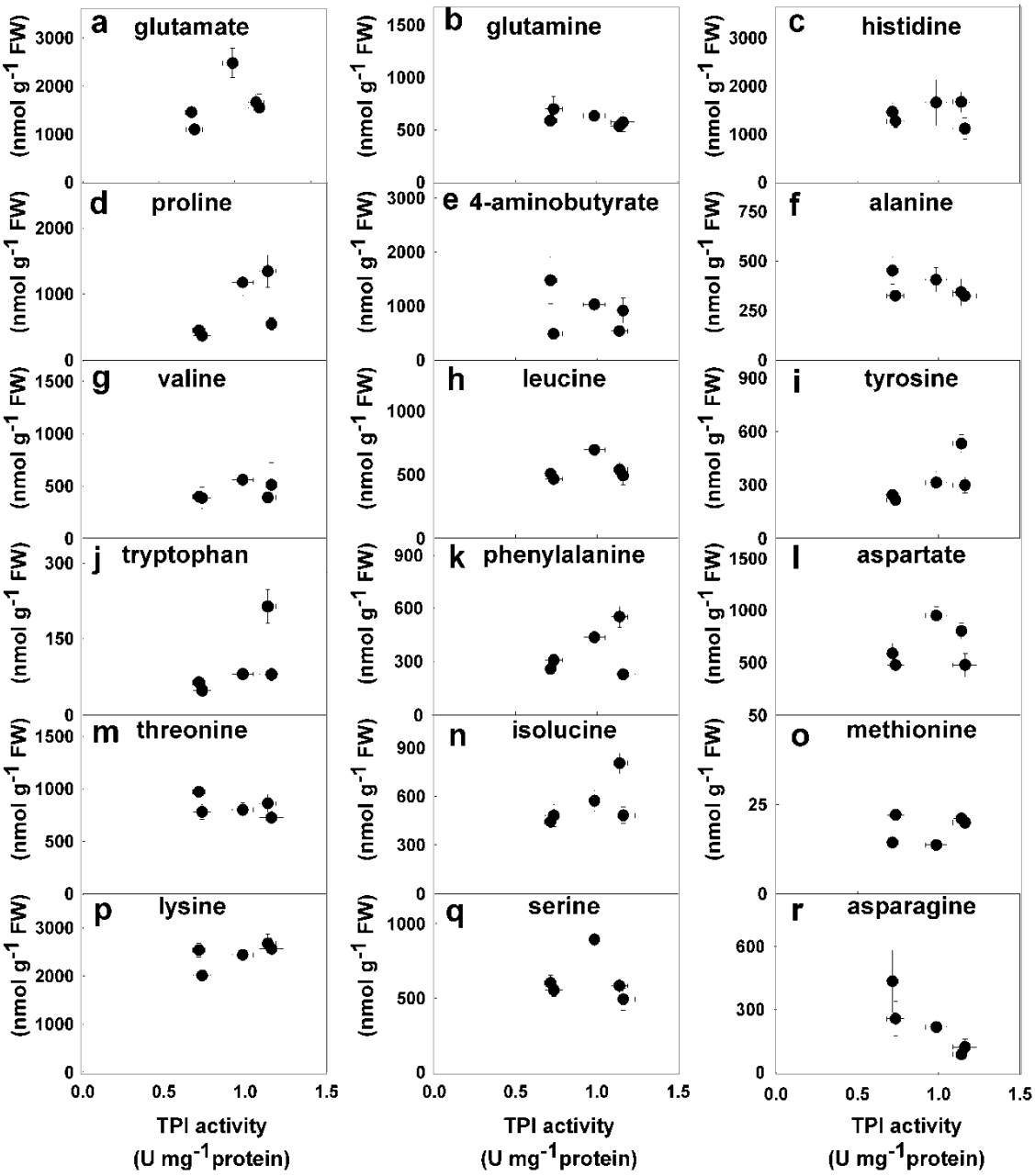

Figure 29. Levels of free amino acid in TPI transgenic roots in N deficient condition. Metabolites were assayed by HPLC. The amino acids contents are plotted as a function of TPI activity. Each dot represents the mean ± SE of 5 biological replicates.

