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"FEDERICO II"**



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Tesi di Dottorato

**Cloning, expression and characterization  
of P2-glucose-6P dehydrogenase from barley (*Hordeum  
vulgare*) and poplar (*Populus trichocarpa*)**

**Effects of Abscissic acid (ABA) on activity, occurrence and  
expression of different isoforms of glucose-6P  
dehydrogenase in barley**

Tutore  
Ch.mo Prof.  
Sergio Esposito

Candidata  
Dott.ssa  
Cardi Manuela

Coordinatore  
Ch.mo Prof.  
Ricca Ezio

Co-Tutore  
Ch.mo Prof.  
Jean-Pierre Jacquot

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*Dedicata a MIO PADRE*

L'amore che ci ha uniti per 27 anni non potrà mai finire...  
continua a vivere in me, nei valori e nell'amore  
per la ricerca che mi hai trasmesso

*Dedicated to the beloved memory of my  
father*

The love that has tied us for 27 years will never end... It still lives  
in me, in the values and in the love for the research that you had  
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*La strada del sapere è in salita perseveranza, costanza, volontà, amore  
(per ciò che si studia) può vincerla; pazienza, UMILTA',  
TENACIA, fiducia e sincerità vicini SEMPRE!  
(Mauro Sirio Cardi – luglio 2008)*

*The knowledge street is an ascent. Only perseverance, endurance,  
willpower and love for what we're studying can beat it; patience,  
humbleness, endurance, tenacity, trust and sincerity at my side ever!  
(Mauro Sirio Cardi – luglio 2008)*

## ABSTRACT

Glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) is the main regulatory enzyme of the oxidative pentose phosphate pathway (OPPP), an important provider of NADPH. The effect of exogenous ABA on the activity, expression level and protein amount of different G6PDH isoforms has been studied in barley plants. G6PDH activity increased by 50% in ABA treated roots for 12h; this increase resembles a similar (+35%) effect observed in P2-G6PDH transcript levels. The transcript Cy-G6PDH levels did not change appreciably upon ABA supply. Similar variations were observed in protein levels in immunoblotting analyses. In leaves, ABA did not affect the cytosolic protein levels, while a sudden decrease was observed for chloroplastic P1-G6PDH protein; therefore the two-fold increase in G6PDH activity observed after ABA treatment is likely related to an increase in mRNA level (+ 50%) and protein amount (+ 85%) of leaf P2-G6PDH. Altogether, these results suggest a specific role for the plastidial P2-isoform in ABA treated barley plants, encouraging a better characterization of this plastidic isoform.

Therefore, genes of the plastidic isoform of G6PDH (P2-G6PDH) from two different organisms, barley (*Hordeum vulgare*) and poplar (*Populus trichocarpa*) have been cloned in two different expression vectors, pET3d and pET15b (His-Tag), and overexpressed in *E.coli*. The purified enzyme was checked by mass spectrometry analysis, and for the reactivity against specific P2—G6PDH antibodies; the main kinetic parameters, and differential sensitivity to reduction by DTT were determined. The recombinant P2-G6PDHs exhibits molecular weights of 55.5 kDa and 61kDa for barley and poplar, respectively. The main kinetic parameters measured for both *Hv*P2-G6DPH and *Pt*P2G6PDH are in agreement (e.g. high  $K_{i_{\text{NADPH}}}$ ) with the values known for the most of other P2-type G6PDHs. The recombinant barley protein is moderately sensitive to reductants (DTT); moreover the *Populus trichocarpa* enzyme presents a redox potential (-280 mV) favourable for control by either thioredoxins *m* or *f*.

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## **ABBREVIATIONS**

ABA: Abscisic acid

ABRE: ABA responsive elements

ATP: Adenosine triphosphate

bZIP: Family transcription factors

CE: Coupling elements

Cys: Cysteine

DTT: Dithiothreitol

E4P: Erythrose 4-phosphate

F6P: Fructose 6-phosphate

Fd: Ferredoxin

F1,6P: Fructose-1,6-bisphosphate phosphatase

G3P: Glyceraldehyde 3-phosphate

G6P: Glucose 6-phosphate

G6PDH: Glucose 6-phosphate dehydrogenase

GPT: Glucose-6-phosphate translocator

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GSH: Reduced Glutathione

GSSG: Oxidized Glutathione

Gln: Glutamine

GS: Glutamine synthetase

GOGAT: Glutamate synthase

GDH: Glutamate dehydrogenase

JA: Jasmonic acid

K<sub>m</sub>: Michaelis-Menten constant

K<sub>i</sub>: Inhibition constant

NADP<sup>+</sup> : Nicotinamide adenine dinucleotide phosphate, oxidized

**NADPH:** Nicotinamide adenine dinucleotide phosphate, reduced

**NAD<sup>+</sup>:** Nicotinamide adenine dinucleotide, oxidized

**OPPP:** Oxidative Pentose Phosphate Pathway

**Pi:** Inorganic phosphate, orthophosphate ( $\text{PO}_4^{3-}$ )

**PPT:** Phosphoenolpyruvate/phosphate translocator

**PEP:** Phosphoenolpyruvate

**PSI:** Photosystem one

**PAGE:** Polyacrylamide gel electrophoresis

**Ru5P:** Ribulose 5-phosphate

**SA:** Salicylic acid

**Triose-P :** Triose phosphate

**Trx:** Thioredoxin

**UA:** Units of Absorbance

**Xlu5P :** Xylulose 5-phosphate

**XPT:** translocator

**6PGDH:** 6-phosphogluconate dehydrogenase

# CHAPTER 1

## INTRODUCTION

### 1.1 The Oxidative Pentose Phosphate Pathway (OPPP)

Glucose 6 phosphate dehydrogenase (EC 1.1.1.49), is the key enzyme of the Oxidative Pentose Phosphate Pathway (OPPP), this cycle providing an alternative route to glycolysis for carbohydrate catabolism (Fig.1).

Both glycolysis and the OPPP share some common intermediates like glyceraldehyde 3-phosphate (G3P), glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) (Dennis *et al.*, 1997).

The OPPP is found in all prokaryotes and eukaryotes, except for Archaeobacteria (Wendt *et al.*, 1999), another important function is the supply of the reducing power and precursors for nitrogen metabolism and for a variety of biosynthetic reactions in higher plants.

The OPPP is divided into two parts: an oxidative phase and a regenerative phase. The oxidative phase begins with G6P being oxidized by glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) producing ribulose 5-phosphate (Ru5P), two molecules of NADPH and one molecule of CO<sub>2</sub>. The two reactions of the oxidative phase are irreversible and tightly regulated within the cell (Fig;1).

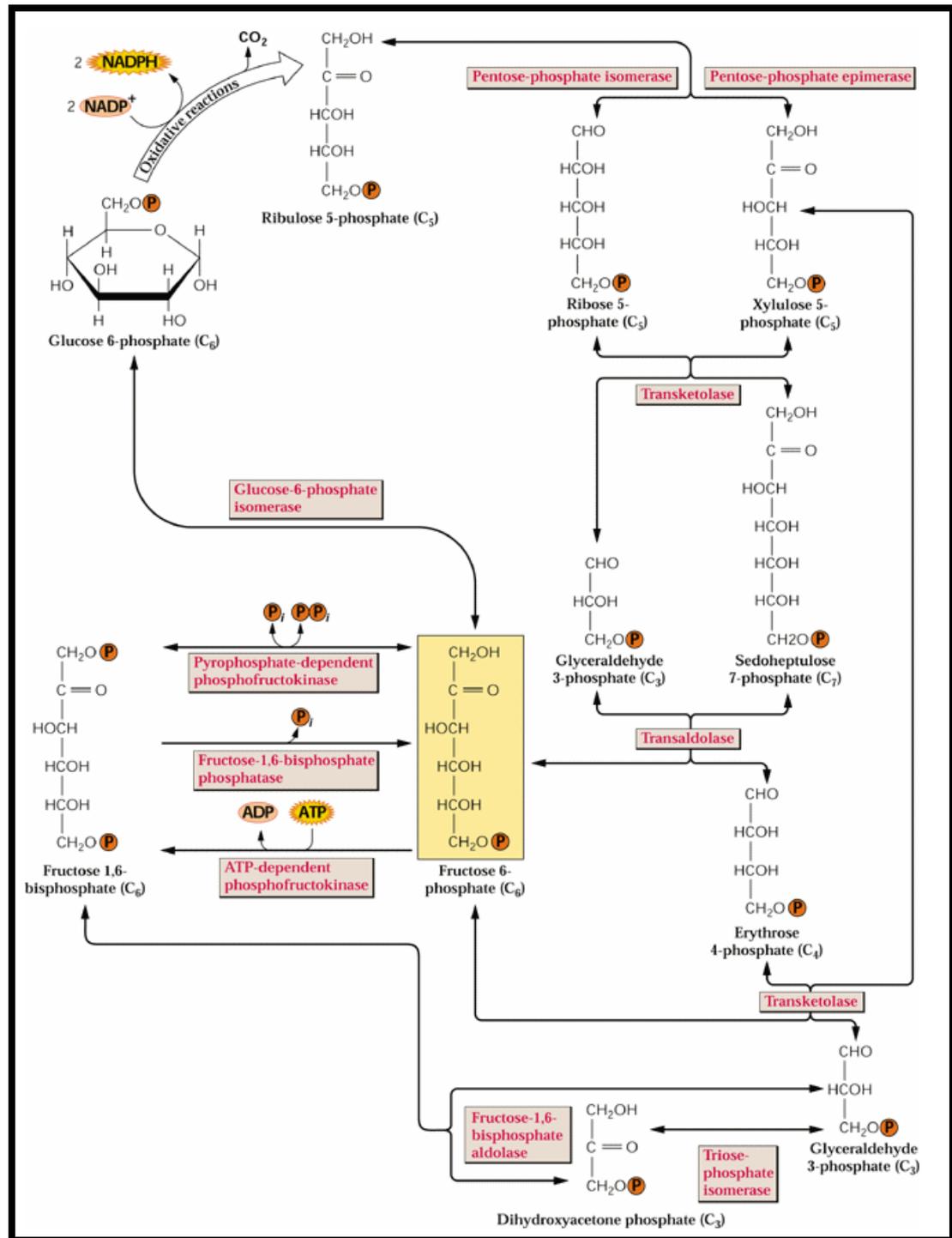
The regenerative phase leads to a series of rearrangements of the pentose phosphates, by various enzymatic reactions including epimerase, isomerase, transketolase and transaldolase. These carbon skeletons may be recycled in the regenerative phase of the OPPP and may return back into the oxidative portion of the pathway, reforming the G6P.

The presence of two oxidative pentose phosphate pathways (OPPP) in plant tissues has been reported for leaves (Schnarrenberger *et al.*, 1973), roots (Emes and Fowler, 1979) and cultured cells (Krook *et al.*, 1998). The oxidative phase takes place in both the plastids and cytosol of plants and photosynthetic algae

(Kruger and von Schaewen, 2003; Schnarrenberger *et al.*, 1995; Klein, 1986). Cytosolic and plastidic isoforms of glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) have been isolated from a sufficiently wide range of photosynthetic and non photosynthetic tissues suggesting that the subcellular duplication of the oxidative section of the OPPP is probably ubiquitous in plants (Debman and Emes, 1999).

The OPPP is regulated by glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49 - G6PDH), (Schnarrenberger *et al.*, 1973; Kruger and von Schaewen, 2003). The cytosolic OPPP cycle represents the major part of the measured activity, about 60-85% of the total (Debman and Emes, 1999), and it seems to be involved to sustain the cell basal metabolism (Esposito *et al.*, 2001). The activity ascribed to compartmented isoforms represents the 15-20% of total (Debman and Emes, 1999; Esposito *et al.*, 2001; Hong and Copeland, 1991; Bredemeijer and Esselink, 1995).

The enzymes of the regenerative phase have been found often only in the plastids of higher plants (Stitt and ap Rees, 1979; Schnarrenberger *et al.*, 1995; Debman and Emes, 1999). The intermediates of the regenerative phase may be shunted to other synthetic pathways. For example ribose 5-phosphate is required for nucleotide synthesis in both plants and animals and erythrose 4-phosphate (E4P), produced by the Calvin cycle in the light and by the OPPP in the dark, is incorporated into aromatic amino acids and phenolic compounds in plants (Dennis *et al.* 1997; Pandolfi *et al.*, 1995).



**Figure 1:** The Oxidative Pentose Phosphate Pathway (OPPP) (Buchanan *et al.*, 2000).

Glucose 6-phosphate can enter plastids in exchange for triose phosphate or orthophosphate (Pi) via the G6P/phosphate translocator (GPT).

The interaction between cytosolic and plastidic processes is possible because the phosphate-translocator family on the plastid inner-envelope has the capability to transport pentose phosphates as well.

Exchange of xylulose 5-phosphate (Xlu5P), triose phosphate (Triose-P) and Pi is catalysed by the Xilulose-5P/Phosphate Translocator (XPT) (Eicks et al. 2002). In the absence of cytosolic transketolase and transaldolase, this XPT activity facilitates further plastidial metabolism of pentose phosphates generated by the oxidative reactions in the cytosol, as well as the provision of pentose phosphates generated for nucleotide synthesis in the cytosol (Eicks *et al.*, 2002).

Another important translocator, the phosphoenolpyruvate/phosphate translocator (PPT) is required to import phosphoenolpyruvate into plastids for the biosynthesis of aromatic acids, through the shikimate pathway. This pathway has been found only in microorganisms and plants and it needs of erythrose 4P (produced during the OPPP) to synthesise phenylalanine, tryptophan and tyrosine.

Therefore, PPT carrier represents a strict link between the cytosolic glycolytic pathway producing PEP and plastidic OPPP, producing erythrose 4P, supplying shikimate pathway of its main precursors with the final goal to synthesise aromatic amino acids within the plastids (Fig.2).



## 1.2 Glucose 6 phosphate dehydrogenase: general properties and regulation

The most important role of OPPP is to generate NADPH to provide reductants for biosyntheses in animals, plants and bacteria (Eggleston and Krebs, 1974; Agrawal and Calvin, 1971). Reductants produced in the OPPP may also be important to contrast oxidative stress (Juhnke *et al.*, 1996; Wang *et al.*, 2003).

The G6PDH is an active homotetramer formed by subunits linked by disulfide bridges formed by cysteines. This structure is stabilized by NADP<sup>+</sup>; when the NADPH/NADP<sup>+</sup> ratio is high, the di-sulphide bridges are reduced and the enzyme splits into inactive single subunits.

G6PDH activity and expression are modulated by a number of factors, such as light, NADPH, and other reductants; moreover the enzyme is involved in response to biotic and abiotic stresses.

The majority of G6PDHs characterized to date show hyperbolic kinetics, but some cyanobacterial enzymes show sigmoidal kinetics (Schaeffer and Stanier, 1978); it should be noted that some bacterial enzymes (e.g. *Leuconostoc mesenteroides* - Cosgrove *et al.*, 1998) are capable of using both NAD<sup>+</sup> and NADP<sup>+</sup> as substrates (Anderson *et al.*, 1997).

In the last years, considerable work has been made to describe the three major types of plant G6PDH: the cytosolic (Cy-G6PDH), chloroplastic (P1-G6PDH), expressed in photosynthetic tissues (Schnarrenberger *et al.* 1973; Anderson *et al.*, 1974) and plastidic (P2-G6PDH) isoforms; but the regulatory properties of each G6PDH type are still poorly characterized (Wendt *et al.*, 2000; Esposito *et al.*, 2001a; 2001b; 2005). These enzymes exhibit different kinetic parameters: it is generally accepted that these isoforms play different roles in plant metabolism; monoclonal antibodies recognizing the different isoforms in potato have been prepared (Wendt *et al.*, 2000). The plastidial G6PDH activity is modulated through dithiol-disulfide exchanges via the ferredoxin/thioredoxin system (Buchanan, 1991; Née *et al.*, 2009), whereas the redox sensitivity of the

cytosolic isoform is still controversial (Graeve *et al.*, 1994). A recent *Arabidopsis* genome-wide analysis indicated the presence of two cytosolic and four plastidial G6PDHs (Wakao and Benning, 2005). The two major classes of plastidial G6PDH (P1 and P2) have been extensively studied in higher plants such as potato (Wendt *et al.*, 2000; Hauschild and von Schaewen, 2003), wheat (Nemoto and Sasakuma, 2000), barley (Esposito *et al.*, 2005) and rice (Huang *et al.*, 2002). P1-G6PDH transcripts and protein are detectable in most of the photosynthetic under illumination (von Schaewen *et al.*, 1995; Esposito *et al.*, 2005), whereas the P2-G6PDH transcripts are detectable throughout the plant, more intensively in stems and roots (Knight *et al.*, 2001; Wakao and Benning, 2005).

The different isoforms of G6PDH can be distinguished by their different affinities for G6P, and different effects of thiol groups on the enzyme activities (Fickenscher and Scheibe, 1986; Scheibe *et al.*, 1989).

The cytosolic enzyme is involved in the production of precursors for nucleic acid biosynthesis (Dennis *et al.*, 1997). Cytosolic G6PDH activity remained constant in the dark; in continuous light or in the presence of metabolizable sugars in the dark, cytosolic G6PDH activity increased 6-fold within 24h (Hauschild and von Schaewen, 2003). Cycloheximide incubation demonstrated that enhanced cytosolic G6PDH activity depends on *de novo* protein synthesis (Esposito *et al.*, 2005). Multiple SURE (SUGar Responsive Elements) elements are present in the promoter region of the Cy-G6PDH gene, suggesting that G6PDH activity is regulated by sugar availability in the cytosol by a sugar sensing mechanism (Hauschild and von Schaewen, 2003).

Both the plastidic P1 and P2 G6PDHs play a major part in the production of reducing power for nitrite reduction (Emes and Fowler, 1983; Oji *et al.*, 1985; Bowsher *et al.*, 1989) and fatty acid biosynthesis (Dennis *et al.*, 1997); hence, G6PDH represents the main site of reducing power for glutamate synthase in non-photosynthetic tissues (Bowsher *et al.*, 1992; Esposito *et al.*, 2003) and possibly in the chloroplast in the dark (Esposito *et al.*, 2005).

### **1.3 The NADPH/NADP<sup>+</sup> regulation and the effect of DTT**

The G6PDH is subjected to a fine control by reducing power: NADPH is a competitive inhibitor in *Ipomea batatas* (Muto and Uritani, 1972), spinach chloroplasts (Wendt *et al.*, 2000), pea chloroplasts (Scheibe *et al.*, 1989), soybean root nodules (Hong and Copeland, 1991), and potato (Knight *et al.*, 2001); this effect could be due to the interaction of reducing power with the tetrameric enzyme structure as described for the light effect (Wendt *et al.*, 1999).

The NADPH interaction occurs in the NADP<sup>+</sup> binding site, therefore making the site less available for the substrate; the inhibition decreases as NADP<sup>+</sup> levels increase, or as the NADPH/NADP<sup>+</sup> ratio decrease.

Therefore, G6PDH inhibition at increasing the NADPH/NADP<sup>+</sup> ratio, suggests a primary role in the regulation of the enzyme is played by NADPH (Esposito *et al.*, 2001; Lenzian and Bassham, 1975); *in vivo*, this ratio could be low during active biosynthetic processes (Huppe and Turpin, 1994) or modulated by the action of the malate valve (Scheibe, 2004).

In chloroplasts the G6PDH is inhibited by DTT (Fickenscher and Scheibe 1986; Srivastava and Anderson 1983; Lenzian, 1980; Wendt *et al.*, 2000; Anderson *et al.*, 1974) whereas the cytosolic G6PDH is less sensitive (Esposito *et al.*, 2001a; Fickenscher and Scheibe, 1986). This inhibition is dependent on the DTT levels and incubation time, and can be completely reversed by oxidants, such as sodium tetrathionate, thus suggesting that the enzyme is reversibly reduced (Esposito *et al.*, 2001a; Wendt *et al.*, 2000).

### **1.4 Effects of light on G6PDH activity. The role of thioredoxins**

Cytosolic isoforms are generally insensitive to light effects (Fickenscher and Scheibe 1986; Anderson *et al.*, 1974). In contrast, chloroplastic P1-G6PDH is inhibited by light to ensure an efficient Calvin cycle for the supply of reducing

power (Kruger and von Schaewen, 2003); in the dark this inhibition is removed and the OPPP is activated to produce reducing equivalents (Anderson *et al.*, 1974; Lenzian, 1980; Graeve and von Schaewen, 1997).

There are three possible mechanisms of light-dependent regulation of chloroplastic enzymes 1, effector-mediated; 2, ion-mediate; 3, protein-mediate.

1. The effector-mediated mechanism leads to an allosteric activation or inactivation of enzymes, based on light-induced change of relevant effector metabolites.
2. Enzymes regulated by an ion-mediated mechanism change their activity by altering pH or regulatory cations like  $Mg^{2+}$ . In chloroplasts, alkaline conditions occur only in the light.
3. The third mechanism of protein-mediated regulation involves enzyme modification by another class of proteins, the thioredoxins (Trx) that alter enzymatic activity by changing the redox status, specifically of disulfide bridges between cysteine residues of target proteins (Fig. 3).

The complete ferredoxin-thioredoxin system consists of ferredoxin, thioredoxin and ferredoxin-thioredoxin reductase (Buchanan *et al.*, 1994; Schürmann and Jacquot, 2000).