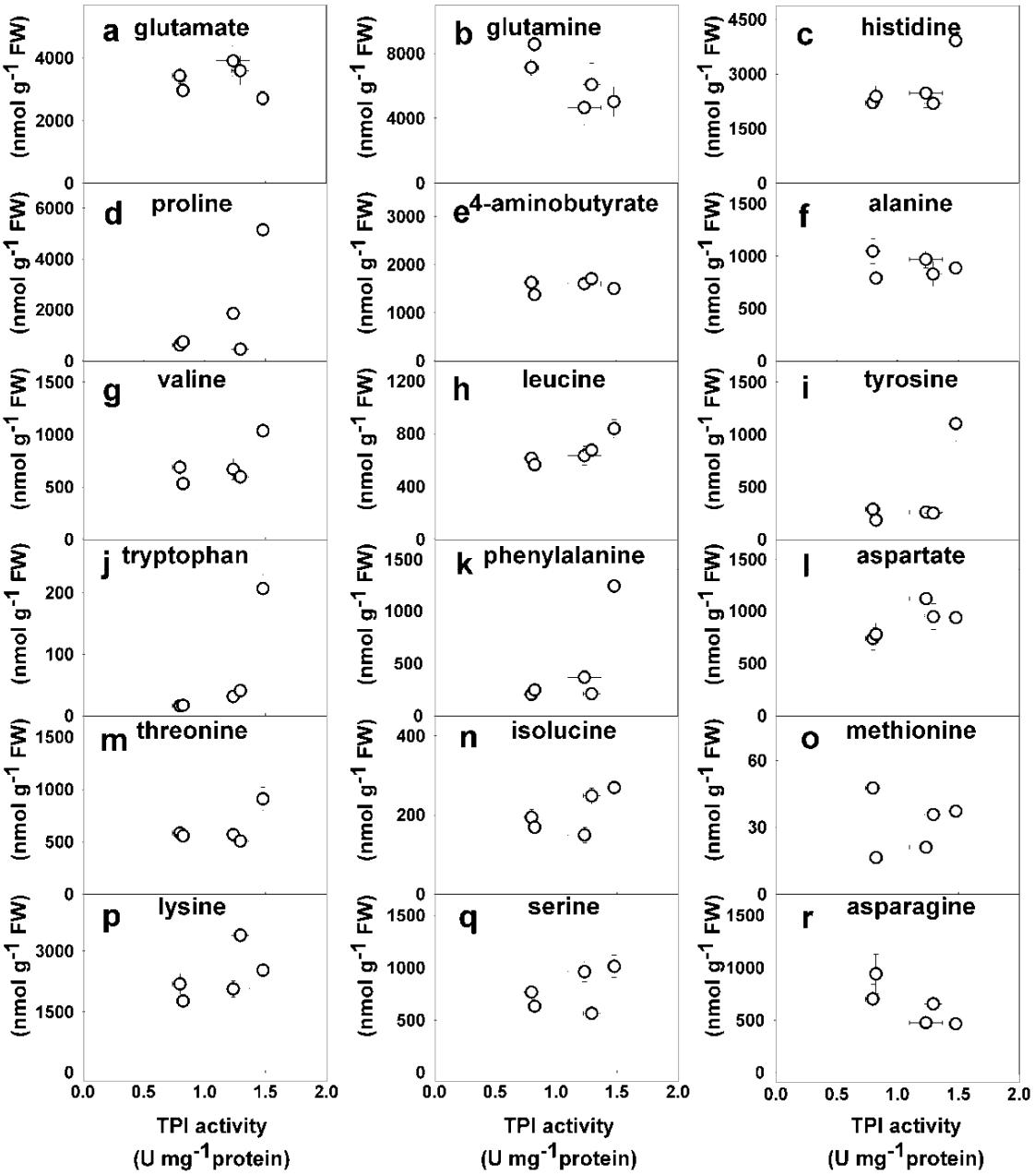

Figure 30. Levels of free amino acid in TPI transgenic roots in N sufficient condition. Metabolite was assayed by HPLC (described in materials and methods). The amino acids contents are plotted as a function of TPI activity. Each dot represents the mean ± SE of 5 biological replicates.

In an attempt to better understand the changes in the distribution of amino acids, the data represented in figure 29 and 30 were used for several other analyses. The total free amino

acids and also the total N present as free amino acids were calculated in both N treatment (Figure 31, 32).

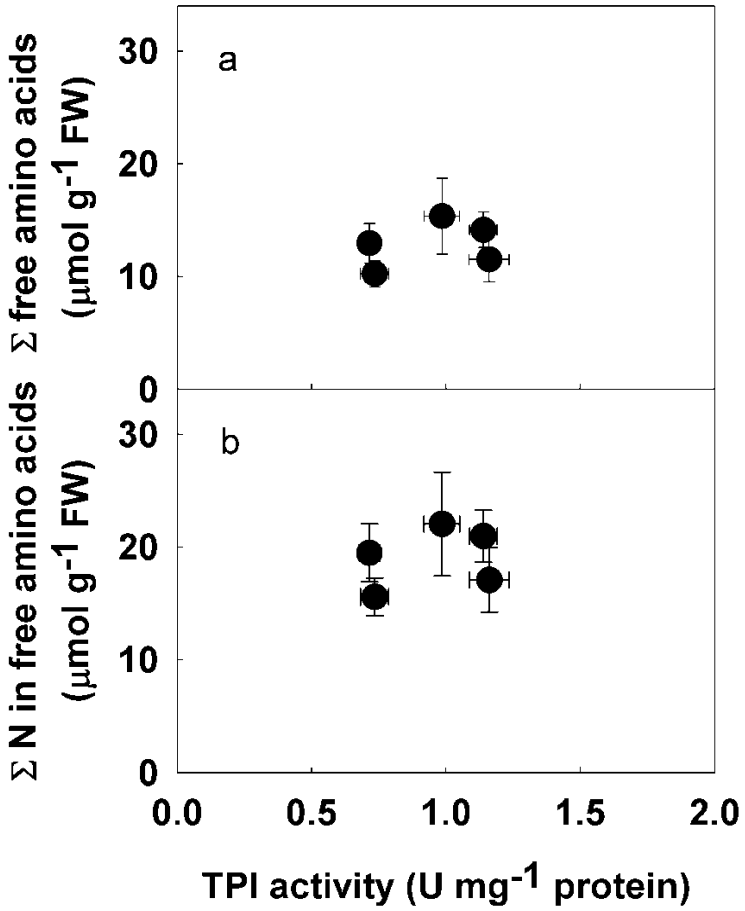

Figure 31. Sum of all free amino acids (a) and N present in amino acids (b) in TPI transgenic roots in N deficient condition. Metabolites were assayed by HPLC. The amino acid contents were calculated from data present in figure 28. Each dot represents a mean ± SE (n = 5).

The amount of total free amino acids and the total N present as amino acids were stable between TPI antisense and control clones in both N deficient (Figure 31) and N sufficient treatments (Figure 32).

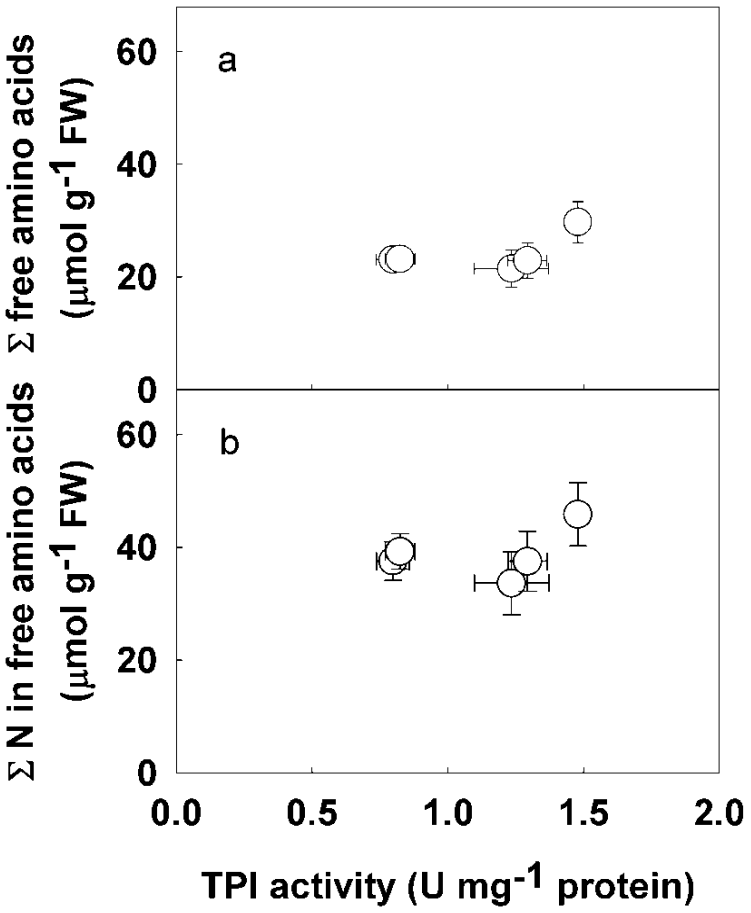

Figure 32. Sum of all free amino acids (a) and N present in amino acids (b) in TPI transgenic roots in N sufficient condition. Metabolite was assayed by HPLC. The amino acid contents were calculated from data present in figure 29. Each dot represents mean ± SE (n = 5).

The Gln/Glu ratio was higher in TPI antisense clones (Figure 33). This negative correlation of Gln/Glu ratio with TPI activity was observed in N sufficient and N deficient condition (Figure 33). The Gln/Glu ratio for both antisense and control clones was higher in N sufficient compare to N deficient condition (Figure 33).