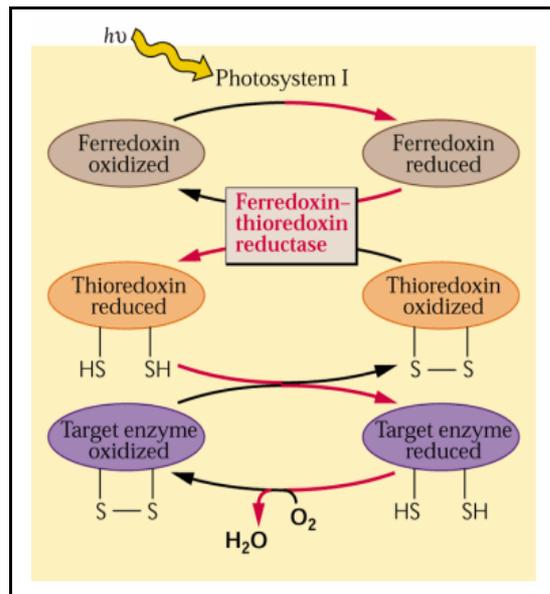
The thioredoxins are highly soluble proteins (12kDa) described in plant, animal and bacterial systems, regulating many cellular processes in photosynthetic and heterotrophic systems (Buchanan *et al.*, 1994; Schürmann and Jacquot, 2000) (Fig. 3). Their reduction is given by the action of a ferredoxin-thioredoxin reductase able to transport electrons from ferredoxin to Trxs.

Ferredoxin is the final acceptor of the photosynthetic electron transport chain: excitation of chlorophyll reaction centers, namely photosystem 1 (PSI) by light, transfers electrons to ferredoxin (and then to target acceptors like Fd-dependent enzymes and  $NADP^+$  which are reduced). Electrons are passed to ferredoxin-thioredoxin reductase reducing a disulfide bond to its dithiol form. In a similar event, thioredoxin acts as the protein mediator of light regulation

of a variety of enzymes, by changing their redox status.

The redox chain pathway guarantees that specific target enzymes are activated and kept in the reduced state in the light.

The thioredoxin regulation can be reversed. The target enzymes must be re-oxidized in the dark in order inactivate the light-dependent pathways and to activate dark-dependent pathways.



**Figure 3:** The ferredoxin – thioredoxin system (Buchanan *et al.*, 2000).

In plants many Trx isoforms are found, especially in plastids where they are subdivided into four types: the *f*- and *m*-types, initially defined by biochemical studies and the *x*- and *y*-type more recently identified by genomics (Lemaire *et al.*, 2007). In the model plant *Arabidopsis thaliana*, nine plastidial Trx isoforms are found.

The molecular basis for this control mechanism is disulfide-dithiol interchange of certain regulatory cysteine residues in the target enzymes.

In photosynthetic systems, reduction by thioredoxin generally leads to activation of an enzyme. One notable, yet not surprising, exception is G6PDH: in the light, reduced plastidial thioredoxin inactivates this enzyme of the OPPP.

*F*-type Trx regulates the activity of all redox-sensitive Calvin cycle enzymes,

in some cases specifically, as for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Marri *et al.*, 2009) and fructose-1,6-bisphosphate phosphatase. *m*-type Trxs regulates chloroplastic G6PDH in potato (Wenderoth *et al.*, 1997), but, quite unexpectedly, Trx *f* efficiently regulate the activity of *At*G6PDH1, in contrast, Trx *x* was a poor regulator of G6PDH activity (Née *et al.*, 2009).

The chloroplastic P1-G6PDH is known to be inactivated in the light by redox modification of the ferredoxin-thioredoxin system (Scheibe, 1990; Buchanan, 1991; Nee *et al.*, 2009).

G6PDH activity is turned off and is only active in its oxidized state in the dark when photosynthetic electron flow ceases. The reason for such regulation prevents carbohydrate breakdown by the OPPP that would work against concurrent CO<sub>2</sub> fixation by the Calvin cycle (Wendt *et al.*, 1999).

It has been demonstrated in potato that this regulation is carried out by at least two cysteine residues, Cys<sub>149</sub> and Cys<sub>157</sub> (Wenderoth *et al.* 1997), which are located in the N-terminus of the monomer; it should be noted that this part of the protein remains near the NADP<sup>+</sup> binding site in the 3D model.

Notably, chimerical enzyme formed by Cy-G6PDH plus the N-terminus containing the cysteines did not show the redox regulation of the chloroplastic isoform, suggesting that further factors are necessary to confer the regulation by reductants to plant G6PDH (Wendt *et al.*, 2000).

## **1.5 G6PDH in non-photosynthetic organisms**

Microbial and mammalian forms of G6PDH have been isolated from a number of species and G6PDH ranges in size from 50000-67000 Da. The enzyme is dimeric, tetrameric or may form higher order oligomeric forms (Levy, 1979).

Enzymatic activity for these forms is generally maximal around pH 7.4 with Michaelis constants of 0.86mM and 0.042mM for glucose-6-phosphate and NADP<sup>+</sup> respectively.

Animal tissues responsible for high levels of fatty acid biosynthesis experience coarse control of the OPPP at the level of G6PDH: in liver tissue, G6PDH is induced in response to high dietary carbohydrate, and repressed in response to starvation (Levy, 1979). The onset of lactation in mammals causes G6PDH activity to increase 60-fold. Elevated G6PDH activity has been noted in tumors of mammary and hepatic tissue.

Oxidative stress responses are also dependent upon G6PDH induction: during normal aerobic metabolism, H<sub>2</sub>O<sub>2</sub> and superoxides are formed, and reduced glutathione (GSH) is required to prevent oxidative damage caused by radical chain reactions; the OPPP generates reductants able to reduce oxidized glutathione (GSSG), as demonstrated in yeast and animal systems.

A particularly interesting example of the importance of G6PDH in oxidative stress is the favism, or G6PD disease in humans. Favism is an X-linked, hereditary genetic defect due to mutations in the G6PD gene. The favism is a form of hemolytic anemia which is induced by the consumption of fava beans, peas and other food that contain a substances like vicin or isouranyl; or molecules as naftalina or trinitrotoluene; or some drugs inhibiting G6PDH activity. These components leads to elevated oxidative stress in erythrocytes, hemoglobin denaturation, and eventual erythrocyte lysis (Pandolfi, 1995).

Many mutations in the G6PDH gene, carried on the X-chromosome cause the lack of functional G6PDH in erythrocytes of victims of favism; anyway the residual G6PDH activity is sufficient for basal metabolism.

During oxidative stresses caused by infections, namely malaria caused by

*Plasmodium falciparum*, the host infects the red blood cells. These cells, lacking of a functional G6PDH, rapidly go to death releasing great amount of potassium which is the first responsible of the parasite death. Therefore humans affected by favism are less susceptible to malaria.

Interestingly, females afflicted with favism but who are heterozygous for the mutant G6PDH allele, are resistant to malaria (Usanga and Luzzatto, 1985).

The mechanism of resistance relies on the phenomenon of random X-chromosome inactivation in females. and the fact that *Plasmodium*, also carries a G6PDH gene.

*Plasmodium falciparum*, the malaria parasite, relies on the host G6PDH, when infecting healthy (favism-free) individuals, but will express its own gene when infecting males afflicted with favism, *i.e.* those who lack functional G6PDH. Induction of G6PDH in *Plasmodium*, however, may take up to 60h, longer than the usual life cycle within an individual host cell (Usanga and Luzzatto, 1985).

## **1.6 G6PDH in photosynthetic organisms**

### **1.6.1 G6PDH in Cyanobacteria**

A single isoenzyme of G6PDH occurs in cyanobacteria; G6PDH was purified and characterized from *Anabaena sp.* (Gleason, 1996). This enzyme is specific for its substrates and inhibited by NADPH. The cyanobacterial enzyme exhibits sigmoidal kinetics with G6P (Rowell and Simpson, 1990; Gleason, 1996).

ATP inhibits the enzyme while glutamine, as well as other free amino acids, enhances activity, (Schaeffer and Stanier, 1978; Gleason, 1996; Rowell and Simpson, 1990). Divalent cations,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  cause an enhancement of activity.

G6PDH from *Anabaena sp.* is redox modulated; activity is inhibited by thioredoxins (Rowell and Simpson, 1990). The interactions with thioredoxins occurs on two cysteins differently located with respect to higher plants

chloroplastic G6PDHs (Wendt *et al.*, 1999). In Cyanobacteria the cysteine residues are positioned one on the N-terminus and the other in C-terminus of the protein sequence suggesting that the redox regulation has been evolved differently in cyanobacteria and higher plants giving a similar results by a molecular evolutionary adaptative convergence (Wendt *et al.*, 1999).

### **1.6.2 G6PDH in Green Algae**

Elevated G6PDH activity has been linked with inorganic nitrogen assimilation in unicellular green algae. The transfer of algal cultures to nitrogen free media results in an increase of G6PDH activity (Hipkin and Cannons, 1985; Huppe and Turpin, 1996).

In green algae cytosolic and plastidial isoforms have been described.

*Chlorella vulgaris* C-27 strain shows two types of G6PDH isozymes, which were considered as cytosolic and chloroplastic G6PDHs, (Honjoh *et al.*, 2003) with different DTT sensitivity and sequence similarity of N-terminal and internal amino acid sequences to other known homologues. Similarly *Chlamydomonas reinhardtii* possesses two G6PDH proteins, supposed to be located in cytosol and chloroplasts, the latter having a subunit molecular weight of 65.5 kDa.

A single glucose-6P dehydrogenase from *Chlorella sorokiniana* (211/8k) was purified by biochemical methods, showing a molecular weight of subunits 76 KDa (Esposito *et al.*, 2006). The kinetic parameters of *C. sorokiniana* G6PDH are similar to those previously measured for potato P2-G6PDH (Wendt *et al.*, 2000) and barley (Esposito *et al.*, 2001a; 2003), and therefore it can be supposed that this algal enzyme might be confined within the chloroplast (Esposito *et al.*, 2006).

## 1.7 Molecular biology of G6PDH in plants

The comparative analysis of G6PDH cDNA sequences from different plants, algae, cyanobacteria, fungi and animals allowed Wendt and co-workers (1999) the construction of a phylogenetic tree, identifying different families of known G6PDHs.

In this tree it is possible to identify a cyanobacterial group of enzymes that evolved independently and with which the G6PDHs from Eubacteria and Fungi share similarities.

Two other main branches are present: the first includes the isoforms from animals and the cytosolic enzyme of higher plants; the second one comprises the isoforms from red, green algae and the plastidic enzymes (Wendt *et al.*, 1999 – Fig.4).

The analysis of G6PDH plastidic sequences leads to a further classification into chloroplastic (P1-G6PDH) and plastidic (P2-G6PDH) isoforms, both encoded by nuclear genes and with a plastidic transit peptide at the N-terminus.

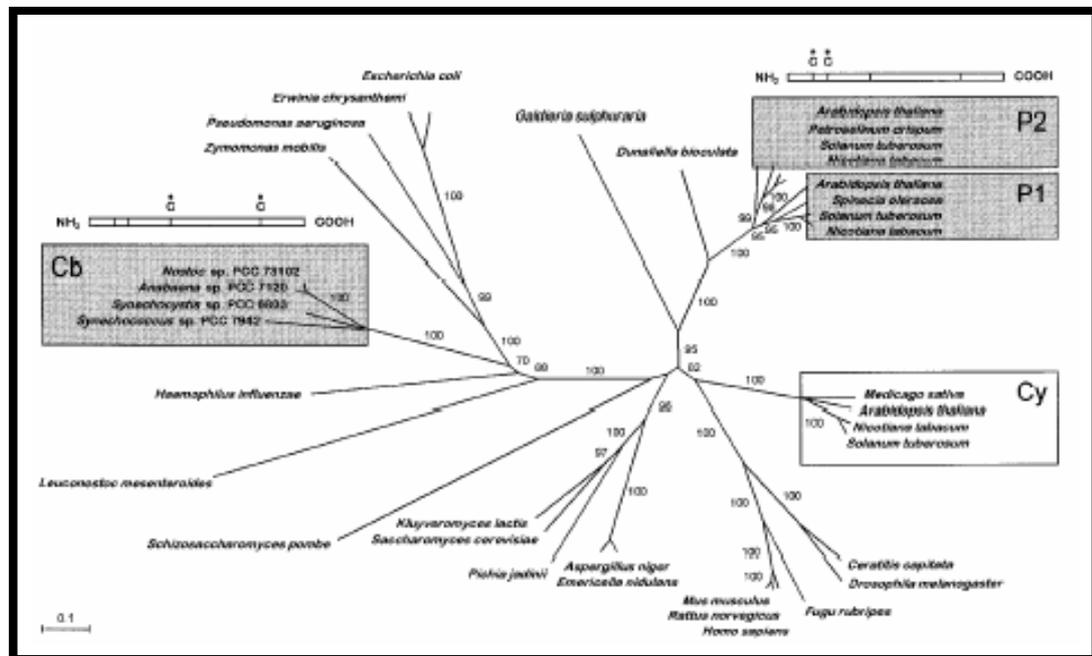
P1-G6PDH (chloroplastic isoform) is mainly expressed in photosynthetic tissues, while P2-G6PDH (plastidic isoform) is expressed in the most of plant tissues (Wendt *et al.*, 2000; Wakao and Benning, 2005).

Six cysteines are present in the plastidic isoforms, all located between the N-terminus and the center of the protein: these residues trigger reversible redox modifications inducing the formation of intra-, inter molecular or mixed-type disulphide bridge (von Schaewen *et al.*, 1995; Wenderoth *et al.*, 1997; Wendt, 1999).

The first known genomic plastidic G6PDH sequence has been obtained for the plastidic isoform from tobacco; it is made up of 10 exons (41.6% G+C) and 9 introns (34% G+C): the whole protein thus consists of 593 amino acids (Knight *et al.*, 2001).

The cDNA sequence of the cytosolic isoform in potato is typical of a eukaryotic gene, as it shows a polyadenylation signal at the 3' untranslated region and a Kozak consensus sequence at the initial codon (Grave *et al.*, 1994).

The cytosolic sequence obviously lacks a signal peptide and shows a 30-40% homology with respect to the cyanobacterial ones (exhibiting a different light/dark modulation with respect to plant P1- and P2-G6PDHs).



**Figure 4:** Phylogenetic tree of different isoforms of G6PDH (Wendt *et al.*, 1999)

## 1.8 G6PDH and nitrogen metabolism

Nitrogen assimilation in plant roots occurs mainly, if not exclusively, through the glutamine synthetase (GS) - glutamate synthase (GOGAT) cycle (Lea and Miflin, 2003), whereas a minor role is attributed to the glutamate dehydrogenase (GDH) reaction (Lewis *et al.*, 1983). The whole process is dependent on the supply of carbon skeletons and ATP, furnished primarily through respiration, and reducing power, which is derived by respiration and the oxidative pentose phosphate pathway (OPPP) (Wright *et al.*, 1997; Esposito *et al.*, 2001b). Therefore it is generally assessed that the oxidative pentose phosphate cycle in plants represents the major pathway in providing NADPH for nitrogen assimilation (Kruger and von Schaewen, 2003; Wang *et al.*, 2003).

The capacity of the OPPP is increased in plant tissues during nitrate assimilation, which requires reducing power for nitrate and nitrite reduction (Emes and Fowler, 1983; Bowsher *et al.*, 1989). Great attention has been given to the supply of the NADPH from OPPP to glutamate synthase (Bowsher *et al.*, 1992, Esposito *et al.*, 2003), and at least in barley roots, the activity of the plastidic G6PDH isoform is induced by ammonium (Esposito *et al.*, 2001b), at the same extent as nitrate, suggesting that this isoform is involved in the supply of reducing power for GOGAT activity (Esposito *et al.*, 2003), particularly the Fd-GOGAT isoform (Esposito *et al.*, 2005).

## **1.9 Abiotic stress in plants**

Plants are frequently exposed to various abiotic stress conditions such as low or high temperatures, salt, drought, oxidative stress and heavy metal pollution. Anthropogenic activity has accentuated some of these existing stress factors, e.g. heavy metals and salinity have begun to accumulate in the soil and often can reach toxic levels.

All these stresses limit the crop productivity: abiotic stress is the main cause of crop failure in the world, decreasing yields for most major crops by more than 50%, threatening the sustainability of agriculture. In response to stress factors several genes are up-regulated, mitigating effects of stress.

In nature many stresses can occur simultaneously; in response to these multiple signals, plants have developed different pathways to induce tolerance and/or resistance to stress.

Previous studies have shown that there is an association in plants between the OPPP and the response to different stresses, namely nutrient starvation, drought, salinity, pathogens, the Cy- and P2-G6PDHs being essential for stress tolerance (Esposito *et al.*, 2003; Esposito *et al.*, 2005; Nemoto and Sasakuma, 2000; Valderrama *et al.*, 2006; Wang *et al.*, 2008; Scharte *et al.*, 2009). The roles and functions of the three G6PDH classes in plants have been debated in the last years (Kruger and von Schaewen, 2003; Hauschild and von Schaewen, 2003; Esposito *et al.*, 2005; Wakao *et al.*, 2008).

A fundamental role in redox regulation for the plastidial P2-G6PDH has been described, this isoform appearing strictly linked to the reductant balance within the plastids and to stress response (Esposito *et al.*, 2003). Besides, the Cy-G6PDH is essential to enhance stress tolerance against oxidative burst and pathogen attacks (Scharte *et al.*, 2009).

## **1.10 G6PDH and plant hormones**

Phytohormones such as abscisic acid (ABA), jasmonic acid (JA), ethylene and salicylic acid (SA) appear to be critical components of the complex signalling

networks established during stress response (Zhu, 2002). ABA plays an important role in a number of physiological processes such as seed maturation and dormancy, stomatal closure, growth and developmental regulations (Zeevaart and Creelman, 1988; Nambara and Marion-Poll, 2005). Application of exogenous ABA is known to regulate a set of different genes suggesting that ABA is involved in the adaptative responses to both abiotic stresses such as cold, drought and salinity (Leung and Giraudat, 1998; Fujita *et al.*, 2006) and biotic stresses (Mantyla *et al.*, 1995; Li *et al.*, 2004; Fujita *et al.*, 2006; Fan *et al.*, 2009). The ABA-mediated gene regulation occurs through the presence of conserved ABA responsive elements (ABREs) in the gene promoters, usually accompanied by coupling elements (CEs). ABRE contains ACGT as a core nucleotide sequence, which acts as a binding site for bZIP family transcription factors governing transcriptional regulation of ABA responsive genes (Hattori *et al.*, 2002). Such an ABRE element has been found in both monocotyledonous and dicotyledonous plants, for example in *Zea mays* and *Arabidopsis thaliana* (Lenka *et al.*, 2009), as well as in the promoter of rice P2-G6PDH (Hou *et al.*, 2006), the ortholog of *Hordeum vulgare* P2-G6PDH ([AM398980](#)).

## **1.11 Research Objectives**

The putative barley roots P2-G6PDH has been isolated through an approach based on degenerated oligonucleotides designed in a highly conserved region. This CDS clone will be sequenced and compared to other known G6PDH sequences available from higher plants, algae, cyanobacteria, using softwares available on the web. Finally, a theoretical protein model will be generated to visualize the P2-G6PDH enzyme. The molecular modeling will be presented based on the coordinates from *Leuconostoc mesenteroides*. The comparative analysis of the sequence with others G6PDH putative genes known from other plants would confirm this belongs to the P2-G6PDH branch, and possibly give informations about the evolution of this particular group of plant G6PDH isoforms.

This sequence will be used to obtain either a recombinant his-tagged protein, or better an untagged version of G6PDH after overexpression in bacteria. This recombinant protein could be purified either by affinity chromatography or by traditional purification. The aim is to obtain a stable P2-G6PDH isoforms, given the partially failed attempts in the past on the behalf of different research groups. This possibly stable recombinant P2-G6PDH protein could be used for an accurate determination of kinetic parameters, immunological characteristics and redox regulation.

The availability of *Populus trichocarpa* genome, the first wood tree whose genome had been sequenced, would furnish a further possibility to clone and overexpress a P2-G6PDH, therefore a similar procedure will be followed to obtain another P2-type recombinant stable enzyme.

The other main point of this research is to investigate the role(s) of the different G6PDH isoforms upon exogenous ABA supply to barley plants grown in hydroponic cultures. The activity, occurrence and expression of the different G6PDH isoforms will be investigated both in roots and leaves. In addition, the importance of the plastidial P2-G6PDH in roots will be specifically discussed.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.2 Cultivation of plants

Seeds of barley (*Hordeum vulgare* L. var. Nure) were supplied by the Istituto Sperimentale di Cerealicoltura di Fiorenzuola d'Arda (PC) and germinated in the dark for 3–5 days on moistened paper. Seedlings were then transferred to a nitrogen-free medium (Rigano *et al.*, 1996a) in a controlled cabinet, according to Esposito *et al.* (2001a), at 21 °C under a 16/8 h light/dark regime with approximately 180  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . Nitrogen was supplied as 10 mM ammonium phosphate after 7d of hydroculture. The nutrient solutions were controlled for pH and daily adjusted to keep the initial ammonium/nitrate concentration.

#### 2.3 RNA preparation and first strand cDNA synthesis

##### 2.3.1 cDNA resource

Total RNAs were prepared from 100mg of barley roots using Trizol Reagent (Invitrogen) according to the manufacture's instructions.

The final RNA pellet was dissolved in DEPC-treated water and quantified reading  $\text{UV}_{260\text{nm}}$  absorbance (Nanodrop – 1000 spectrophotometer - Thermo Scientific).

cDNA synthesis was carried out using ThermoScript RT-PCR System (Invitrogen).

The first strand cDNAs were synthesized with 2ng of purified total RNAs using the RT-PCR system (Invitrogen).