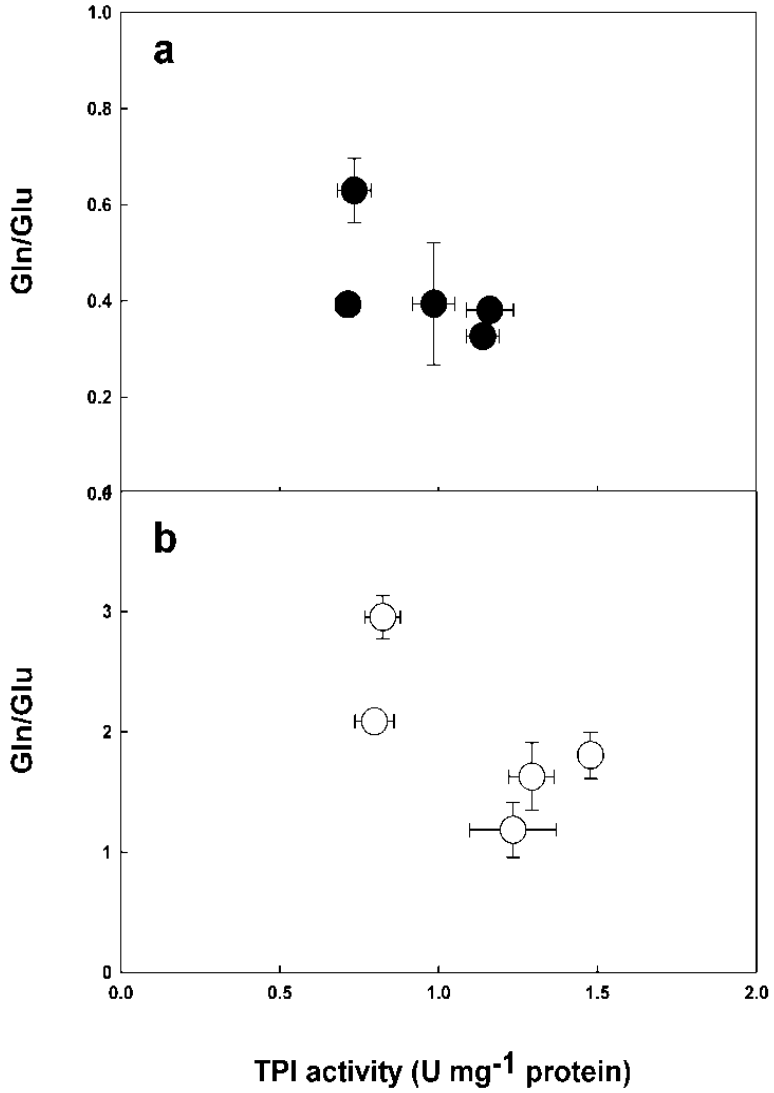

Figure 33. Interaction between the pools of Glu and Gln in transgenic TPI roots. Data for Glu/Gln in N deficient (a) and in N sufficient (b) were calculated from data present in figure 28 and 29. Each dot represents mean ± SE (n = 5). Symbols are used: white circles; high N condition, black circles; low N condition.

The Asn/Asp ratio also demonstrated a negative correlation with TPI activity in both N sufficient and deficient treatments (Figure 34). The Asn/Asp ratio was higher in N sufficient compare to N deficient conditions (Figure 34).

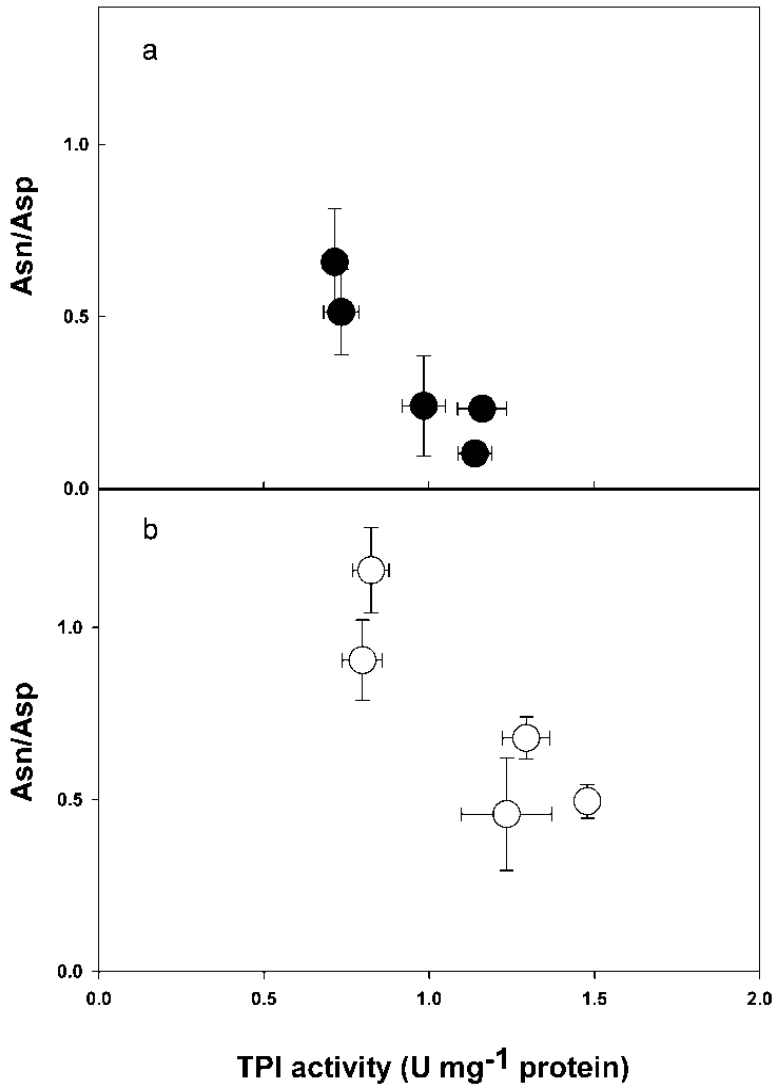

Figure 34. Interaction between the pools of Asn and Asp in transgenic TPI roots. Data for Asn/Asp in N deficient (a) and in N sufficient (b) were calculated from data present in figure 28 and 29. Each dot represents mean ± SE (n = 5). Symbols are used: white circles; high N condition, black circles; low N condition.

4. Discussion

4.1. Stability of G6PDH and 6PGDH after extraction

We investigated the stability of the oxPPP enzymes in potato cell extracts immediately, 2 and 4 hours after extraction by using a fresh dilution for each time point (Figure 13). The activity of enzymes was also measured after 0, 30 and 60 min of dilution (Figure 14). The specific activity of cytosolic G6PDH and that of 6PGDH were stable for up to 4 hours after extraction but the plastidial activity was extremely unstable after extraction and upon dilution (Figure 6). The labile nature of the plastidial G6PDH isoform has been previously reported in the literature. This instability of the enzyme makes it nearly impossible to study its kinetics or purify the isoform (Simcox 1997, Wright et al 1997). Therefore, the accurate assay of plastidial G6PDH requires rapid extraction conditions and modification of the redox status in order to minimize the loss of activity during the experiment. We were able to measure the plastidial G6PDH activity in potato cell extracts (Figure 14). Therefore, in transgenic roots cultivated in vitro, the lack of a sizeable activity for the plastidial isoform that we observed (see below) must reflect the fact that this material contains an extremely low level of this enzyme.

4.2. Cytosolic G6PDH and 6PGDH are induced by N availability in HK and TPI transgenic roots

The specific activity of the oxPPP enzymes was measured in 7 d old HK and TPI transgenic roots (Figures 16, 17, 19 and 20). The examination of cytosolic G6PDH and 6PGDH specific activities shows an increase in specific activity with high amounts of N, regardless of the genetic background. The higher specific activity of oxPPP enzymes during high N supply is consistent with the activation of the pentose phosphate pathway in these conditions. Higher activity of oxPPP enzymes in high N conditions is consistent with results

that were previously observed in barley roots and maize (Averill et al 1998, Esposito et al 1998, Esposito et al 2005, Wright et al 1997).

4.3. Higher activity of cytosolic G6PDH in HK sense clones

The activity of cytosolic G6PDH was positively correlated with GK in both low and high N conditions (Table III). However, no correlation was detected between GK specific activity and 6PGDH. The positive correlation of GK and cytosolic G6PDH suggests a higher capacity of the first step of the oxPPP in HK sense clones compare to antisense clones (Table IV). There is little information available in the literature concerning the regulation of HK enzymes in detail, so the explanation for these results is not known at this time. The characterization of transgenic HK roots reported by (Claeyssen et al 2012) demonstrated that manipulation of only HK led to very few metabolic alterations. Among these, the levels of ATP, the ATP/ADP ratio, the adenylate energy charge and the levels of free Pi were shown to be lower in clones overexpressing HK. Growth of HK overexpressors was also impaired. It is possible that the increase in cytosolic G6PDH activity that we measured here is indirectly related to one or several of these factors. Another possibility to explain the alteration of G6PDH activity in HK sense roots is that HK (which is known to act as a hexose sensor in plants) interferes with the normal sugar level regulation of *g6pdh* genes (Hauschild & von Schaewen 2003). Indeed, it was previously shown that cytosolic G6PDH activity is inducible in the dark by Glc and other hexoses in a process that depends on protein synthesis and that involves increased *g6pdh* mRNA steady states (Hauschild & von Schaewen 2003).

4.4. Activities of cytosolic G6PDH and 6PGDH are correlated with TPI activity in transgenic roots

The activity of the two oxPPP enzymes were positively correlated with TPI activity in transgenic roots (Figure 22). It is possible that the lower activities of cytosolic G6PDH and 6PGDH found in roots with lower amounts of TPI are linked to some of the metabolic changes

that occur in these clones. Since the metabolic changes due to the down regulation of cTPI are quite numerous (Dorion et al 2012), it is difficult to point to anything in particular without doing further studies. In this regard, one of the priorities should be to examine whether the observed changes in cytosolic G6PDH and 6PGDH result from differences in the expression of the corresponding genes.