The cDNA from poplar roots was prepared in the Laboratory of Professor Jean-Pierre Jacquot - University “Henri Poincaré” – Nancy – France.

In order to isolate the plastidic G6PDH cDNA from barley and poplar roots,

protein sequences of several G6PDHs available on GenBank were analysed and compared. The prediction protein websites (<http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html>; <http://urgi.versailles.inra.fr/predotar/predotar.html>; <http://psort.hgc.jp/form.html>; <http://www.cbs.dtu.dk/services/TargetP/>; <http://wolfsort.org/>) were utilised to identify the correct transit peptide, and to define the functional plastidic enzyme.

### 2.3.2 Design of specific primers

Based on the several plastidic G6PDH sequences available, a pair of specific primers were designed for cloning of the plastidic G6PDH gene from barley (*Hv*) and poplar (*Pt*) roots, and their sequences were as follows:

NAME	NUCLEOTIDE SEQUENCE
<i>Hv</i> P2-G6PDH-99-1-for (NcoI)1	5' CCCCCCATGGCGTCTGTTAGCATCACTGTG 3'
<i>Hv</i> P2-G6PDH-99-2-for (NdeI)	5'CCCCCCCCCATATGGCGTCTGTTAGCATC 3'
<i>Hv</i> P2-G6PDH-reverse (BamHI)	5' CCCC GGATCCCTAGTGTTC CGAGCCGCCCAG 3
<i>Pt</i> P2-G6PDH-66-1-for (NcoI)	5'CCCCCATGGCTCAAGATGGTGCAGTGGCC 3'
<i>Pt</i> P2-G6PDH-66-2-for (NdeI)	5'CCCCCCCCCATATGCAAGATGGTGCAGTGGCC 3
<i>Pt</i> P2-G6PDH reverse (BamHI)	5'CCCCGGATCCTTATTGCTCTATACCAAGGTC 3'

*Hv.*, *Hordeum vulgare*; *Pt.*, *Populus trichocarpa*

### 2.3.3 Cloning of plastidic G6PDH sequences

The first strand cDNA prepared from barley roots and poplar roots were used as a template of PCR. The PCR conditions were as follows: a pre-denaturation of 5 min at 94°C; 40 cycles of 94°C denaturation for 30s, 60°C annealing for 45s, 72°C for 2 min and final extension at 72°C for 15 min.

PCR products were separated on 1% agarose gel and a single band was purified using Amersham GE Healthcare kit according to the manufacture's protocol. Isolated DNA was digested with restriction enzymes NcoI/NdeI and BamHI,

by incubation for 1 hours at 37°C. The digested DNA was purified using a Amersham GE Healthcare kit according to the manufacture's protocol; then fragments were ligated into NcoI/NdeI and BamHI restriction digested vectors, pET3d and pET15b respectively. Ligation mix was incubated over night at 4°C and then utilised to transform *E.coli* DH5 $\alpha$  strain thermo-competent cells. Transformed colonies were grown on LB medium, upon ampicillin antibiotic selection. The colonies were selected and screened by PCR with specific primers, to check the positive clones.

The positive colonies were grown over night in 3ml of LB with ampicillin and then the pure plasmid was isolate using an Amersham GE Healthcare miniprep kit following manufacturer's instructions.

The pure plasmids were digested with NcoI/NdeI - BamHI then an agarose gel electrophoresis was performed to determine identify which the plasmids containing the correct inserts, based on their expected bp weight. Recombinant plasmid was prepared and sequenced to check the sequence and the correct frame.

## 2.4 Sequence analysis and production test of recombinant protein

The protein sequence for root barley (*Hordeum vulgare*) plastidic P2-G6PDH and for root poplar (*Populus trichocarpa*) plastidic P2-G6PDH were retrieved from the NCBI database (<http://www.ncbi.nlm.nih.gov>) and used to search against other genomes using BlastP or tblastn.

All the sequences are available at the following websites:

*Arabidopsis thaliana* (<http://www.arabidopsis.org/>)

*Oryza sativa* (<http://rice.plantbiology.msu.edu/>)

*Sorghum bicolor* (<http://genome.jgipsf.org/Sorbil/Sorbil.home.html>)

*Nicotiana tabacum* (<http://www.ncbi.nlm.nih.gov>)

*Populus trichocarpa* ([http://genome.jgi-psf.org/Poptr1\\_1/Poptr1\\_1.home.html](http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html))

*Solanum tuberosum* (<http://www.ncbi.nlm.nih.gov>)

*Triticum aestivum* (<http://www.ncbi.nlm.nih.gov>)

*Spinacia oleracea* (<http://www.ncbi.nlm.nih.gov>).

The amino acid alignments were performed using ClustalW ([www.ebi.ac.uk/Tools/clustalw2/index.html](http://www.ebi.ac.uk/Tools/clustalw2/index.html)) and the phylogenetic tree was constructed using the neighbour-joining tree algorithm in MEGA version 4 (Tamura *et al.*, 2007).

Plasmids (pET3d and pET15b) containing the correct and in frame P2-G6PDH sequences were used to transform *E.coli* BL21(DE3) (Novagen) for overexpression of the recombinant enzymes. Electroporation was made using Bio-Rad electroporation cuvettes (0.2 cm gap) under standard conditions for *E. coli* strains. Transformant bacteria were selected by growing on media containing ampicillin .

Individual positive colonies were picked and grown in liquid culture (3ml) for 4h, until the OD<sub>600</sub> was 0,6 then the synthesis of recombinant protein was induced by addition of Isopropyl B-D galactoside (100mM IPTG) for 3h.

The bacterial suspension was centrifuged for 1' at 13000 rpm and the pellet resuspended in BBP (Bromophenol blue: 1% (w/v) sodium dodecyl sulfate

(SDS) and 5% (v/v) 2-mercaptoethanol) and samples (5  $\mu$ l) were run on 10-12% SDS-PAGE to check the occurrence of the recombinant protein.

The positive clones were incubated in 100 ml of LB medium overnight at 37°C with aeration; then the culture was transferred in 2,5 l of LB liquid medium. When OD<sub>600</sub> the medium reach about 0.6, UA the expression of target gene was induced by adding 100mM isopropyl-thiogalactopyanoside (IPTG). After incubation at 20°C overnight under continuous shaking, cells were collected by centrifugation for 20' at 5.000rpm (7000g) at 6°C in a Beckman JA25 centrifuge equipped with a JA14 rotor. The pellet was resuspended in Buffer TE [30 mM Tris-HCl (pH 8.0), 1 mM EDTA] and cells ruptured by sonication 10' 30s ON / 30s OFF 10% amplitude. The cells lysates were then centrifuged 60' at 14.000 rpm (20000g) at 4°C in a Beckman JA25 centrifuge equipped with a JA25.50 rotor. All the subsequent steps were performed at 2-6°C.

### 2.5 Electrophoresis and Western blotting analysis

All electrophoresis and western blotting analyses were performed with the GE Healthcare Bio-Sciences Mini VE System equipped with EPS 301 power supply.

SDS-PAGE was performed using 10% acrylamide resolving gel with a 4% stacking gel. Before loading, samples were boiled for 10 min, in the presence of BBF to ensure protein denaturation. Proteins were subject to electrophoresis under a constant voltage of 180 V, 40mA until the bromophenol blue dye front was at bottom of the gel.

After electrophoresis, proteins were stained using Coomassie brilliant blue, 10% (v/v) acetic acid and 40% (v/v) methanol. To visualize protein spots the gel was quickly destained with 10% (v/v) acetic acid in 25% (v/v) methanol for 60 seconds to remove excessive dye and then destained in 25% (v/v) methanol for another 24 hours under gentle orbital shaking.

For western blotting analysis, the separated polypeptides were transferred (2h – 25V, 300 mA) on a Hybond membrane (Ge Healthcare). After the transfer,

the membrane was incubated with primary G6PDH antibody from potato for P1-, P2- and Cy-G6PDH isoforms (Wendt *et al.*, 2000). These antibodies have proven to react with and discriminate the different barley G6PDH isoforms in previous papers (Esposito *et al.*, 2001b, 2003, 2005). After washing, the membranes were incubated with secondary antibodies coupled to alkaline phosphatase as previously described (Esposito *et al.*, 2005).

## **2.6 Purification of recombinant P2-G6PDH (pET3d)**

### **2.6.1 Soluble protein**

The supernatant of transformed bacteria lysates was retained as soluble protein fraction; then desalted on a GE- Healthcare PD10 column (Sephadex G25), and assayed for G6PDH activity. The desalted extract was applied to a GE Healthcare Resource Q column (6ml) connected to a GE- Healthcare AKTA prime plus system (Amersham Biosciences – GE Healthcare). UV absorbance, temperature, ionic strength and pressure over the chromatographic column were continuously monitored using a personal computer connected to the AKTA Prime and equipped with Primeview<sup>®</sup> software (Amersham Biosciences – GE Healthcare).

The column was washed with the buffer TE and the proteins were eluted applying a 0-400mM NaCl linear gradient at a flow rate of 1 ml min<sup>-1</sup>. and 2ml eluted fractions were assayed for G6PDH activity.

The active fractions were collected and desalted as described above. The partially purified enzyme sample was applied to a HiTrap-Blue HP (1ml) column, previously equilibrated with buffer TE connected with the AKTA<sup>®</sup> prime plus system. The NAD<sup>+</sup> utilising enzymes were eluted with Buffer TE plus 5mM NAD<sup>+</sup>. The NADP<sup>+</sup> utilising enzymes were eluted with Buffer TE plus 1mM NADP<sup>+</sup>; the recombinant P2-G6PDH was eluted with Buffer TE plus 5mM NADP<sup>+</sup> and 1.5M NaCl, and fractions showing G6PDH activity were pooled to estimate the kinetic parameters.

### **2.6.2 Insoluble recombinant protein (inclusion bodies)**

The inclusion bodies was thus resuspended in buffer [Tris-HCl 50mM, 5mM MgCl<sub>2</sub>, 1mM EDTA, 10 % glycerol] in the presence of 20mM DTT and 7M Guanidine-HCl pH 7.9, sonicated as previously described, centrifuged, kept under nitrogen, and samples were shaken 4h at 4°C.

The denatured protein solution was diluted 20-fold in renaturing buffer [Tris-

HCl 50mM, 5mM MgCl<sub>2</sub>, 1mM EDTA, 10% glycerol] in the presence of 200μM NADP<sup>+</sup>, 0.5M arginine, 10mM DTT, pH 7.9. The solution was kept under N<sub>2</sub> at room temperature for 5-10 days (usually one week) and then the renatured protein concentrated about 8-10-fold by ultrafiltration under N<sub>2</sub> (Aminco PW10 filters). The final solution was desalted and assayed for G6PDH activity and for the determination of kinetic and immunological properties.

## **2.7 Purification of soluble P2-G6PDH (pET15b).**

### **2.7.1 Soluble protein**

The supernatant of transformed bacteria lysates in buffer H (10mM imidazole, KH<sub>2</sub>PO<sub>4</sub> 20mM(pH 7.4), NaCl 0.5M) was retained as soluble protein fraction; was desalted on a GE- Healthcare PD10 column (Sephadex G25), and assayed for G6PDH activity.

The desalted extract was filtered (0.22 μm) and applied to a GE Healthcare HiTrap FF crude column (1ml) connected to a GE- Healthcare AKTA Prime plus system (Amersham Biosciences – GE Healthcare). UV absorbance, temperature, ionic strength and pressure over the chromatographic column were continuously monitored using a personal computer connected to the AKTA Prime and equipped with Primeview<sup>®</sup> software (Amersham Biosciences – GE Healthcare).

The column was washed with the buffer H and proteins were eluted applying Buffer I [0.5M Imidazole, KH<sub>2</sub>PO<sub>4</sub> 20mM (pH 7.4), NaCl 0.5M]. Eluted fractions (1ml) were assayed for G6PDH activity.

The active fractions were collected and desalted as described above and used to estimate the kinetic parameters.

### **2.7.2 Insoluble recombinant protein (inclusion bodies)**

The recombinant protein from *E. coli* inclusion bodies was refolded and purified using a 1ml HisTrap FF column. The inclusion bodies was resuspended in cold isolation buffer: 2M urea, 20mM Tris-HCl, 0.5M NaCl, 2% Triton-X 100, pH

8.0, sonicated as described before, and centrifuged at high speed for 10min at 4°C. After removing supernatant the pellet was resuspended in 3ml of cold isolation buffer. After a further sonication and centrifugation, the inclusion bodies were washed in binding buffer (lacking urea) composed with 6M guanidine hydrochloride, 20mM Tris-HCl, 05M NaCl, 5mM imidazole, 1mM  $\beta$ -mercaptoethanol, pH8; after 1h at room temperature on shaker the sample was centrifuged for 15' at 13000rpm at 4°C, the supernatant was filtered ( 0.45  $\mu$ m); the pH was adjusted at 8.0 and loaded on column connected to an AKTA Prime plus system, using stored purification programs and following manufacturer's instructions. The buffer used for elution the protein was composed of: 20mM Tris-HCL, 0,5MNaCl, 0,5M imidazole, 1mM  $\beta$ -mercapthoethanol, pH8, and the refolding buffer was composed of 20mM Tris-HCL, 0,5M of NaCl, 5mM imidazole, 1mM  $\beta$ -mercapthoethanol, pH 8.

## **2.8 G6PDH activity assay**

G6PDH activity was assayed by monitoring NADP<sup>+</sup> reduction at 340nm. The assay mixture contained: 50mM Tris-HCl pH 8.0, 50mM MgCl<sub>2</sub>, 1.5mM NADP<sup>+</sup>, 30mM glucose-6P, and extract (10-100  $\mu$ l; 3-60  $\mu$ g prot). For enzyme activity measurements against a blank without G6P; three different replicates were performed.

The activity was expressed as nmol NADP<sup>+</sup> reduced min<sup>-1</sup> mg<sup>-1</sup> prot.

## **2.9 Determination of the kinetic constants of the recombinant P2-G6PDH**

Purified enzymes were incubated in the G6PDH assay mixture described above, and  $Km_{G6P}$  measured by varying the concentrations of glucose-6P from 0 to 30mM, changing the NADP<sup>+</sup> concentration from 0 to 150 $\mu$ M; results are plotted, and kinetic parameters calculated, using Graph Pad Prism 4.0 software.

## **2.10 Midpoint redox potential**

Oxidation–reduction titrations were carried out to monitor the redox state of the regulatory disulfide in the presence of mixtures of oxidized and reduced DTT to poise the ambient-redox potential. The recombinant protein (*PtP2-G6PDH* (pET15b), 50 $\mu$ g) in its initial redox state was incubated at 25 °C in 30 mM Tris-HCl, 1mM EDTA pH 7, with a different concentration mix of DTT<sub>ox</sub>/DTT<sub>red</sub> for 4h, after then 1.84mM of mBBr (monobromobimane) was added, and samples incubated 20' in the dark; after spinning 4', 20% TCA was added, samples cooled on ice for 30', and centrifuged for 15' at 13000rpm.

1% of TCA was added to the pellets, centrifuged 15' at 13000rpm, after removing the supernatant, 0,1M Tris-HCl (pH8) containing 1%SDS was added, vortexed and after further 30', fluorescence was measured in a spectrofluorimeter (Cary Eclipse Fluorescence Spectrophotometer). Experiments were repeated two times. The data fit the Nernst equation for a single two-electron process ( $n = 2$ ).

## **2.11 Determination of protein content**

Protein concentrations were determined using the Bio-Rad protein assay based on the Bradford method (1976) with bovine serum albumin as the standard. During the chromatography steps proteins were monitored at 280nm.

## **2.12 Enzyme units and data statistics**

Enzyme rates were measured as nmoles of product formed in 1min under the conditions of assay; enzyme activities are expressed per ml or per mg of protein in the extract.

All the data presented are representative of the experimental behaviour and are means of at least three separate determinations  $\pm$  standard error (SE).

## **2.13 ABA experiments**

### **2.13.1 Cultivation of plants**

Plants were cultivated as described before. After seven days of growth (“0” experimental time), 0.1 mM ABA was supplied to the nutrient medium. Plants were harvested at different times of exposure to ABA (3h, 6h, 9h, 12h, 24h, 48h), and G6PDH activity was measured as described in Esposito et al., (2001a). Data shown in figures or tables are representative of five separate experiments.

### **2.13.2 Preparation of crude extracts for enzyme activities**

Plants were collected 2 h after the beginning of the light period (16h), thus any group of plants was taken under illumination. Each replicate is done from a group of 10-40 plants to generate at least 2 gr (usually 3-5 gr) of roots or leaves. Plant material was quickly frozen in liquid nitrogen, and powdered in a mortar with a pestle and proteins were extracted in 100 mM Tris-HCl pH 7.9, 10 mM MgCl<sub>2</sub>, 4 mM EDTA, 1 mM PMSF, 10% glycerol, 15 μM NADP<sup>+</sup>. The homogenate was then filtered through four layers of muslin and centrifuged at 20000xg for 20 min at 4 °C. The supernatant (fraction designated as the crude extract) was used for G6PDH assays.

### **2.13.3 Semi-quantitative RT-PCR assay**

Semi-quantitative RT-PCR experiments were done to estimate the expression of G6PDH transcripts in leaves and roots. Total RNAs were extracted from 100 mg of barley roots and leaves using Trizol Reagent (Invitrogen) according to manufacturer's instructions. The final RNA pellet was dissolved in DEPC-treated water and quantified with UV spectrophotometry. cDNA synthesis was carried out using ThermoScript RT-PCR System (Invitrogen). PCR amplification was performed with 10ng/μl of cDNA as PCR-template and 2.5 pmol of the primers in a final volume of 25 μl. Syntheses of cDNA and PCR amplification were carried out with a thermal cycler (GeneAmp PCR System 2700, Applied Biosystems). The program used was as follows: 3 min denaturation at 94° C, and 40 denaturation cycles of 1' at 94° C, 30 s annealing at 58° C, 30 s extension at 72° C; extension in the last cycle was prolonged for 10 min. Primers were designed for plastidial P2-G6PDH:

forward: 5'-GGGAAAGGAGCTGGTGGAGAAC-3'

reverse: 5'-TATTCTCAGAAGACTTTGGCAC -3'

and for cytosolic Cy-G6PDH:

forward: 5'-ATACGAGCGCCTCATTTTGG-3'

reverse: 5'-ACAACATCGACGCTGGCAA-3'.

As an internal control the constitutively expressed ribosomal 18S gene was amplified from various samples to generate a 600 bp fragment. The PCR conditions for amplifying 18S gene were a pre-denaturation of 5 min at 94° C; 35 cycles of 30 s at 94° C, 45 s at 50° C, 30 s at 72° C and an extension for 10 min at 72° C.

The sequences of the primers for 18S gene are as follows :

forward: 5'- GGAGAAGTCGTAACAAGGTTTCCG 3'

reverse: 5' -TTCGCTCGCCGTTACTAAGGG 3'.

As control, PCR analyses were made on roots and leaves extracts from untreated plants at the same given times; in these samples the expression levels of both Cy-G6PDH and P2-G6PDH remained constant throughout the experiments (not shown).

#### **2.13.4 Analysis of PCR products**

The amplified products were resolved on a 1.5% agarose gel, and the DNA was visualized by ethidium bromide, using an UV trans-illuminator and acquired by a camera. Each gel photo was processed using Image J (NIH) to obtain a threshold black and white pixel map. The spots were quantified as arbitrary units (pixel) in an Excel worksheet, and used to construct a graph using the Sigmaplot (Jandel) software. The experiment shown is representative of three experiments which showed the same general behaviour, the average values and the standard errors are given in the chart below the gel photos in the figures.

## CHAPTER 3

### RESULTS

#### 3.1 Bioinformatic analysis of G6PDH isoforms in plants

In plants different G6PDH isoforms can be described using a phylogenetic analysis.

Among 72 cDNA sequences found in database encoding for G6PDH, 22 were from higher plants (*Medicago truncatula*, *Populus trichocarpa*, *Sorghum bicolor*, *Spinacea oleracea*, *Arabidopsis thaliana*, *Nicotina tabacum*, *Hordeum vulgare*, *Oryza sativa*), 7 in Briophyta (*Physcomitrella patens*), 9 from green algae (*Dunaliella bioculata*, *Coccomyxa sp.C-169*, *Chlorella vulgaris*, *Volvox carteri*, *Ostreococcus lucimarinus*, *Ostreococcus tauri*), 1 from the red alga *Galdieria sulphuraria*, 15 from Fungi (*Agaricus bisporus*, *Alternaria brassicola*, *Aspergillus niger*, *Aspergillus carbonarius*, *Batrachochytrium dendrobatidis*, *Chaetomium globosum*, *Cochiliobolus heterostrophus*, *Coprinopsis cinerea*, *Cryptococcus niger*, *Haeterobasidion annosum*, *Laccaria bicolor*, *Melampsora laricis-populina*, *Emericella nidulans*, *Kluyveromyces lactis*, *Saccharomyces cerevisiae*), 7 from Bacteria (*Erwinia crysanthemii*, *Escherichia coli*, *Haemophilus influenzae*, *Leuconostoc mesenteroides*, *Zymomonas mobilis*, *Rhodopsedomonas palustri*, *Chlorobium tepidum*), 5 from Cyanobacteria (*Anabaena cylindrica*, *Gloeobacter violaceus*, *Mycrocistis aeruginosa*, *Synechococcus elongatus*, *Nostoc*), 1 from the diatom *Phaeodactylum tricornutum*, 5 from Metazoa (*Drosophila melanogaster*, *Homo sapiens*, *Fugu rubripes*, *Mus musculus*, *Rattus norvegicus*).