Our data demonstrate a higher capacity of oxPPP enzymes in control clones compared to TPI antisense clones in both N sufficient and N deficient conditions (Figure 22). Previous work done on roots cultivated with a much higher N supply (100 mM total N) (Dorion et al 2012) reported that roots with low TPI activity had a higher C flux through the pentose phosphate pathway when fed with radioactive glucose. If the correlation between oxPPP and TPI enzyme activities observed in the present study also occurs at 100 mM total N, it would mean that the capacity of the pentose phosphate pathway (measured as extractable enzyme activity) and the flux through the pathway (measured as moles of C processed per unit of time) are not obligatorily tightly correlated. This is not surprising considering the large body of evidence indicating that the control of flux though metabolic pathways results from a multitude of little effects rather than simply turning on or off the activity of a single enzyme (Stitt et al 2010). One of the first published examples of this comes from the study of the manipulation of 6-phosphofructokinase in potato (Burrell et al 1994). A more than ten-fold increase in the expression of this enzyme, previously recognized as a 'pacemaker' in glycolysis, had no effect on pathway flux. Indeed, more and more evidence point to the *in vivo* regulation of enzymes by their effectors as important factors in the control of metabolic fluxes (Stitt et al 2010).

4.5. HK activity in relation to N status

The activity of HK was assayed with Glc and Fru as substrates in HK transgenic root extracts in low and high N conditions (Figure 18). These data show that GK activity was not significantly different in N deficient compared to N sufficient conditions in antisense and control clones. Interestingly, in N deficient conditions, GK activity of sense clones was higher

than in N sufficient conditions. This might suggest the possibility of differential regulation of HK in sense clones.

One possibility to explain these results is that they could be due to the regulation of 35SCaMV by N availability. Although The 35SCaMV promoter is usually believed to be constitutive in different tissues and in several different species (Odell et al 1985), it has been suggested that the 35SCaMV promoter is not expressed in all plant cell and tissue types or it is expressed at different levels (Sunilkumar et al 2002, Terada & Shimamoto 1990, Williamson et al 1989, Yang & Christou 1990). However, the regulation of 35SCaMV promoter has not previously been reported to be affected by different amount of nutrients, including N.

Another possibility to explain the higher activity of HK in sense clones might be due to N- dependent posttranslational regulation of HK in sense clones. One of the posttranslational modification mechanisms that can regulate the function of proteins is the S-nitrosylation of Cys residues in target proteins. S-nitrosylation, the covalent addition of nitric oxide (NO) to a Cysthiol (Huber & Hardin 2004), regulates all classes of functional proteins and plays a regulatory role in almost all cell types (Hess et al 2005). The occurrence of protein S-nitrosylation has been investigated in plants (Huber & Hardin 2004). In the deduced *Arabidopsis* proteome (found in the SwissProt database), several metabolic enzymes contain sequences that matched the degenerate motif [GSTCYNQ]-[KRHDE]-C-[DE] thought to be S-nitrosylation targets (Huber & Hardin 2004). These possible S-nitrosylation targets include galactokinase and hexokinase1 (Huber & Hardin 2004). NO can be produced in plants by NADH-dependent nitrate reductase (NR) (Rockel et al 2002). Since this activity is affected by the production of reducing equivalents, it could possibly have an effect on HK activity in different N treatments. S-nitrosylation is sometimes involved in the crosstalk with other posttranslational modification mechanisms including phosphorylation, acetylation, ubiquitylation and related modifications, as well as alternative Cys-based redox modifications (Hess & Stamler 2012). Among them, phosphorylation is one of the most important posttranslational modifications (Kwon et al 2006). This mechanism could possibly affect the function of HK in plants. In the recent characterization of purified potato HK1 enzyme by MS/MS sequencing, no such posttranslational modification was detected (Moisan & Rivoal 2011). It should be pointed out however, that the purification procedure of HK1 takes over a

week to complete. Therefore, labile posttranslational modifications occurring *in vivo*, such as phosphorylation, could have been missed during this analysis.

4.6. TPI activity in relation to N status

The TPI specific activity was measured in cTPI transgenic roots (Figure 21). The data did not show a systematic change in TPI activity between N sufficient compared to N deficient conditions. This means that N treatment does not greatly affect TPI specific activity. There are few literature reports concerning TPI in relation to changes in the environment. It has been suggested that TPI might be important under stress since TPI activity was induced by Fe starvation in *Arabidopsis thaliana* shoots (Thimm et al 2001). *Pteris vittata* TPI is involved in resistance to arsenate (Rathinasabapathi et al 2006) while in *Arabidopsis,* TPI is negatively regulated by glutathionylation under oxidative stress condition (Ito et al 2003). TPI is also affected by stressing agents like herbicides interfering with photosynthesis (Del Buono et al 2009). Concerning N availability, literature reports on TPI is somewhat contradictory. (Castillejo et al 2010) Reported that TPI protein increase under low N condition in triticale flag leaf. In maize leaves subjected to N starvation (Prinsi et al 2009) showed that TPI was increased after resupply with N. (Møller et al 2011) showed increase in TPI in barley shoots by long N starvation. Considering our data and these results, it may be suggested that the effect of N status on TPI activity differs depending on tissue and different systems.

4.7. The manipulation of HK did not alter amino acid metabolism

The primary data obtained in HK transgenic roots by (Claeyssen et al 2012) showed that HK had a high control over the phosphorylation of Glc, but no control over glycolytic flux. We hypothesized that the higher conversion of Glc to G6P due to increased HK expression could translate to a higher C flux through the oxPPP in HK sense clones in both N conditions (Table I). This, in turn could lead to higher assimilation rates for N in HK sense clones (Table I). However the measurement of free amino acids in HK transgenic roots grown under the two N regimes tested here do not support these hypotheses. We did not observe any

large difference in amino acid contents or in N contained in amino acids between roots expressing different levels of HK (Figures 23-26). The level of total free amino acids as well as total N reported in our study are similar to the values reported previously in HK sense and antisense clones (Claeyssen et al 2012). Except in the case of Glu, 4-aminobutyrate, leucine and proline, the effect of HK activity on free amino acid levels was negligible (Figure 23). Proline increased marginally with HK activity leads in both N conditions (Figure 23 a) (Claeyssen et al 2012). Proline is a marker of environmental stress and it might increase in a variety of conditions (Szabados & Savoure 2010). Likewise, no trend in Gln/Glu or Asn/Asp was observed between the roots having different levels of HK activity (Figures 27, 28). The Gln/Glu and Asn/Asp ratios together with the pools of Gln and Asn are tightly linked to primary N assimilation (Lam et al 1996). In particular, the size of the Gln pool and the Gln/Glu ratio are considered as good indicators of the activity of nitrogen assimilation (Foyer et al 2003). Our data therefore indicate that no difference exists in N assimilation among root clones expressing different levels of HK. As expected, our analyses show that roots cultivated on high N also have higher amino acid contents. For example, the Gln content increased 4-5 fold in N sufficient roots compared to N deficient while Glu content increased marginally in response to high N treatment (figure 24b). Although Glu content can vary in response to alteration of N supply, these changes are usually not as important as what is observed for Gln (Forde & Lea 2007). For example, in a comparison between tobacco plants grown on 12 mM and 0.2 mM nitrate, a 10-12 fold increase of Gln was observed in the high N treatment while the concentration of Glu never exceeded twice that of N deficient plant (Fritz et al 2006a, Fritz et al 2006b). These data lead us to the conclusion that the manipulation of HK activity in roots grown under different N regimes does not lead to changes in the C flux carried by the oxPPP. Consequently, it is reasonable to conclude that under the different N regimes tested, the manipulation of HK affects the rate of conversion of Glc to G6P, but has no impact on the glycolytic flux (Claeyssen et al 2012) or on the flux through the oxPPP (this study). Therefore, the main conclusion of the study by (Claeyssen et al 2012), that, in transgenic roots a futile cycle returns C from the hexose-P pool to the carbohydrate pool appears to be supported by the results presented here. These results contrast with those obtained by (Menu et al 2004) in tomato fruits, where the levels of free amino acids were increased by HK overexpression. This

disparity might be explained by the existence of differences in the function or regulation of hexokinase and/or the futile cycling of hexose Ps in various plant organs.

4.8. The manipulation of cTPI alters amino acid metabolism

The results previously obtained by (Dorion et al 2012) led us to hypothesize that a higher flux of oxPPP in TPI antisense clones, could in turn affect N assimilation and amino acid metabolism. In antisense clones grown under N deficiency treatment, a higher activity of the oxPPP could thus have led to increases in higher N assimilation and consequently, to higher free amino acid levels. Some of the results obtained in this study support the validity of this hypothesis. For example, the cTPI antisense clones had significantly higher Gln/Glu and Asn/Asp ratios (Figures 33 and 34). Interestingly, this situation was observed in both N conditions. As emphasized in the section above, an increase in Gln/Glu and Asn/Asp ratios indicate higher rates of N assimilation because Gln and Asn are the primary acceptors of mineral N and are formed from Glu and Asp respectively (Foyer et al 2003). These apparently higher assimilation rates of mineral N were found in antisense roots grown under low and high N conditions, indicating that a higher C flux through the oxPPP is likely to occur over a wide range of N regimes. However, in both conditions, the total free amino acid pools and total N present as amino acids were unaffected by TPI levels (Figures 31 and 32). Even though some variation in the pools of a few amino acids was observed. For example, in N deficient and N sufficient treatments the levels of Gln, and Asn were found to be slightly negatively correlated with TPI activity (Figure 29 and 30). Positive correlations with TPI activity were found for Glu, Pro, Lys, Ile and the aromatic amino acids Phe, Tyr and Trp.