All the sequences present the Rossman fold “ASGDLAKKK” and the central part of NADP<sup>+</sup> binding site “NELVI” (Asn-Glu-Leu-Val-Ile), where often Leu is changed in Phe.

Interestingly, the active site of cytosolic and plastidic isoforms is slightly different: in fact the Cy-G6PDH presents the peculiar sequence: YRIDHYLGK; all the plastidic isoforms present a change in the first amino

acid of the active site sequence: FRIDHYLGK. It should be underlined that this difference can be found in both algae, Bryophyta, and higher plants.

The redox sensitivity is a feature of P1 and P2 G6PDH from algae, bryophyte and higher plants, and of cyanobacteria enzymes. Although this regulation can be ascribed to the presence of regulatory cysteines, it is noteworthy that the positions of the cysteines is different in cyanobacteria, where one cys residue is located on the N-terminal and the other on the C-terminal; and eukaria plastidic enzymes, in which both cys residues are located on the N-terminus in the positions 145-155 or 151-156, distanced of exactly 6 amino acid residues.

The analysis of all the sequences using different prediction web site (material and methods) suggested the subcellular localisation of the isoforms.

A further analysis was made to check the occurrence, number and position of cysteines.

Only 2 cys residues were found in the bacterium *Zymomonas mobilis* (P21907), in cyanobacteria *Synechococcus* (P29686) and *Mycrocystis aeruginosa* (MAE43640), in the fungi *Heterobasidion annosum* (jgi|Hetan1|146671|estExt\_fgenesh3\_kg.C\_40156) and *Melampsora laricis-populina* (jgi|Mellp1|42219|estExt\_Genewise1.C\_60001), (jgi|Mellp1|74091|estExt\_fgenesh1\_pm.C\_30094).

Only one cys residue was identified in *Saccharomyces cerevisiae* (P11412).

A group comprising all the cytosolic G6PDHs from higher plants is clearly associated with cytosolic Bryophyta and algal enzymes, and all these sequences appear correlated with Metazoa G6PDHs. All these sequences are devoid of transit peptides and at the same time present a reduced number of cysteines, none in the N-terminal part of the protein in the zone 125-180 aa. It should be noted that all the fungal G6PDHs (which possess cy-G6PDH solely) are on the same branch of the evolutionary tree. Looking the tree clockwise, (Fig. 5) follow the Rhodophyta branch, the Diatoms, and then Prokaryotes: Green bacteria, Bacteria and cyanobacteria.

On the left side of the tree lie all the plastidic isoforms being characterized by

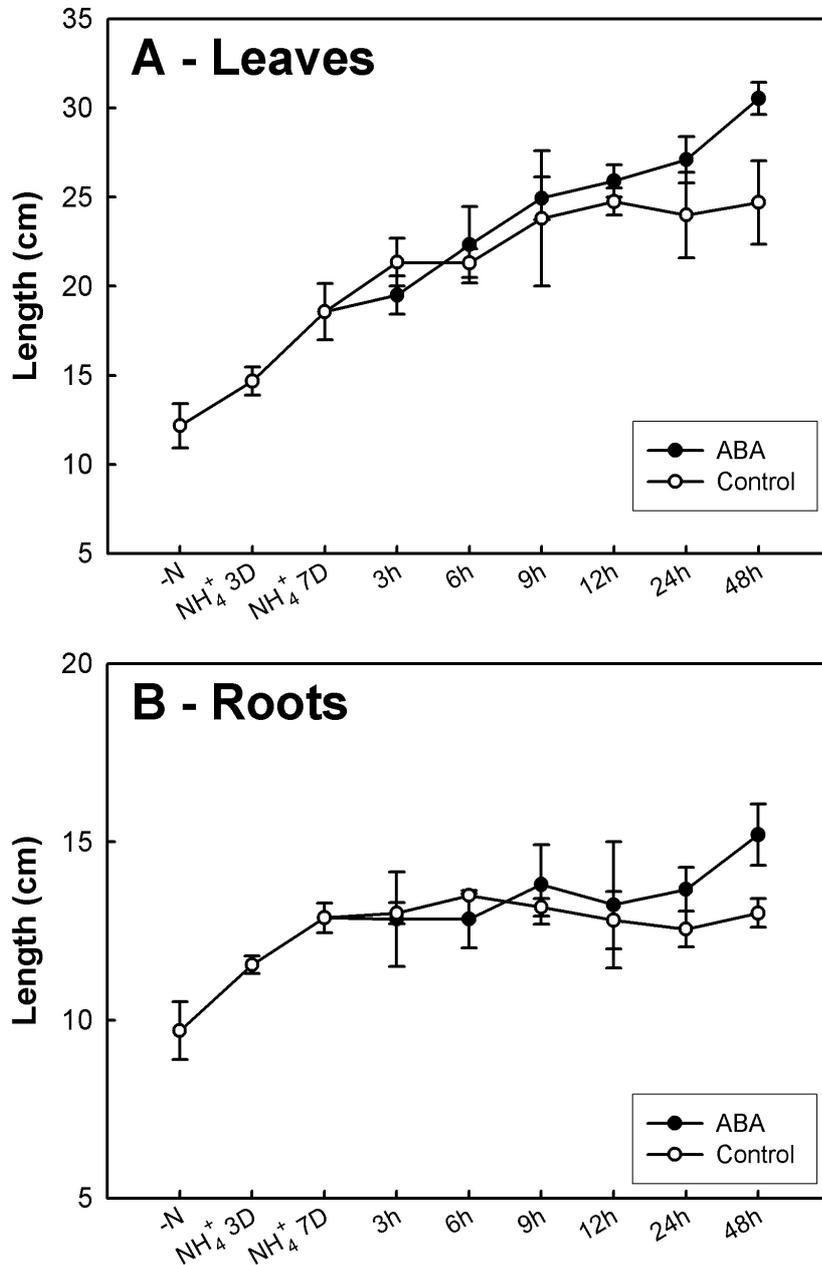
the presence of a plastidic transit peptide and two regulatory cysteines on the N-terminus. It is very interesting to note that the branch with plastidic higher plants G6PDH present single ramifications for dicotyledons P2-G6PDH and monocotyledons P2 G6PDH, and a similar description can be made for P1-G6PDH branch. Note that Bryophyta present two groups of compartmented G6PDH, one more similar to cytosolic G6PDH, the other is halfway between P1- and P2-G6PDH from higher plants.



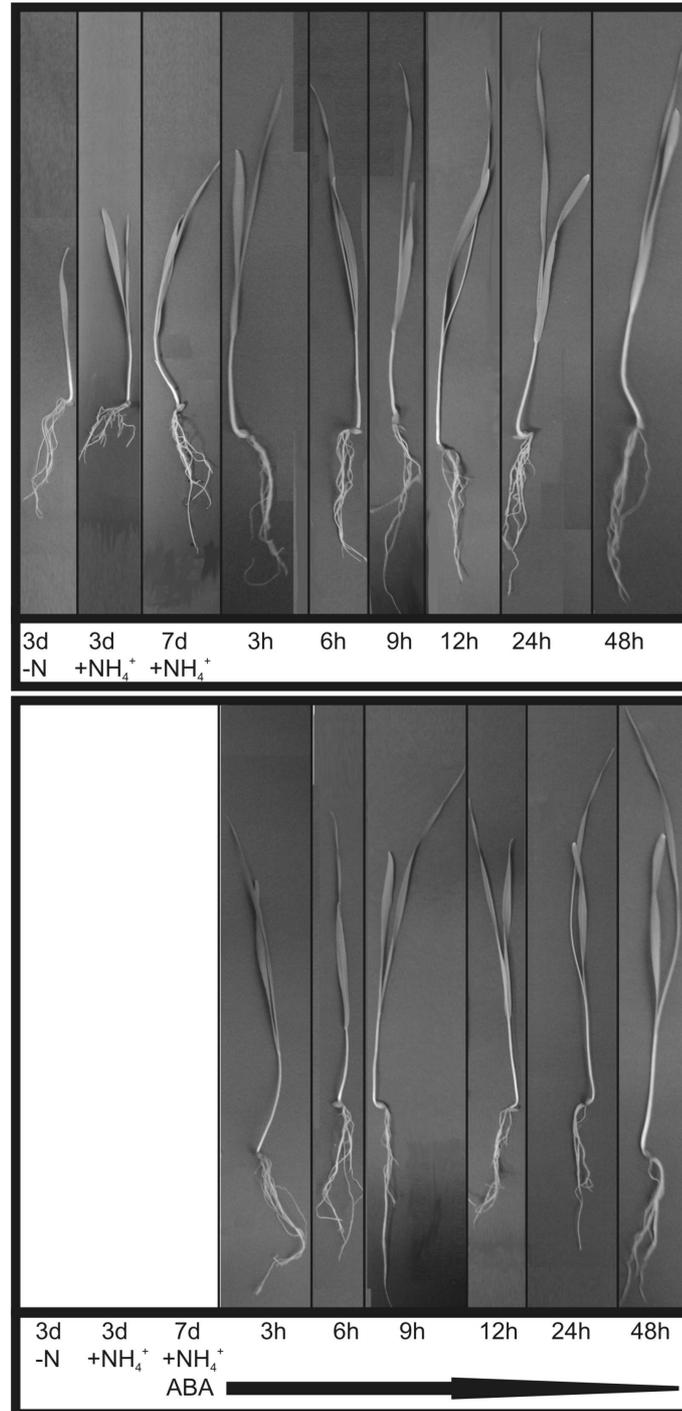
## **3.2 The effects of Abscissic acid on activity and occurrence of G6PDH isoforms in barley**

### **3.2.1 Effects of ABA supply on barley plants**

Application of ABA to the barley seedlings during the first 24h of experimental treatment had no visible effect on the root length, and slightly increased the length of the leaves (Fig. 6). After 48h of ABA treatment, a 23% increase in leaf and a 17% in root lengths were observed with respect to control plants. Pictures of ABA supplied plants and control plants at different times during experiments are shown in supplemental data (Fig. 7).



**Figure 6:** Relative growth rate of barley roots and leaves after 0.1mM ABA addition. Barley seedlings were grown for 3 days in hydroculture without any nitrogen source, then grown under 5mM ammonium phosphate for 7 days; then seedlings were supplied with 0.1mM of ABA and representative samples collected at given times. Measurements are average from three different plants  $\pm$  s.e.

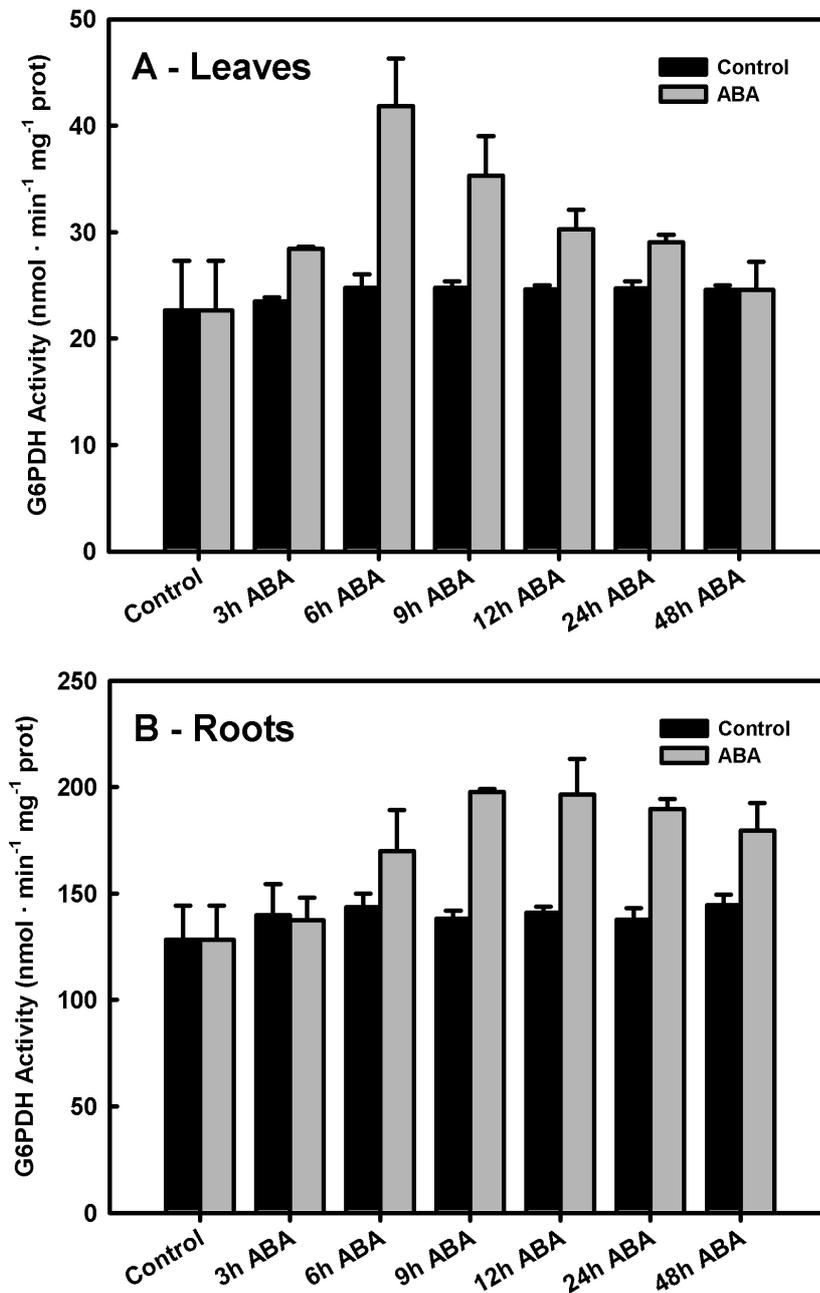


**Figure 7:** Relative growth rate of barley seedlings upon 0.1mM ABA. Barley seedlings were grown for 3 days in hydroculture without any nitrogen source, then grown under 5mM ammonium phosphate for 7 days; then seedlings were supplied with 0.1mM of ABA and representative samples collected at given times. Appearance of control plants is shown above in comparison with ABA treated plants (below).

### **3.2.2 Total G6PDH activity in roots and leaves upon ABA treatment**

To study the effect of ABA on G6PDH activity, exogenous 0.1mM ABA was applied to the seedlings and the total G6PDH enzymatic rates were measured in root and leaf extracts. ABA (0.1mM) caused a rapid increase (+85%) in G6PDH activity in leaves within 6h. Thereafter enzyme activity rates decreased constantly until they reached the control values within 48h (Figure 8A). Control plants (no ABA supply) showed no appreciable variation (less than 9%) in G6PDH activity throughout the experiments (Fig. 8A).

Application of ABA caused a gradual increase in total G6PDH activity between 3h and 12h in roots (+54% with respect to activity detected at experimental starting conditions (plants grown for 7d in hydroponic culture). In the following days, G6PDH activity remained at the same high values (Fig. 8B). In control plants (without ABA supply), enzyme activity did not appreciably change ( $\pm 10\%$ ) throughout the experiments (Fig. 8B).



**Figure 8:**Total G6PDH activity in barley leaves (A) and roots (B). Barley seedlings were grown for 3 days in hydroculture without any nitrogen source, then under 5mM ammonium phosphate for 7 days; then seedlings were supplied with 0.1mM of ABA and samples collected at given times and crude extracts of leaves and roots were assayed for G6PDH activity. Data shown are average  $\pm$  standard error of three different determinations measured at least in duplicate.

### 3.2.3 Effect of ABA on the occurrence of the different G6PDH isoforms

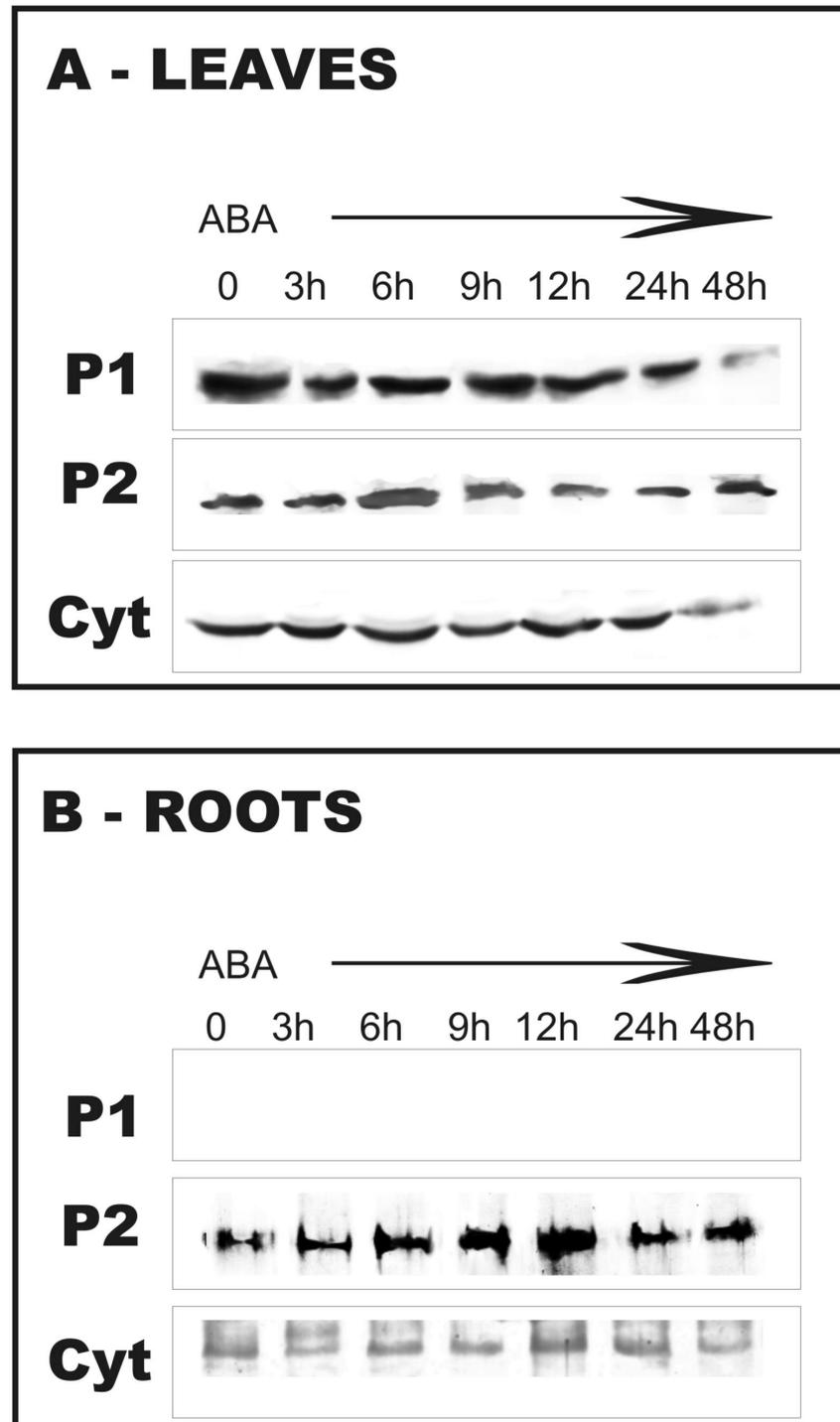
The abundance of G6PDH proteins was investigated by Western blotting using antibodies able to discriminate the different isoforms (Wendt *et al.*, 2000; Esposito *et al.*, 2005).

The Cy-G6PDH protein content in leaves did not apparently change within the first 12h of ABA treatment. In leaf crude extracts, the amount of P2-G6PDH strongly increased (1.9-fold) within 6h following the ABA treatment, and then it returned to levels comparable to those of control plants in the following hours/days. In leaves, the amount of P1-G6PDH decreased rapidly 3h after ABA treatment and this decrease level remained constant (Fig. 9A).

These results suggest also in green tissues the involvement of plastidial (and possibly chloroplastic) isoforms in the changes in G6PDH activity observed in crude leaf extracts (compare with Fig. 8A).

In roots, the Cy-G6PDH protein content showed a slight increase within 12-24h following ABA treatment (Fig. 5B). In roots, supply of exogenous ABA increased progressively the P2-G6PDH signal with respect to control plants within 12h. The densitometric analysis (Image J software – NIH – USA) indicates an increase of 70% after 3h until 12h and up to 2.8-fold at 12h. Therefore, changes in abundance of P2-G6PDH (Fig. 9B) suggest that there is a gradual and consistent increase of P2-G6PDH protein levels in roots, mimicking that observed for total activity (Fig. 8B). This increase of P2-G6PDH protein content in roots following ABA supply strongly supports the involvement of this isoform in the response to abiotic stress in plants.

The absence of signal for the chloroplastic P1-G6PDH in barley roots confirmed that it is not or very weakly synthesized in non photosynthetic tissues, making the plastidial P2-type isoform the only G6PDH representative for this compartment in roots (Fig. 9).



**Figure 9 :** Western blots of G6PDH isoforms from crude extracts of leaves (A) and roots (B) of barley plants subjected to an ABA treatment. The seedlings were grown on a medium supplied with 0.1mM of ABA and samples collected at given times. Detection of different G6PDH isoforms was made using antibodies raised against potato cytosolic Cy-G6PDH; and plastidial P2-G6PDH; chloroplastic P1-G6PDH enzymes (Wendt *et al.*, 2000).