The fact that TPI antisense roots displayed high rations of Gln/Glu (and Asn/Asp) but did not have an increase in the total amino acid content may seem surprising at first. Two possible explanations can be advanced to account for this. The first is linked to the fact that our measurements determine only the steady state levels of free amino acids per units of g FW, and excess of amino acids resulting from increased N assimilation used immediately to sustain growth would not be detected. Indeed, recently, (Valancin et al 2012) showed that the TPI antisense root clone G1.8 used in this study grew better than control clones when cultured in liquid MS media. The higher growth rates detected by (Valancin et al 2012) was coupled to

a higher uptake of O_2 (Dorion et al 2012, Valancin et al 2012) and by higher glycolytic rates (Dorion et al 2012), which could also serve to sustain growth. It would therefore be interesting to evaluate the total nitrogen budget of control and antisense roots to determine whether the evidence for higher N assimilation rates detected here in antisense clones is correlated with increased levels of organic N (total proteins, polyamines, etc) during the course of a culture.

A second explanation for the lack of correlation between elevated Gln/Glu (and Asn/Asp) ratio and total N present as amino acids is that there may be a shortage of C skeletons to accept amine groups present in Glu. Indeed, after the assimilation steps that yield Gln and Glu, organic nitrogen is normally distributed within the different amino acids by transamination reactions (Wilson et al 1954). As emphasized earlier (see section 1.5), these reactions require C skeletons (e.g. in the form of keto-acids) that serve as acceptors for the transfer of the amine group from a donor (e.g. Glu) to generate a new amino acid. Therefore, C and N metabolism interact closely (Foyer et al 2003). In non-photosynthetic tissues, respiratory metabolism provides energy and C skeletons required for amino acid biosynthesis (Foyer et al 2003). Indeed, C skeletons are normally derived from primary metabolism pathways glycolysis and the TCA cycle. It is possible that in the conditions that were used in our experiments, these pathways do not have a sufficient output to sustain the production of keto-acids used in transamination reaction. A careful evaluation of glycolytic and anaplerotic fluxes using metabolic tracers should therefore be done to evaluate this possibility.

5. Conclusion

In this study, we evaluated the effects of various N regimes on the capacity and activity of the oxPPP in transgenic roots modified to contain different levels of HK and cTPI. The capacity of the oxPPP was estimated from the extractible activities for the enzymes cytosolic G6PDH and 6PGDH. The activity of the pathway was then assessed relative to the contents of the different root clones in amino acids because it is the oxPPP that provides the reducing power necessary to assimilate mineral nitrogen in non photosynthetic tissues.

We found a higher capacity of the oxPPP and higher amino acid content in response to the provision of high levels of N in both HK and cTPI transgenic roots. These data confirm that transgenic roots grown in vitro respond to N in a manner similar to other plant organs.

The transgenic plants designed to overexpress HK resulted in an increased capacity for the first step in the oxPPP but did not change the assimilation of N. Our initial hypothesis that higher levels of assimilation could occur in HK sense clones thus does not agree with the results described here. This supports the view that G6P resulting from higher conversion of Glc to G6P in HK sense roots is not preferentially used by the oxPPP. These data emphasize the possible futile cycling of hexose-Ps and/or synthesis/degradation of Suc previously detected in this material. Our data also demonstrate an apparent up-regulation or activation of HK in HK sense clones subjected to low N conditions. These findings will be further investigated by testing the constitutive aspect of promoter in N deficient condition and by searching for possible post-translational modifications of HK under low N condition. To date, surprisingly little is known about the regulation of plant HKs, and further investigation into HK regulation in cases of nutrient deficiency needs to be evaluated.

The capacity of the oxPPP was investigated in potato roots with different levels of cTPI. We found a relatively strong correlation between cTPI activity and the activities of cytosolic G6PDH and 6PGDH. Our results also provide evidence of higher mineral N assimilation in clones with low cTPI, since the Gln/Glu and Asn/Asp ratios were significantly higher in roots with low cTPI than in control roots. However, the total amino acid content was not increased. This may represent the result of an increased use of amino acids to sustain higher growth rates in clones with lower cTPI. It may also reflect an incapacity of anaplerotic

C fluxes to match the demand in C skeletons necessary for the synthesis of amino acids. These two possibilities provide suggest research directions to investigate the function of cTPI in plants. It will be of interest to examine the fluxes of C in primary metabolism and the provision of C skeleton for N assimilation in roots grown under low N conditions.

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