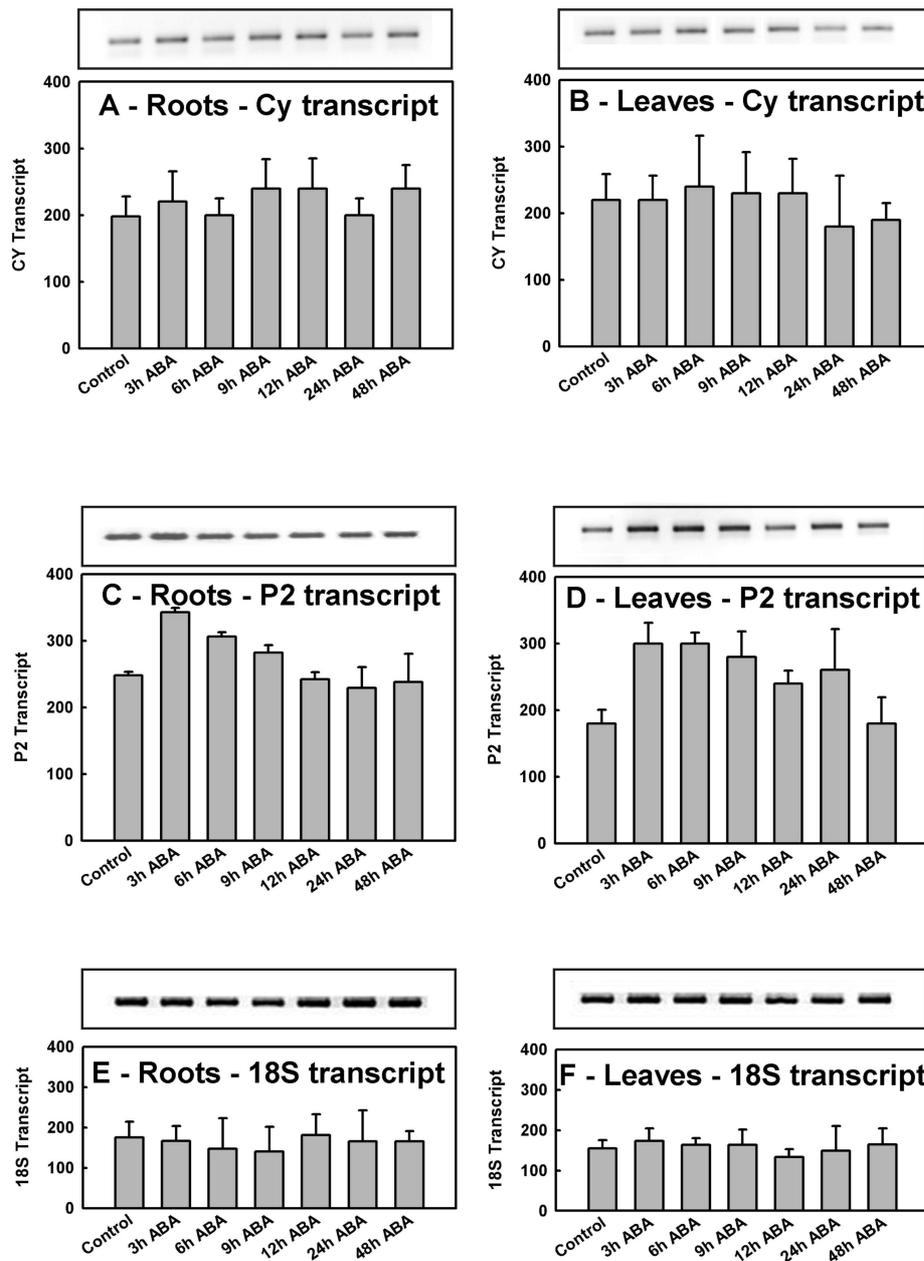
### **3.2.4 Expression of the Cy- and P2-G6PDH genes in ABA-treated plants**

The transcript level of Cy-G6PDH in roots did not appreciably change after ABA supply fluctuating within 20% all over the experiment (Figure 10a).

Regarding P2-G6PDH transcript levels, a 40 % increase is observed in roots within 3 hours of ABA supply as attested by the densitometry analysis. Afterwards, the transcript levels slowly declined until reaching the initial levels at 12 h, then remaining unchanged at 24h and 48h (Figure 10c). Thus, the progressive increase in total G6PDH activity measured in the first 12h could be due at least in part to an increase in transcription and synthesis of P2-G6PDH protein induced by the hormone in the roots (Figure 9).

Similarly, in leaves, the amount of Cy-G6PDH transcripts remained unchanged with respect to the control levels in the first 12h (Fig 10b). This stability of the Cy-G6PDH transcript in leaves, corresponded to the Cy-G6PDH protein amount detected in western blots (Fig 9).

In leaves, ABA supply caused a rapid and significant increase (+66%) of the P2-G6PDH transcript abundance within 3h, after which the levels remained higher than initial levels until 48h when it returned to the initial value (Figure 10d).



**Figure10:** G6PDH transcript expression profiles after ABA treatment. Semi-quantitative RT-PCR were performed with RNA extracted from roots (left) and leaves (right) of samples collected at given times from seedlings supplied with 0.1mM of ABA. A and C: Cy-G6PDH transcript levels; B, D: P2-G6PDH transcript levels; E, F ribosomal 18S transcript levels used as control for roots and leaves, respectively.

The graphs show the quantification of transcript obtained using Image J software (NIH – USA) indicated by bars. Data shown are average  $\pm$  standard

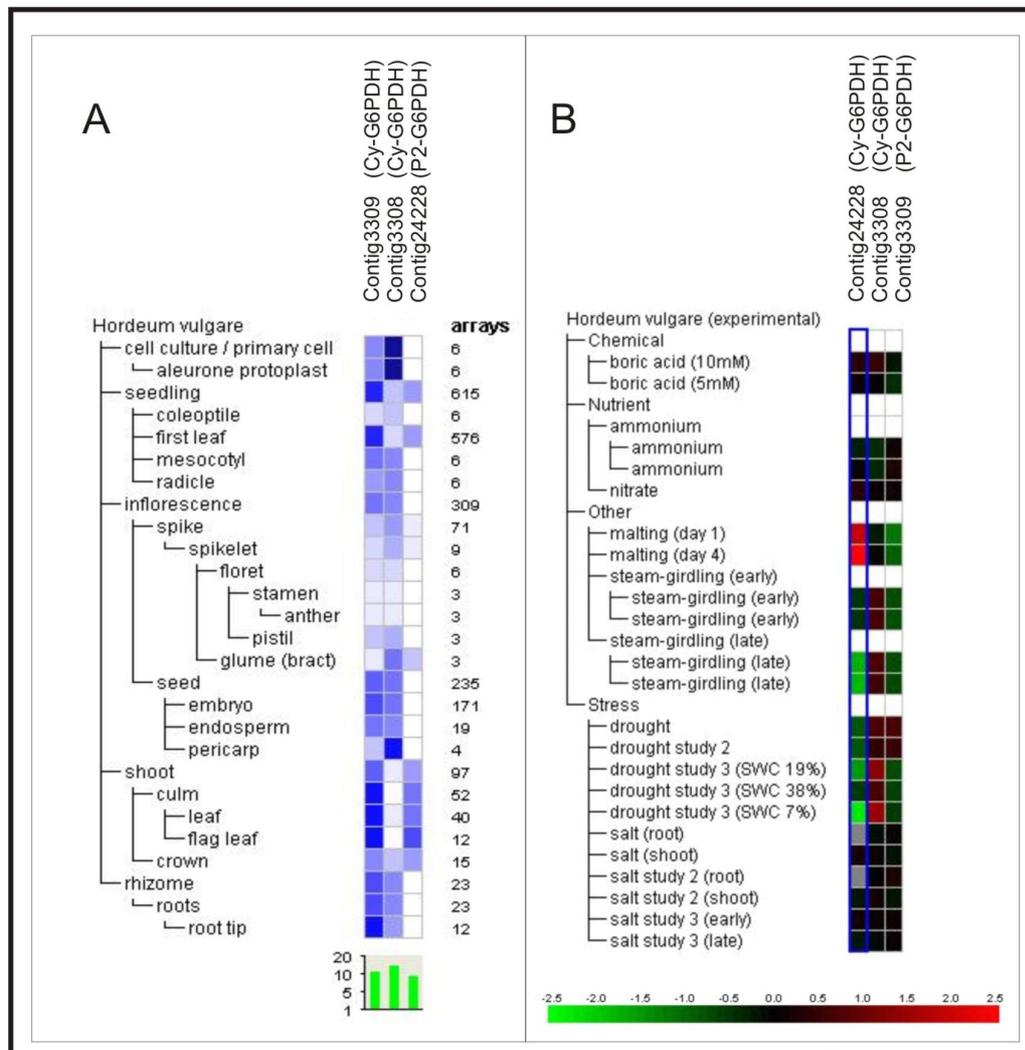
error of three different determinations

### 3.2.5 The Genevestigator data

In order to study in more detail the regulation of G6PDH genes in the different plant organs and under stress conditions, we took advantage of the barley affymetrix arrays results available in the Genevestigator website (<https://www.genevestigator.com>). The genevestigator database contains quality-controlled and well-annotated microarray experiments of barley (and rice as well) (Zimmermann *et al.*, 2008).

The contig 24228 corresponds to a plastidial barley G6PDH (P2-type), whereas the contigs 3308 and 3309 correspond to two different Cy-G6PDH (similar to Cy-G6PDH from *Triticum aestivum* and *Oryza sativa* respectively). The contig 3308 is highly expressed in primary cell and aleurone protoplast, whereas the contig 3309 is expressed in shoots and leaves of barley (Fig. 11A). It should be noted that the two different cytosolic isoforms described in *Arabidopsis thaliana*, AtG6PDH5 and AtG6PDH6 (Wakao and Benning, 2005), are similarly differentially distributed, the former being expressed mainly in roots, and the latter constitutively expressed in leaves (Wakao and Benning, 2005; Wakao *et al.*, 2008). Concerning the contig 24228, a relatively high expression is detected in shoots (leaf and flag leaf) compared to the other organs (Fig. 7A). Besides, Knight *et al.* (2001) found that the P2-G6PDH was expressed in all tissues, including roots, leaves, stem and flowers of *N. tabacum*. This result is in accordance with the result of the semi-quantitative RT-PCR in barley leaves and roots presented in figure 10A.

The contig 3308, representing barley Cy-G6PDH, is strongly up-regulated by biotic stress, in particular during the infection by *Blumeria graminis* and slightly up-regulated by drought (Fig. 11b). In contrast, the contig 3309 (barley Cy-G6PDH) is not regulated in the stress conditions tested except for a slight down-regulation during malting and drought stress (Fig. 11b). The P2-G6PDH (contig 24228) is heavily down-regulated by drought stress and up-regulated during malting (Fig. 11b).



**Figure 11:** A) G6PDH transcript analysis in different organs of barley using the meta-profile analysis tool in Genevestigator V3 ([www.genevestigator.ethz.ch](http://www.genevestigator.ethz.ch)). Data are log<sub>2</sub>.

B) G6PDH transcript abundance analysis using the meta-profile analysis tool in Genevestigator V3 ([www.genevestigator.ethz.ch](http://www.genevestigator.ethz.ch)). Data are log<sub>2</sub>. Red color marks up-regulation and green color down-regulation.

Legend: Contig 24228 corresponds to P2-G6PDH and contigs 3308 and 3309 to Cy-G6PDHs.

### 3.3 Overexpression and characterisation of plastidic P2-G6PDH from higher plants

#### 3.3.1 Primers design for barley and poplar P2-G6PDH

Barley roots P2-G6PDH had been previously sequenced from *Hordeum vulgare* cv. Alfeo (Accession AM398980). Based on this sequence, comprehending the plastidic transit peptide, specific primers were designed and used to clone and sequence the same protein from *H.vulgare* cv. Nure.

A comparison was made among barley roots P2-G6PDH, different homologous plastidic sequences, and human G6PDH; all the sequences were submitted to Psort website, to indicate define where each protein is targeted. All the sequences are predicted to be sent to the chloroplast except, of course, human G6PDH. Despite of the evidence that all the sequences possess a plastidial transit peptide in the N-terminus, the cleavage sites are given at different positions: few identities in the N-terminus were observed; a significant homology was observed in the region starting at amino acid 99 containing the sequence ASVSITV. It should be underlined that the human G6PDH protein starts in this region.

It may be thus assumed that the functional structure of the enzyme will be respected even if we cut it severely at the N-terminus, starting it with the sequence MASVSITV and ending it with LGGSEH.

This sequence would encode for an enzyme truncated for the first 99 aminoacids, therefore the protein is devoid of the transit peptide supposed long at least 38aa and of a 60aa fragment on the N-terminus of the native P2-G6PDH.

Therefore oligonucleotides were designed according to all the conserved regions in the plastidic G6PDHs cDNA sequences from several higher plants.

The barley P2-G6PDH sequence was cloned in two different plasmids, pET3d, and pET15b, being the latter a plasmid adding an his-tag tail for a possibly faster and better purification.

In both cases the sequence inserted were sequenced to check their correct

insertion an correct frame to obtain a functional enzyme.

In Figure 12 are shown the alignments of the barley P2-G6PDH sequence with other known G6PDH isoforms sequences present in database, and with the sequence of the cytosolic barley G6PDH (Accession ACV97161) recently obtained in our laboratory. The barley sequences are identical for *H.vulgare* cv. Alfeo and cv. Nure; the amino acid homology with P1-G6PDH from potato is 81% of identity. These calculation were made considering only the functional protein coding sequence (starting at aa n° 99) and not considering the transit peptides that have a great variability among different organisms (Fig. 12).

*Populus trichocarpa* genome has been completely sequenced, and the gene for P2-G6PDH was annotated (estExt\_Genewise1\_v1.C\_LG\_I7789). Based on this sequence, and applying the same strategy previously used for barley P2-G6PDH, specific primers were designed. For poplar the forward primer started at amminoacid 66 from the beginning of the sequence, in the aim to obtain a recombinant enzyme more similar to the native protein.

The poplar P2-G6PDH was cloned in pET3d and also in pET15b.

In Fig. 13 are shown the alignments of the *Populus trichocarpa* P2-G6PDH sequence with *Solanum tuberosum* G6PDH isoforms sequences present in database. The amino acid homology with P1-G6PDH from potato is 72% of identity (Fig. 13).

Hv.P2 (Nure)	-----	
Hv.P2 (Alfeo)	MALSCMRCP SVAAGP AVRRP SSSVALSLARCGRPA AVASGGWRIH AVAGKGVVKGPMDT	60
StP2	MVTLYSSPSTNCSGAFSSYSNSISGFYNYHYNFP ISSRKFSGSHKISLQIN--AVRMDGA	58
StP1	MGVQLRLNF--CSSSSAATSPSTFHNGTPLYCKKFNFLPFRTOPLNWSG--IYSRIQP	55
HvCy	-----MAGTDSASSSRQSSFNLSLAK-----	20
StCy	-----MAAS-WCIEKGRSIRNDSFR-----	19
Hv.P2 (Nure)	-----ASVSTIVVGSAGDLAKKRIFPA	22
Hv.P2 (Alfeo)	AVENTVAPAAPSPVENGTSSAITVEEYEDLASLAKDDEASVSTIVVGSAGDLAKKRIFPA	120
StP2	VVAQP SKTQDETP LKKLKDGLSKEQKHTFDLDCNKDKSTVSTIVVGSAGDLAKKRIFPA	118
StP1	RKHFEVFSNGFPLN----AVSVQDVQVPLTELGSDDTVSTIVVGSAGDLAKKRIFPA	110
HvCy	-----DLELPLEQGLTIVVGSAGDLAKKRIFPA	50
StCy	-----DNDNIPETGCLTIVVGSAGDLAKKRIFPA	49
Hv.P2 (Nure)	LFALYYEGCLP-KHE-SIFGYARSKMTDABLRHMVSKLTCRIDKRENCSEKMEFLKRCF	81
Hv.P2 (Alfeo)	LFALYYEGCLP-KHE-SIFGYARSKMTDABLRHMVSKLTCRIDKRENCSEKMEFLKRCF	179
StP2	LFALYYEGCLP-EHE-TIFGYARSKMSDDDLRNMVSKLTCRIDKRENCSEKMEFLKRCF	177
StP1	LFALYEDCLP-ENEVVFYGSRTKLSDELRNMTITLTCRIDKRENCSEKMEFLKRCF	169
HvCy	LYHLEQGLTIVVGSAGDLAKKRIFPA-----ASEEHVSEFLQLR	104
StCy	LFNLYRSGF-SNLEHIFGYARTKLSDDLRSMIRGELSQGK----ENEGEVEFLQLR	105
Hv.P2 (Nure)	YFSCGYDS EEDPREL GKKIELHQGPRVS-----NRLFYLSIFPNIQLDVKKAS---KSA	133
Hv.P2 (Alfeo)	YFSCGYDS EEDPREL GKKIELHQGPRVS-----NRLFYLSIFPNIQLDVKKAS---KSA	231
StP2	YFSCGYDS DEHPAELDKKLEHEAGRFS-----NRLFYLSIFPNIQLDVKKAS---LSA	229
StP1	YFSCGYDS EDDPAELDYKLEKEGGRVS-----NRLFYLSIFPNIQLDVKKAS---LKA	221
HvCy	YFSCGYDS SECEKLNKEISDYEMSNNS--GSSRRLFYLALPFSVVCVKMIRTVCMP	162
StCy	YFSCGYDS AEGTSLDKAISSEHEFSKNSTEGSSRRLFYLALPFSVVCVKMIRKYSYCMNK	165
Hv.P2 (Nure)	SSASGWTRVIVEKPPGRDSESSAALIRGLKEFTAEQIIRIDHYLQKELVNLVLRFSN	193
Hv.P2 (Alfeo)	SSASGWTRVIVEKPPGRDSESSAALIRGLKEFTAEQIIRIDHYLQKELVNLVLRFSN	291
StP2	SSAAGWTRVIVEKPPGRDSESSAALIRGALKQYIKEDQIIRIDHYLQKELVNLVLRFSN	289
StP1	SSTS GWTRVIVEKPPGRDSESSSELIRSLKRYITAEQIIRIDHYLQKELVNLVLRFSN	281
HvCy	TSRTGWTRVIVEKPPGRDLSAEELSSQLCELEQDQLRIDHYLQKELVNLVLRFSN	222
StCy	SDLGGWTRVIVEKPPGRDLSAEELSSQLCELEQDQLRIDHYLQKELVNLVLRFSN	225
Hv.P2 (Nure)	LVFEP LWSRQIRNNVCLIFSEDFTGTCGRGGYFDVYGIIRDIMONHLLQQLALFAMETPTIS	253
Hv.P2 (Alfeo)	LVFEP LWSRQIRNNVCLIFSEDFTGTCGRGGYFDVYGIIRDIMONHLLQQLALFAMETPTIS	351
StP2	LVFEP LWSRQIRNNVCLIFSEDFTGTCGRGGYFDVYGIIRDIMONHLLQQLALFAMETPTIS	349
StP1	LVFEP LWSRQIRNNVCLIFSEDFTGTCGRGGYFDVYGIIRDIMONHLLQQLALFAMETPTIS	341
HvCy	RIFLPLWNRDNDNLCIVREDFTGTCGRGGYFDVYGIIRDIMONHLLQQLALFAMETPTIS	282
StCy	RIFLPLWNRDNDNLCIVREDFTGTCGRGGYFDVYGIIRDIMONHLLQQLALFAMETPTIS	285
Hv.P2 (Nure)	LEAEDIRNEKVKVLRSMKFLRLEDVVIQYKSHTRGGITYPGYTDKTVPKKSLTPTFAA	313
Hv.P2 (Alfeo)	LEAEDIRNEKVKVLRSMKFLRLEDVVIQYKSHTRGGITYPGYTDKTVPKKSLTPTFAA	411
StP2	LDAEDIRNEKVKVLRSMRELQLDDVVIQYKSHTRGGVNYPGYTDKTVPKKSLTPTFAA	409
StP1	LDAEDIRNEKVKVLRSMRELQLDDVVIQYKSHTRGGVNYPGYTDKTVPKKSLTPTFAA	401
HvCy	LKPEIRDEKVKVLRSMRELQDEEVVLQYQG-----YKDEIVPDDSNITPTFAA	332
StCy	QKPEIRDEKVKVLRSMRELQDEEVVLQYQG-----YKDEIVPDDSNITPTFAA	335
Hv.P2 (Nure)	AALFINNARWGVFPLMKACKALHTKCAEIRVQFRHVPENLTKGSFGTDLDRATNEIVR	373
Hv.P2 (Alfeo)	AALFINNARWGVFPLMKACKALHTKCAEIRVQFRHVPENLTKGSFGTDLDRATNEIVR	471
StP2	AALFIDNARWGVFPLMKACKALHTKCAEIRVQFRHVPENLTKGNFGTDLDRATNEIVR	469
StP1	AALFIDNARWGVFPLMKACKALHTKCAEIRVQFRHVPENLTKGNFGTDLDRATNEIVR	461
HvCy	IYLVVHNRWGVFPLMKACKALNSRKAIRVQFKDVPCEIIRKCKK----QSRNEIVR	387
StCy	MVLRHNRWGVFPLMKACKALNSRKAIRVQFKDVPCEIIRKCKK----QSRNEIVR	390
Hv.P2 (Nure)	VQPEEGTYIKINNKIFGLGMRLDRSNNLNHYAARVP-IPDAYERLLDATTGERRLFT	432
Hv.P2 (Alfeo)	VQPEGGTYIKINNKIFGLGMRLDRSNNLNHYAARVP-KEIPDAYERLLDATTGERRLFT	530
StP2	VQPEEATYIKINNKIFGLGMRLDRSNNLNLYSARVA-IPDAYERLLDATTGERRLFT	528
StP1	VQPEEATYIKINNKIFGLGMRLDRSNNLNLYKAKVR-IPDAYERLLDATTGERRLFT	520
HvCy	LQPEEAVYKMLTVKKEGLEMATQCELDLSYGMRYQDVKRIPDAYERLLDATTGDOOHVF	447
StCy	LQPEEAVYKMLTVKKEGLEMATQCELDLSYGMRYQGVVIPDAYERLLDATTGDOOHVF	450
Hv.P2 (Nure)	RSDELLAAWELFTPLIKKLEQRMAPELVYVYSGRGPVCAHYLAAR--YVNRVCDLGGSEH	490
Hv.P2 (Alfeo)	RSDELLAAWELFTPLIKKLEQRMAPELVYVYSGRGPVCAHYLAAR--YVNRVCDLGGSEH	588
StP2	RSDELLAAWELFTPLIKKLEQRMAPELVYVYSGRGPVCAHYLAAR--YVNRVCDLGGSEH	582
StP1	RSDELLAAWELFTPLIKKLEQRMAPELVYVYSGRGPVCAHYLAAR--HNVRVCDLGGSD	577
HvCy	RSDELLAAWELFTPLIHNLDACKLKAVALYVYSGRGPVCAHYLAAR--SEKVGVMQTHGYIWPPT	507
StCy	RSDELLAAWELFTPLIHRIDNCEVKEIPYVYSGRGPVCAHYLAAR--LQNGYVQTHGYIWPPT	510
Hv.P2 (Nure)	--	
Hv.P2 (Alfeo)	--	
StP2	--	
StP1	--	
HvCy	LA 509	
StCy	L- 511	

**Figure 12:** Comparison of P2-G6PDH deduced amino acid sequences from *H. vulgare* vs other known G6PDH sequences. The strictly conserved sequences are in black color, and in grey the aa conserved in compartmented isoform and in cytosolic isoform only. *Hordeum vulgare* HvP2 (Nure); HvP2 (Alfeo - AM398980); Cytosolic isoform, HvCy (ACV97161.1); *Solanum tuberosum*, StCy (CAA52442.1); StP1 (CAA58775.1); StP2 (CAB52708.1). Other details in the text.



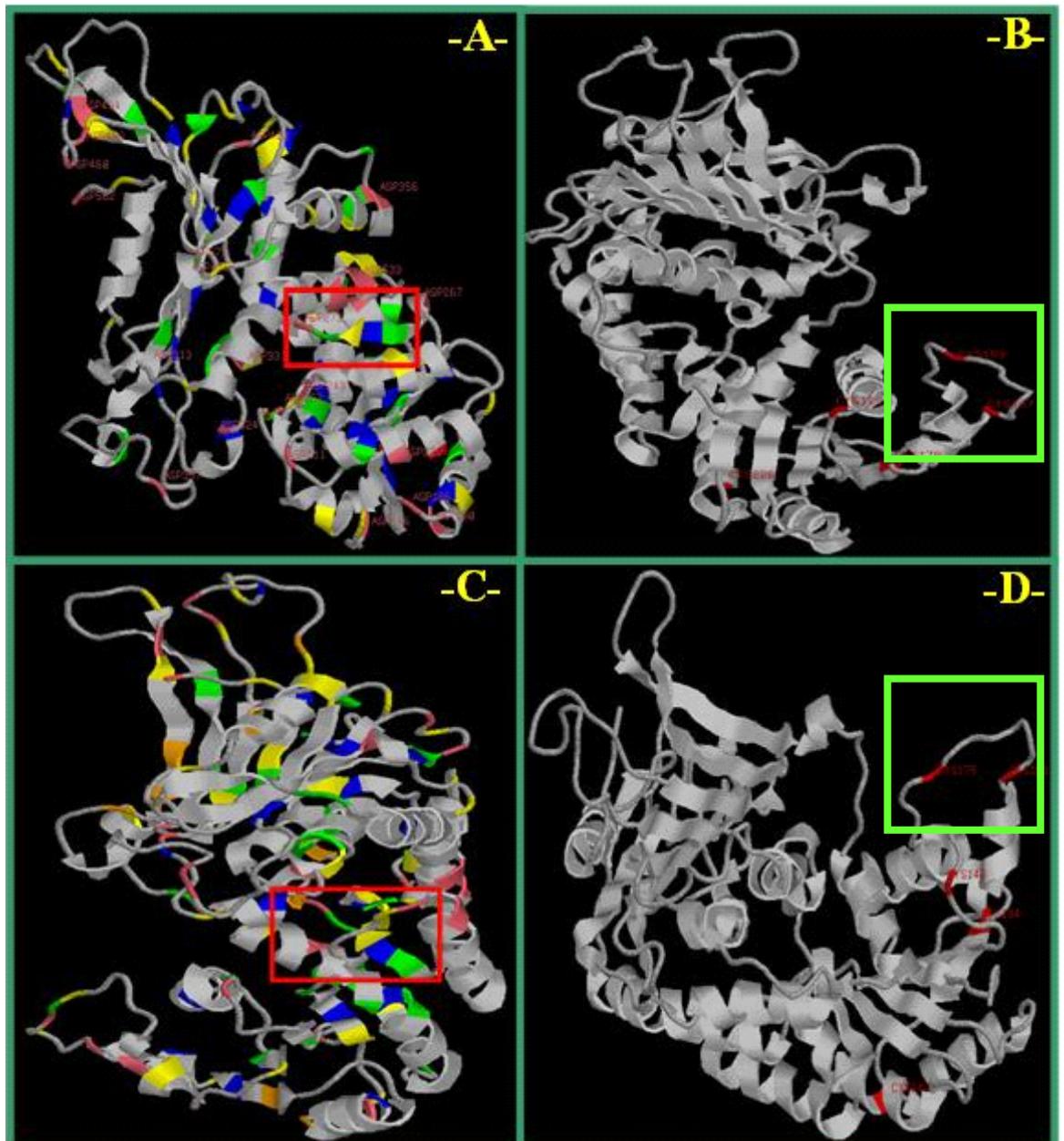
### 3.3.2 Barley and poplar P2-G6PDH 3D modeling

P2-G6PDH constitutes a distinct group in the G6PDH family with an undefined 3D structure. Our previous comparative genome analyses revealed the presence of one isoform in *Hordeum vulgare* and two isoforms in *P. trichocarpa*. Poplar and barley P2 proteins share 72 % identity. P2G6PDH is a protein of around 60 kDa and around 600 amino acids (aa), characterized by the presence FRIDHYLGKE active site (Fig. 14A-*Hordeum vulgare*; Figure 14B-*Populus trichocarpa*) and 5 cysteins at position 145aa, 175aa, 183aa, 194aa, 242aa in populus and at position 128aa, 159aa, 167aa, 178aa, 226aa in barley (Fig. 14B and 14D).

The predicted structure is based on the crystallographic determination of G6PDH from the bacteria *Leuconostoc mesenteroides* (Cosgrove *et al.*, 1998); it is very important to note that this prokaryotic organism possesses a bi-specific G6PDH, able to utilize both NAD<sup>+</sup> and NADP<sup>+</sup>.

Previous studies on the potato chloroplastic isoform of G6PDH have shown that its redox regulation involves two conserved Cys-residues located at position cys<sub>149</sub> and cys<sub>157</sub>. Potato P1 and barley P2 proteins share 70% identity; and potato P1 to poplar P2 proteins share 72% identity. Their sequence alignment identifies two possible regulatory cysteines at positions 159 and 167 in the *Hordeum vulgare* isoform and at position 175 and 183 in the *Populus trichocarpa* isoform (Fig 14B (*Hordeum vulgare*) Fig.14D (*Populs trichocarpa*)).

The software structure suggests that the redox regulating cysteines are positioned in an unstructured, probably flexible loop, located near the active site (Fig. 14. A-B) *Hordeum vulgare* – C-D) *Populus tricocharpa*). Again it is important to underline that bacteria do not possess the regulatory cycteines on the N-terminus of the protein; only cyanobacteria do possess a redox regulation but this properties is due to by the present of two cysteines in the N-terminus an C terminus of the protein (Wenderoth *et al.*, 1997).



**Figure 14:** A – B) modelling of *HvP2-G6PDH*; C-D) modelling of *PtP2-G6PDH* structure. Modelling was made using the Swiss-Model workspace (<http://swissmodel.expasy.org/workspace/>) Structured parts of the protein appear in grey while A-C) The active site sequence (FRIDHYLGKE) is in red rectangle, for barley and poplar respectively. B-D) The 2 cys residues (green rectangle) involved in the redox regulation of chloroplastic G6PDH isoform, are located at a surface-exposed unstructured loop close to the active site and cofactor binding domain.

### 3.4 Purification of *E.coli* BL21(DE3) G6PDH

As preliminary result, to ensure that the purified G6PDH from transformed bacteria was not the native *E.coli* enzyme, a purification and biochemical characterization of *E.coli* G6PDH were performed using the BL21(DE3) strain transformed with the native plasmid pET3d.

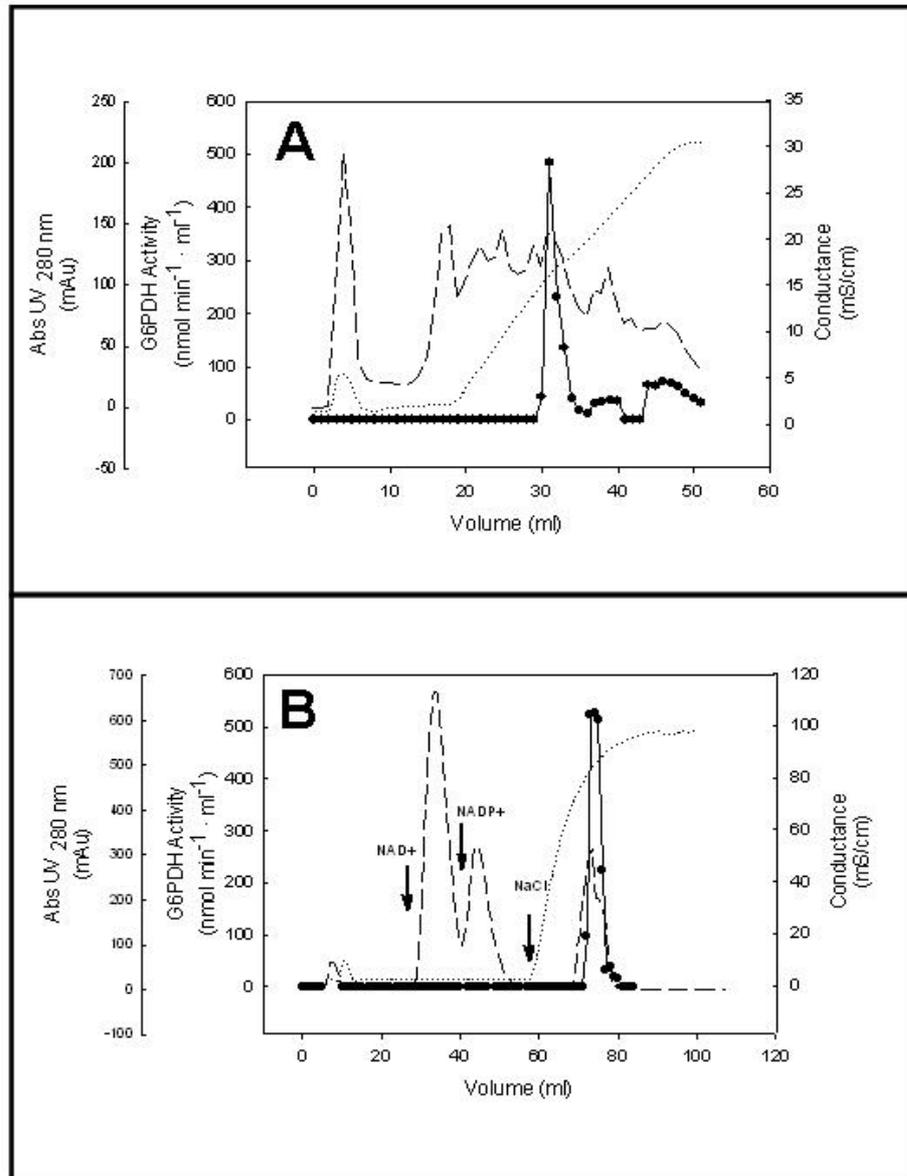
The G6PDH activity in soluble fraction of *E.coli* BL21 (DE3) pET3d was  $400 \pm 25 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ prot.}$

The bacterial enzyme peak eluted at 250mM NaCl in a single peak (Fig. 15A). The active fractions were pooled and loaded on Blue-Sepharose column (Ge Healthcare Hi-Trap Blu FF) and G6PDH activity was eluted in a single peak applying 1.5M NaCl and 200 $\mu$ M NADP<sup>+</sup>.(Fig. 15B)

The western blotting demonstrated that the reactivity of *E.coli* G6PDH vs plant G6PDH antibodies was null against all the different isoforms antibodies raised vs recombinant potato enzymes (Wendt *et al.*, 2000) (Fig. 16).

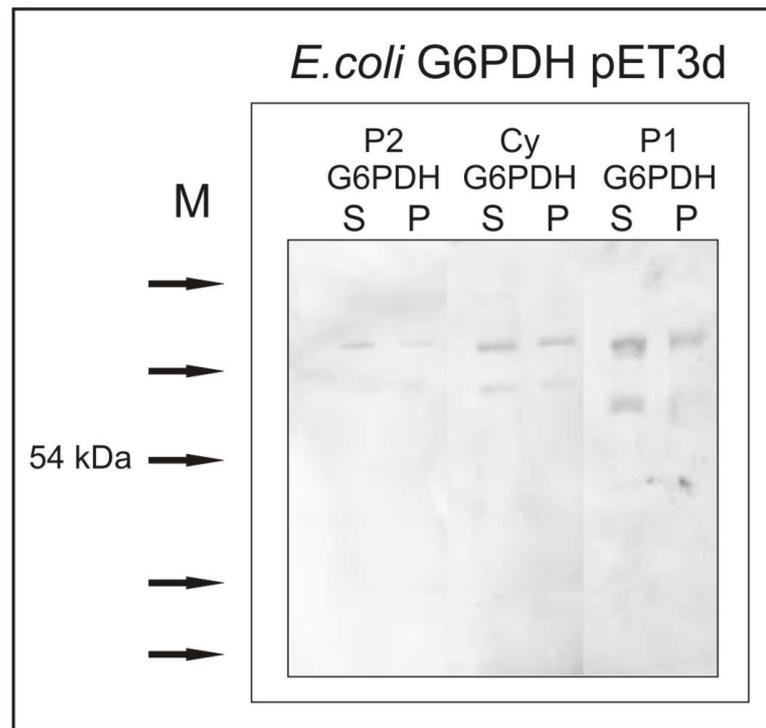
The main kinetic properties of *E.coli* BL21(DE3) G6PDH were determined. The bacterial enzyme showed hyperbolic saturation curve with both substrates (Fig. 17a and B).

This parameters are clearly different with respect to those known for higher plant plastidic G6PDHs, e.g.  $K_m \text{ G6P} = 0.175\text{mM}$  (0.6-1mM in higher plants);  $K_m \text{ NADP}^+ = 2.1\mu\text{M}$ ;  $K_i \text{ NADPH}$  of 8.8 $\mu\text{M}$  (50-90 $\mu\text{M}$  in higher plants) (Fig. 17C).

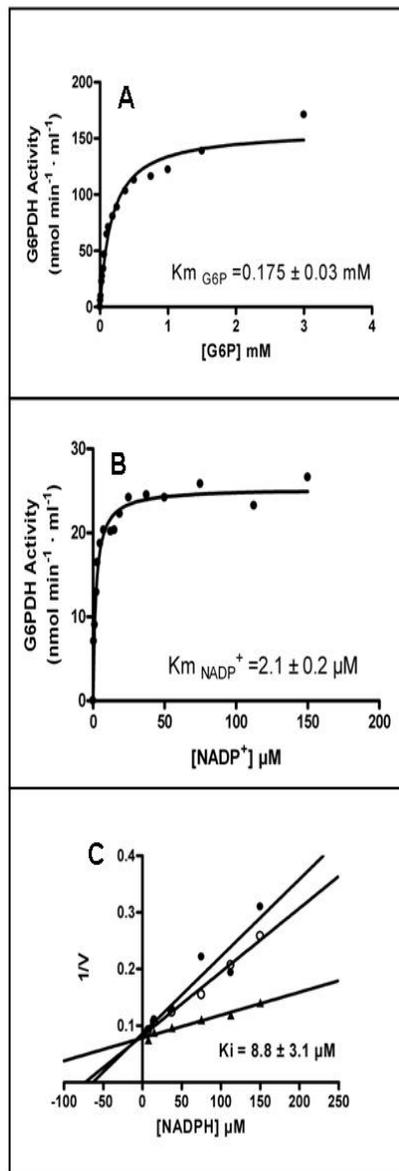


**Figure 15:** A) G6PDH activity profile (●) measured in fractions eluted from a Resource Q-Sepharose column (6ml) of the supernatant of the *E. coli* BL21(DE3) lysate, transformed with native pET3d plasmid. B) G6PDH activity profile (closed circles) measured in fractions eluted from a Blue-sepharose column of the active fractions from ion-exchange chromatography.

UV absorbance (280 nm) indicating total protein elution profile is indicated by the dashed line (- -), the dotted line (···) indicates the NaCl gradient. Other details in the text.



**Figure16:** Western blotting of crude extracts of *E. coli* G6PDH transformed with native pET3d plasmid. Western blotting was made utilising antibodies raised against potato chloroplastic (P1); plastidic (P2) and cytosolic (Cyt) G6PDH (Wendt *et al.*, 2000). The molecular weight of the *E. coli* G6PDH protein (54 kDa) is indicated by an arrow. Legend: M, molecular weight markers; P, pellet; S, supernatant. Other details in the text.



**Figure 17:** Kinetic properties of purified *E. coli* BL21(DE3) G6PDH. Bacteria transformed with native pET3d plasmid. A) Effect of varied G6P concentration. B) Effect of varied NADP<sup>+</sup> concentration. C) Inhibition constant for NADPH ( $K_{i\text{ NADPH}}$ ) calculated by the Dixon plot analysis. The  $K_i$  was determined at 3mM G6P using non saturating NADP<sup>+</sup> concentrations: (●) 15μM NADP<sup>+</sup>; (○) 30μM NADP<sup>+</sup>; (▼) 50μM NADP<sup>+</sup>; regression lines show a  $r^2$  value at least of 0.94. The graphs are representative of at least three independent G6PDH purifications and kinetic parameters determinations. Other details in Materials and methods.

### 3.5 Purification of recombinant protein *HvP2-G6PDH* (pET3d)

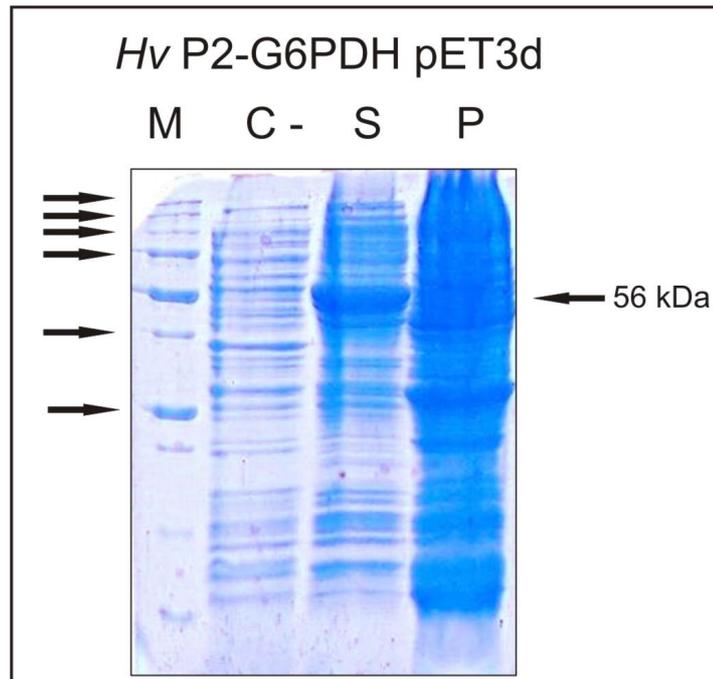
#### 3.5.1 Purification of recombinant protein from soluble fraction

Batch cultures of *E.coli* BL21(DE3) containing *HvP2-G6PDH* (pET3d) plasmid were grown and bacteria lysates were analysed for the presence of the recombinant protein. Although well over-expressed, the recombinant *HvP2-G6PDH* protein accumulated mainly in the inclusion bodies; anyway an appreciable amount of enzyme was present in the soluble fractions (Fig.18). The estimated molecular weight of the G6PDH monomers was 56 kDa.

Recombinant P2-G6PDH was partially purified from soluble fraction of bacteria lysates (*E.coli* BL21(DE3) *HvP2-G6PDH* (pET3d)) by anion exchange chromatography. Samples were desalted and applied to a Q-Sepharose column (Resource Q). The bound proteins were eluted by applying a 0 – 400mM NaCl gradient (Fig.19A). A main peak of G6PDH activity was eluted at 100mM NaCl.

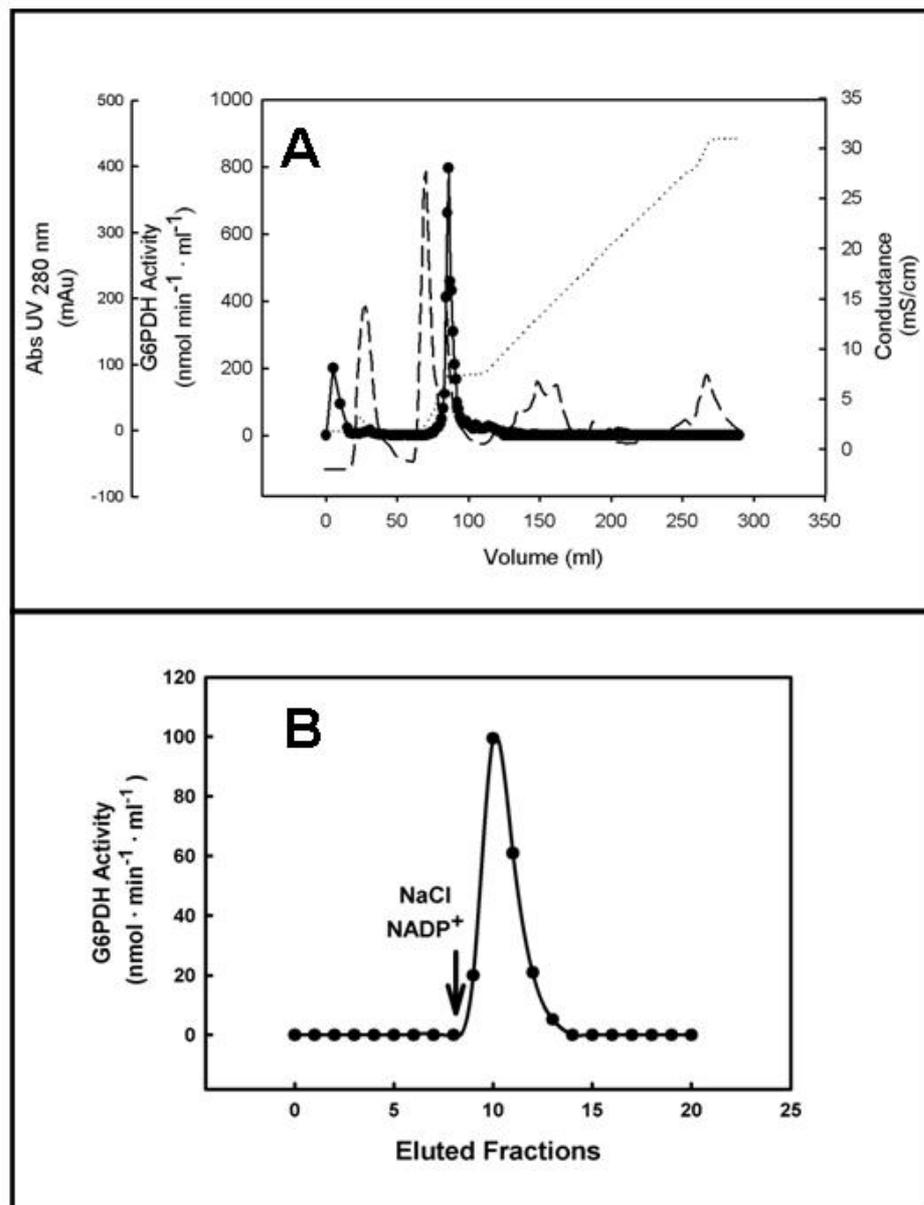
The active fractions from Resource Q-Sepharose step were applied to a Blue- Agarose column and the recombinant G6PDH was eluted with 5mM NADP<sup>+</sup> plus 1M NaCl (Fig. 19B).

The G6PDH activity eluted in a single peak, the active fractions were pooled and utilised for further biochemical characterization.



**Figure 18:** SDS-PAGE of lysate samples of *E.coli* BL21(DE3) transformed with *Hv*P2-G6PDH (pET3d) plasmid. The molecular weight of the overexpressed G6PDH protein calculated with the relative mobility factor method is indicated.

Legend: M, molecular weight markers; C-, negative control (bacteria transformed with an native plasmid); S, Surnatant; P, pellet.

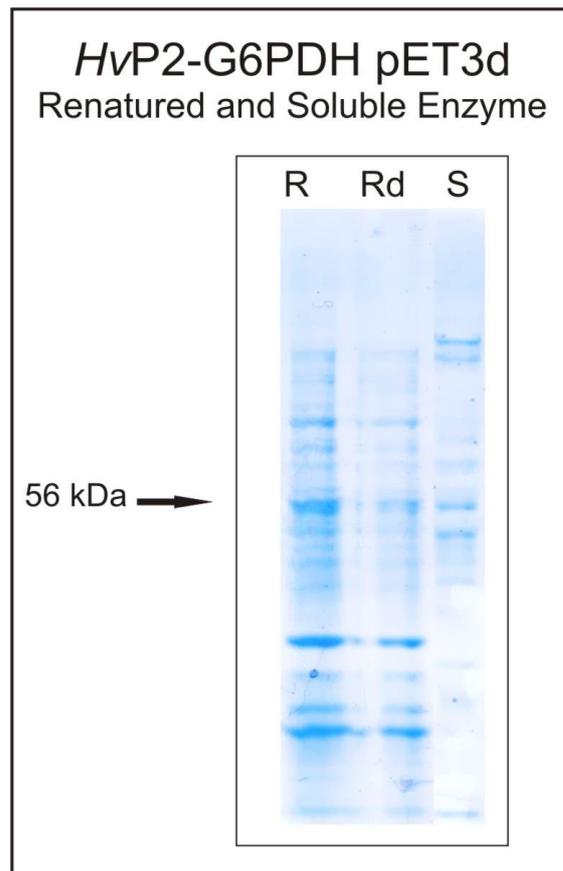


**Figure 19:** A) G6PDH activity profile (●) measured in fractions eluted from a Resource Q-Sepharose column (6ml) of the supernatant of the *E. coli* BL21(DE3) lysate, transformed with *HvP2-G6PDH* (pET3d). B) G6PDH activity profile (closed circles) measured in fractions eluted from a Blue-Agarose column of the active fractions from ion-exchange chromatography.

UV absorbance (280 nm) indicating total protein elution profile is indicated by the dashed line (--), the dotted line (···) indicates the NaCl gradient. Other details in the text.

### **3.5.2 Purification of recombinant protein from inclusion bodies**

A large part of the recombinant protein *HvP2-G6PDH* (pET3d) was present in the insoluble fraction (inclusion bodies) of bacterial lysates. Therefore, an appropriate strategy of denaturing followed by renaturation of the protein was employed. The inclusion bodies were ruptured by sonication in 7M Guanidine-HCl and the recombinant protein was denaturated for 2-4h at room temperature. After that period, the solution was diluted 20-fold in renaturing buffer, containing arginine, NADP<sup>+</sup> and DTT (see Materials and Methods). After 7 days (Wang and Engel, 2009) the solution was concentrated by ultrafiltration at a final volume of 30ml, desalted on Sephadex G25, and used for the determination of kinetic parameters. A SDS-PAGE gel confirmed that the refolded protein has the same molecular weight of the soluble recombinant enzyme (56 kDa) (Fig. 20).



**FIGURE 20:** SDS-PAGE of refolded G6PDH *HvP2-G6PDH* purified from the pellet of the *E.coli* BL21(DE3) lysate, transformed with *HvP2-G6PDH* (pET3d) plasmid. Legend: R, refolded protein; Rd, refolded protein desalted on a G25 column; S, surnatant of the bacteria lysate. The molecular weight of the refolded protein calculated with the relative mobility factor method is indicated. Other details in the text.

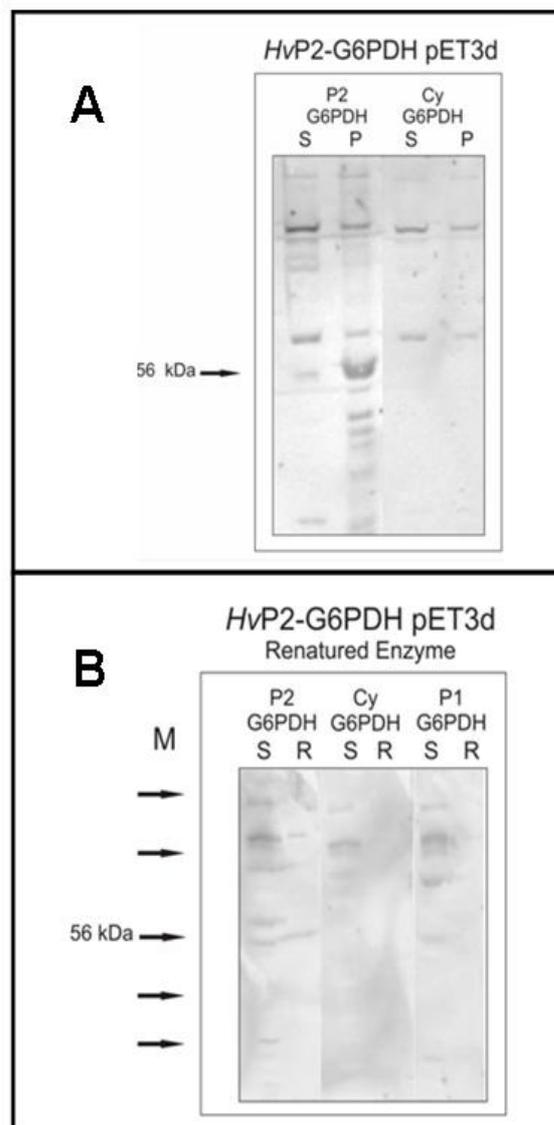
A western analysis carried out using specific antibodies raised against potato cytosolic (Cy-G6PDH), chloroplastic (P1-G6PDH) and plastidic (P2-G6PDH) (Wendt *et al.*, 2000) confirmed the presence of a single G6PDH solely reacting versus potato P2-G6PDH antibodies mainly present in the pellet of bacteria lysates (Fig. 21A). No reaction was observed using Cy-G6PDH antibodies.

Both purified G6PDH from soluble fraction of lysates and refolded protein reacted vs potato P2-G6PDH antibodies on western blots (Fig. 21B): it should be noted that no reactions were observed using potato Cy-G6PDH; P1-G6PDH antibodies gave a faint band with soluble protein (Fig. 21B), and a mixture of the three antibodies gave a single stained band corresponding to that one reacting with P2-G6PDH antibodies alone (not shown). The estimated molecular weight of the G6PDH subunits, calculated by the relative mobility factor, was 56 kDa.

### **3.5.3 Kinetic properties of recombinant *Hv*P2-G6PDH (pET3d)**

The main kinetic parameters of recombinant *Hv*P2-G6PDH (pET3d) purified from soluble fraction or refolded from inclusion bodies are very similar, giving a  $Km_{G6P}$  of 0.8-1mM (Fig 22 A-D), a  $Km_{NADP^+}$  of 6-8 $\mu$ M (Fig. 22 B-E).

Most important the inhibition by NADPH was measured at different saturating  $NADP^+$  concentrations of 15, 30, and 50 $\mu$ M. The Dixon plot analysis allowed the estimation of a very high  $K_{iNADPH}$ , 75-80  $\mu$ M (Fig. 22 C-F).



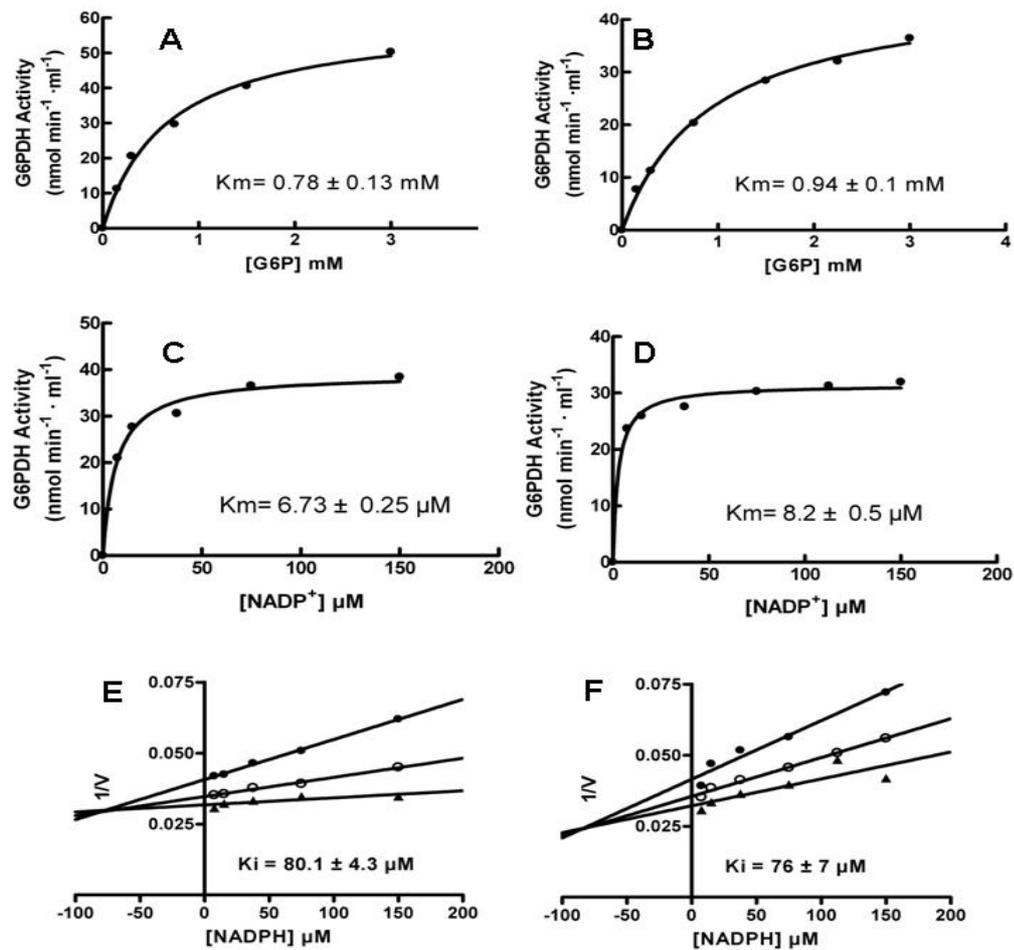
**Figure 21:** Western blotting of different samples of bacteria lysates and refolded enzyme.

Panel A: P, pellet; S, supernatant of bacteria B121(DE3) lysate, transformed with *Hvp2-G6PDH* (pET3d) plasmid.

Panel B: S, Supernatant; R refolded protein from inclusion bodies.

The molecular weight of the overexpressed G6PDH protein, calculated with the relative mobility factor method, is indicated.

Western blotting was made utilising antibodies raised against potato chloroplastic (P1); plastidic (P2) and cytosolic (Cy) G6PDH (Wendt et al., 2000). Other details in the text.



**Figure 22:** (A-B-C), Kinetic properties of *HvP2-G6PDH* purified from the supernatant of the *E.coli* BL21(DE3) lysate, transformed with *HvP2-G6PDH* (pET3d) plasmid. (D-E-F) Kinetic properties of *HvP2-G6PDH* renatured from inclusion bodies of *E.coli* BL21(DE3). A-D) Effect of varied G6P concentration. B-E) Effects of varied NADP<sup>+</sup> concentration. C-F) Inhibition constant for NADPH ( $K_{i_{\text{NADPH}}}$ ) calculated by the Dixon plot analysis .

The  $K_i$  was determined at 3mM G6P using non saturating NADP<sup>+</sup> concentrations: (●) 15μM NADP<sup>+</sup>; (○) 30μM NADP<sup>+</sup>; (▲) 50μM NADP<sup>+</sup>; regression lines show a  $r^2$  value at least of 0.96. The graphs are representative of at least three independent G6PDH purifications and kinetic parameters determinations. Other details in Materials and methods.

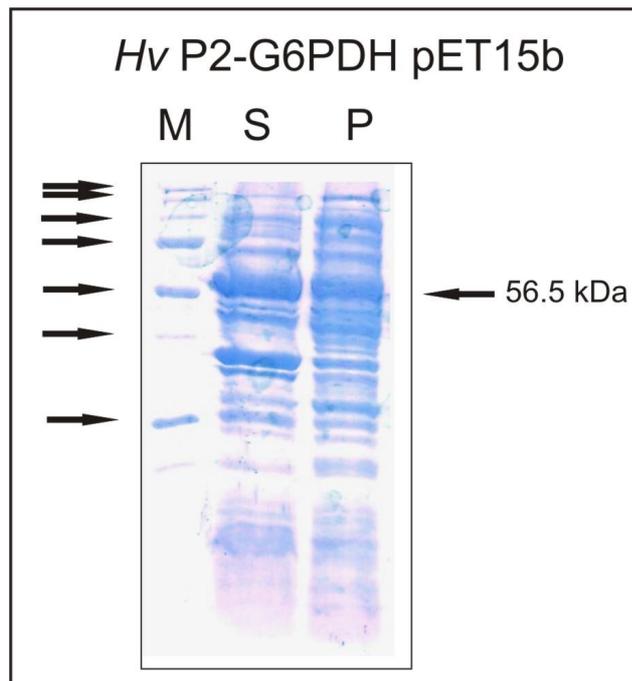
### 3.6 Purification of recombinant *HvP2-G6PDH* (pET15b)

#### 3.6.1 Purification of recombinant protein from soluble fraction

Batch cultures of *E.coli* BL21(DE3) containing *HvP2-G6PDH* (pET15b) plasmid were grown and bacteria lysates were analysed for the presence of the recombinant protein. The recombinant *HvP2-G6PDH* protein accumulated both in the inclusion bodies and in the soluble fraction. The estimated molecular weight of the G6PDH monomers was 56.5 kDa. (Fig. 23)

Recombinant P2-G6PDH from soluble fraction of bacteria lysates (Bl21-DE3 pET15b) was rapidly purified using an IMAC column; the purified fractions were used for Mass Spectrometry analysis (see after).

The recombinant *HvP2-G6PDH* (pET15b) was purified also using HiTrap FF crude column connected to an AKTA prime plus system. The recombinant protein eluted in a single G6PDH activity peak in the presence of 0.5M imidazole (Fig. 24). The eluted fractions were used, after desalting, for the determination of the kinetic parameters of the recombinant enzyme.

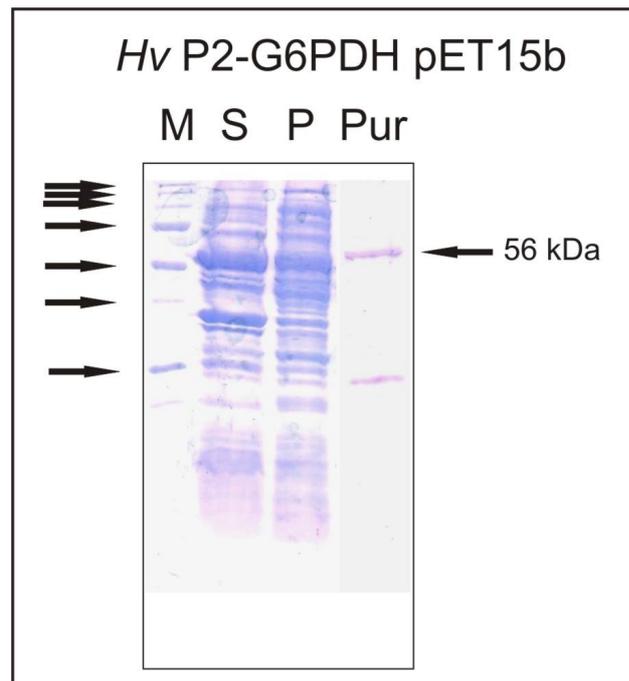


**Figure 23:** SDS-PAGE of samples from *E.coli* BL21(DE3) lysate, transformed with *Hv*P2-G6PDH(pET15) plasmid. The molecular weight of the overexpressed G6PDH protein, calculated with the relative mobility factor method, is indicated. Legend: M, molecular weight markers; S, supernatant; P, pellet.

**Figure24:** G6PDH activity profile (closed circles) measured in fractions eluted from a His-Trap column (6ml) of the supernatant of the *E.coli* BL21(DE3) lysate, transformed with *HvP2*-G6PDH (pET15b). UV absorbance (280 nm) indicating total protein elution profile is indicated by the dashed line (- -), the dotted line (···) indicates the imidazole gradient. Other details in the text.

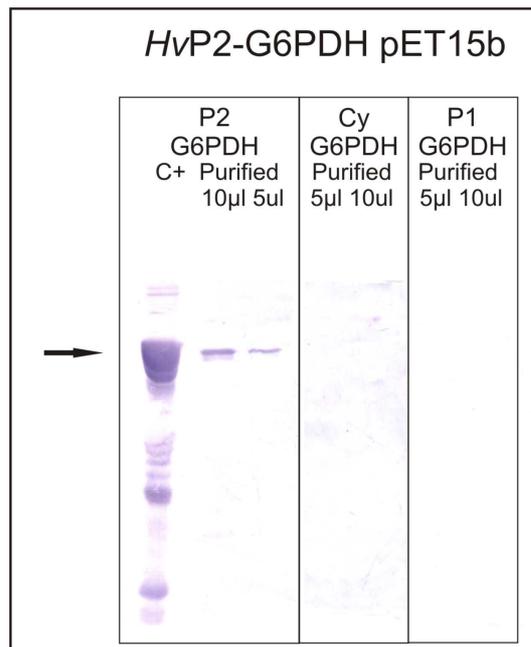
The purified protein showed two bands on SDS-gels, one has the expected molecular weight (56 kDa), determined for the over-expressed protein in both pellet and supernatant of bacteria lysates (Fig; 25). A second protein of about 20 kDa was present in the purified fractions.

These purified fractions were utilised for Mass spectrometry analysis (see after). A western analysis carried out using specific antibodies raised against potato cytosolic (Cy-G6PDH), chloroplastic (P1-G6PDH) and plastidic (P2-G6PDH) (Wendt *et al.*, 2000) confirmed that the purified protein G6PDH solely reacted versus potato P2-G6PDH antibodies (Fig. 26). No reaction was observed against both P1-G6PDH and Cy-G6PDH antibodies.



**Figure 25:** SDS-PAGE of samples from *E.coli* BL21(DE3) lysate, transformed with *Hv*P2-G6PDH (pET15) plasmid. The molecular weight of the overexpressed G6PDH protein, calculated with the relative mobility factor method, is indicated.

Legend: M, molecular weight markers; S, supernatant; P, pellet; Pur, purified protein.



**Figure 26:** Western blotting of bacteria BL21(DE3) lysate, transformed with *HvP2-G6PDH* (pET15b) plasmid. Legend: C+ positive control (pellet of bacteria transformed with *HvP2-G6PDH* (pET15b)); other lanes were loaded with purified protein. The arrow indicates the expected molecular weight of the overexpressed G6PDH protein (56 kDa).

Western blotting was made utilising antibodies raised against potato chloroplastic (P1); plastidic (P2) and cytosolic (Cy) G6PDH (Wendt *et al.*, 2000). Other details in the text.

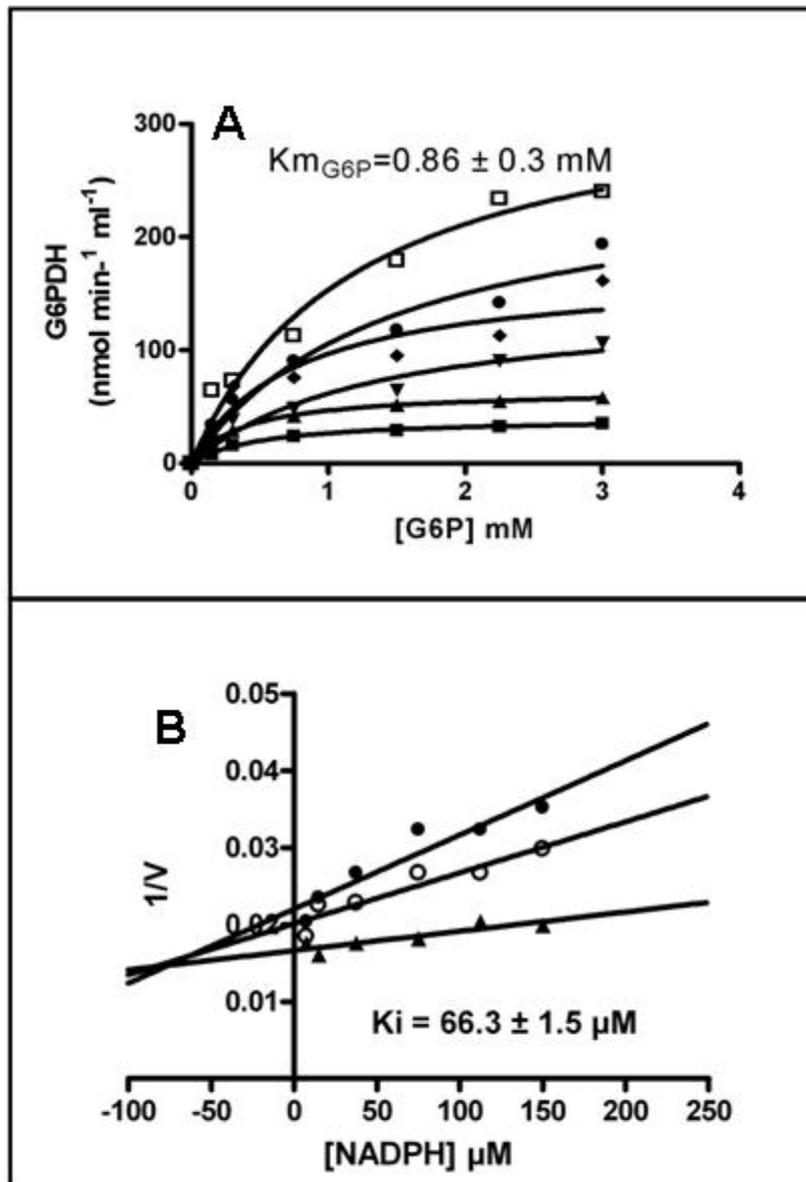
### 3.6.2 Kinetic properties of recombinant *HvP2-G6PDH* (pET15b)

Recombinant His-tagged P2-G6PDH (pET15b) from soluble fraction of bacteria lysates *E.coli* B121(DE3) showed a hyperbolic kinetics vs G6P, exhibiting a  $K_m$  G6P of 0.86mM (Fig. 27A).

The saturation curve for NADP<sup>+</sup> showed a similar hyperbolic kinetics, with a calculated  $K_{m\text{NADP}^+}$  = 15 $\mu$ M (not shown). The inhibition by NADPH was measured at different sub-saturating NADP<sup>+</sup> concentrations of 15, 30, and 50 $\mu$ M. The Dixon plot analysis allowed the estimation of a  $K_i$  NADPH = 66 $\mu$ M (Fig. 27 B).

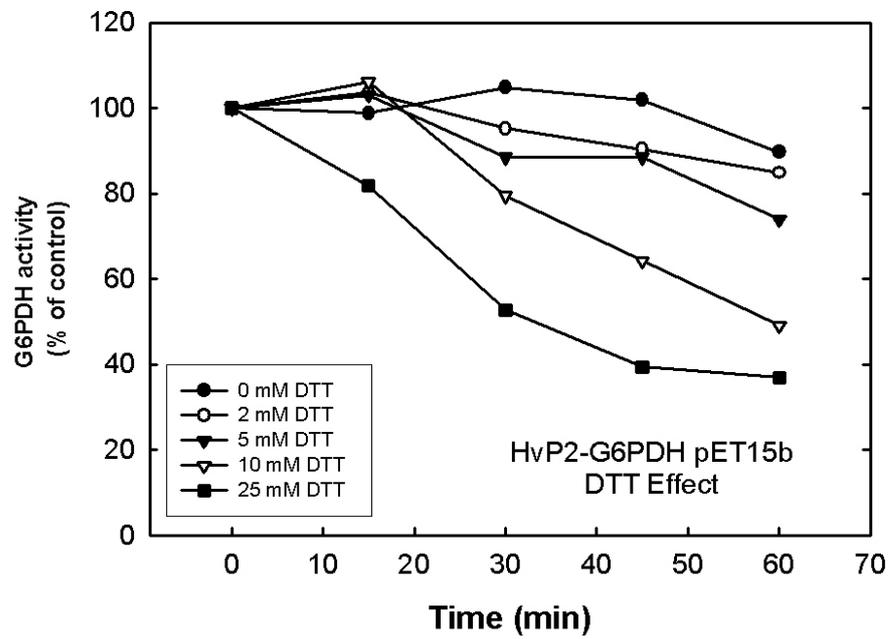
### 3.6.3 Effects of DTT on recombinant *HvP2-G6PDH* activity

The recombinant *HvP2-G6PDH* (pET15b) was moderately inhibited by reductants: a 50% inhibition after 1h at 10mM DTT, and a 60% inhibition after 1h was measured in the presence of 25mM DTT. This results are in accord with previous results obtained for native P2-G6PDH (Esposito *et al.*, 2001) (Fig. 28).



**Figure 27:** Kinetic properties of *HvP2-G6PDH(pET15b)* A) Effect of varied G6P concentration. B) Inhibition constant for NADPH ( $K_i$  NADPH) calculated by the Dixon plot analysis.

The  $K_i$  was determined at 3mM G6P using non saturating NADP<sup>+</sup> concentrations: (■) 15 $\mu$ M NADP<sup>+</sup>; (▲) 30 $\mu$ M NADP<sup>+</sup>; (▼) 50 $\mu$ M NADP<sup>+</sup>; regression lines show a  $r^2$  value at least of 0.96. The graphs are representative of at least three independent G6PDH purifications and kinetic parameters determinations. Other details in Materials and methods.

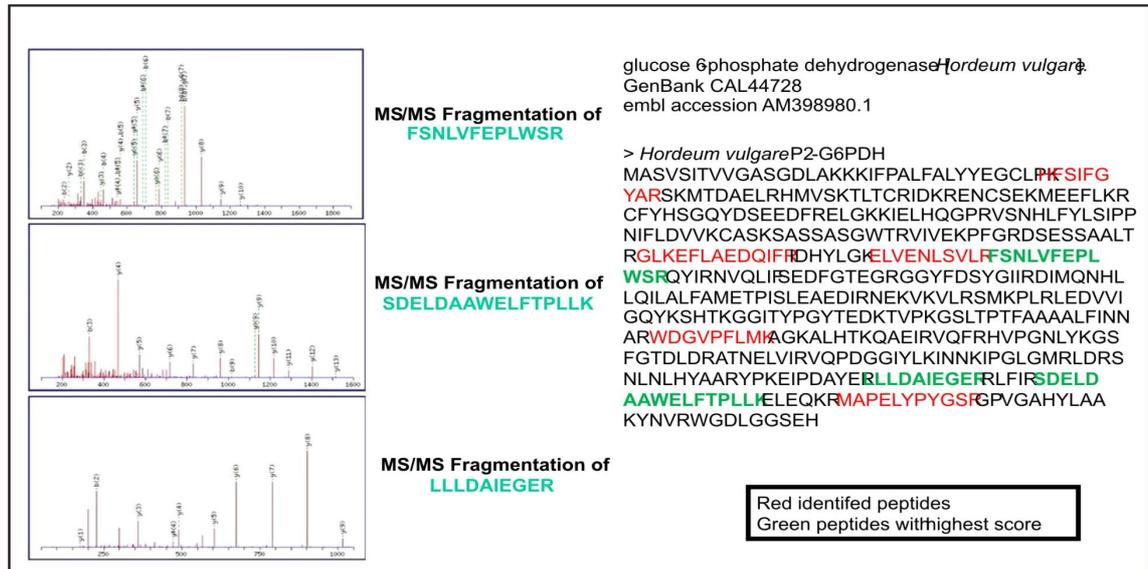


**Figure 28:** Dependence on DTT reduced of recombinant *HvP2-G6PDH* (pET15b) activity. Purified G6PDH was used for the assay. Each point represent an average of three replicates. Other details in the text.

### **3.6.4 Mass Spectrometry analysis of the purified *HvP2-G6PDH* (pET15b)**

The *HvP2-G6PDH* (pET15b) was purified by IMAC chromatography and used for Mass Spectrometry determination.

The MS analysis clearly demonstrates that the purified protein corresponds to barley P2-G6PDH: three peptides (12, 16 and 10 aa length) correspond to sections of the aminoacid sequence of the barley G6PDH (Accession AM398980); several peptides identify other parts of the sequence (Fig. 29).

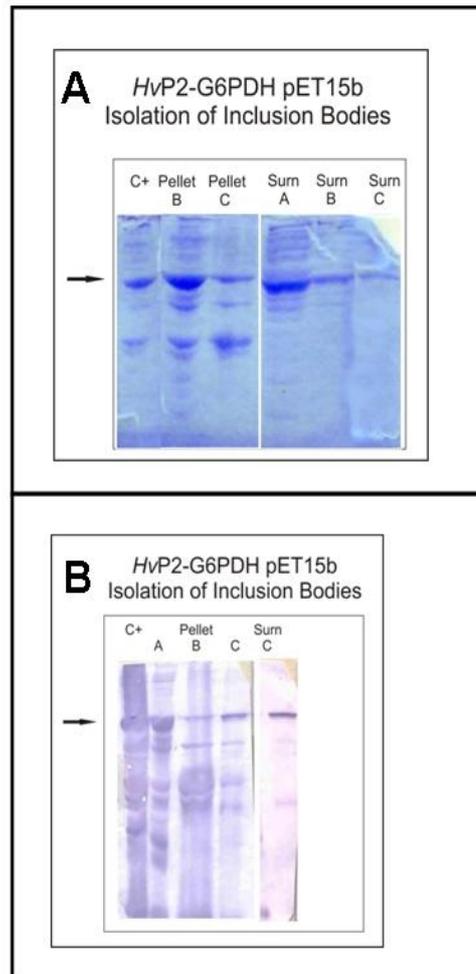


**Figure 29:** Mass spectrometry analysis of purified *Hv*P2-G6PDH (pET15b). The digested fragments with the highest score with *Hv*P2-G6PDH sequence (Accession #AM398980.1) were identified by MS (left, center); their position is highlighted in green on the right, together with other identified fragments are in red.

### **3.7 Isolation of recombinant protein *HvP2-G6PDH* (pET15b) from inclusion bodies**

A part of the recombinant protein was present in the insoluble fraction (inclusion bodies - IB) of bacterial lysates. The inclusion bodies were isolated by centrifugation and ruptured by sonication in 6M Guanidine-HCl, and the recombinant proteins were loaded on a HisTrap FF column connected to an AKTA Prime plus chromatographic system. The protein eluted with 0.5M imidazole according manufacturer's instructions. The active fractions were collected and utilised for kinetic properties determination and for the identification of the purified protein by western blotting .

A SDS-PAGE gel confirmed that the refolded protein has the same molecular weight of the soluble recombinant enzyme (56 kDa) (Figure 30A). A western analysis carried out using specific antibodies raised against potato cytosolic (Cy-G6PDH), chloroplastic (P1-G6PDH) and plastidic (P2-G6PDH) (Wendt *et al.*, 2000) confirmed the presence of a single G6PDH solely reacting versus potato P2-G6PDH antibodies mainly present in the pellet of bacteria lysates (Fig. 30B). No reaction was observed using Cy-G6PDH antibodies.



**Figure 30:** A) SDS-PAGE of samples from *E.coli* BL21(DE3) lysate, transformed with *Hvp2-G6PDH* (pET15b) plasmid, during the isolation procedure of inclusion bodies (IB). B) Western blotting of bacteria BL21(DE3) lysate, transformed with *Hvp2-G6PDH* pET15b plasmid, during the isolation procedure of inclusion bodies (IB).

Legend: C+ positive control (pellet of bacteria transformed with *Hvp2-G6PDH* pET15b); Pellet and surnatant A and B, successive centrifugations of IB in Urea 2M; Pellet and Surnatant C, sonicated IB in Guanidine HCl.

The arrow indicates the expected molecular weight of the overexpressed G6PDH protein (56 kDa).

Western blotting was made utilising antibodies raised against potato chloroplastic (P1); plastidic (P2) and cytosolic (Cyt) G6PDH. (Wendt *et al.*, 2000). Other details in the text.

## 3.8 Purification of recombinant protein *PtP2-G6PDH* (pET3d)

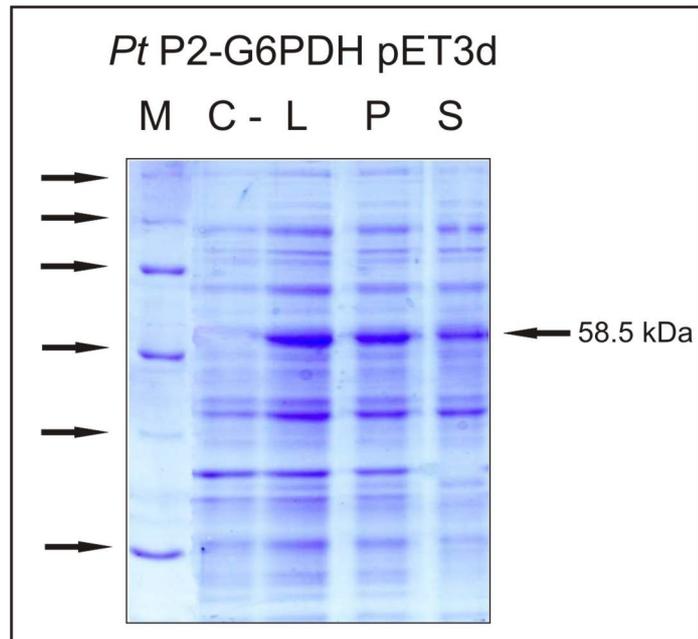
### 3.8.1 Purification of recombinant from soluble fraction

Batch cultures of *E. coli* BL21(DE3) containing *PtP2-G6PDH* (pET3d) plasmid were grown and bacteria lysates were analysed for the presence of the recombinant protein. The recombinant *PtP2-G6PDH* protein accumulated both in the pellet and in the soluble fractions. The estimated molecular weight of the G6PDH monomers was 58,5 kDa. (Fig. 31).

Recombinant P2-G6PDH was partially purified from soluble fraction of bacteria lysates (*E. coli* BL21(DE3) *PtP2-G6PDH* (pET3d)) by anion exchange chromatography. Samples were desalted and applied to a Q-Sepharose column (Ge Healthcare - Resource Q). The bound proteins were eluted by applying a 0 - 400mM NaCl gradient (Fig. 32A). A main peak of G6PDH activity was eluted at 100mM NaCl.

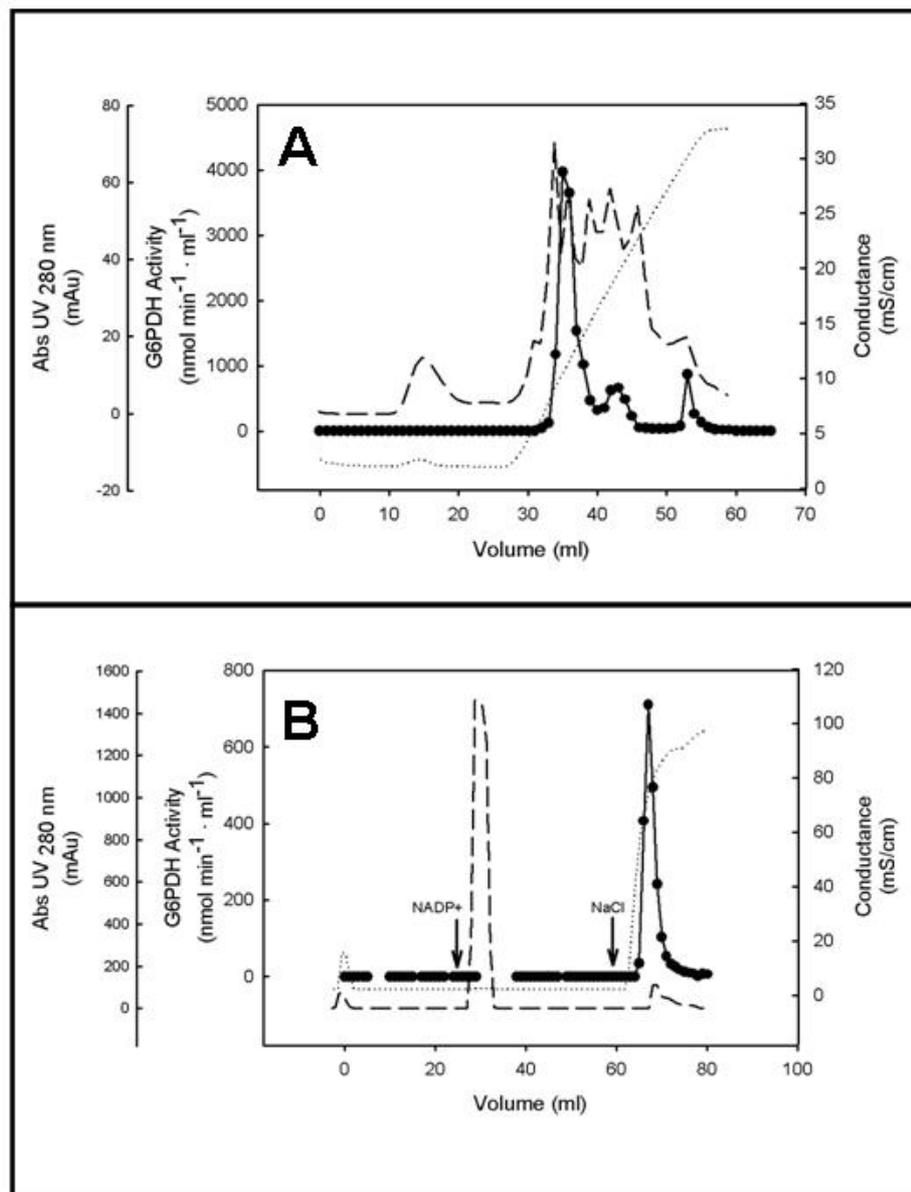
The active fractions from Resource Q-Sepharose step were applied to a Blue-sepharose column (Amersham Biosciences – Ge Healthcare Hi-Blu FF) and the recombinant G6PDH was eluted with 1.5M NaCl (Fig. 32B).

The G6PDH activity eluted in a single peak, the active fractions were pooled and utilised for further biochemical characterization.



**Figure 31:** SDS-PAGE of samples from *E.coli* BL21(DE3) lysate, transformed with *PtP2-G6PDH* (pET3b) plasmid. The molecular weight of the overexpressed G6PDH protein, calculated with the relative mobility factor method, is indicated.

Legend: M, molecular weight markers; C-, negative control (bacteria transformed with an empty plasmid); L, bacteria lysate; P, pellet; S, surnatant.



**Figure 32:** A) G6PDH activity profile (closed circles) measured in fractions eluted from a Resource Q-Sepharose column (6ml) of the supernatant of the *E. coli* BL21(DE3) lysate, transformed with *PtP2-G6PDH* (pET3d). B) G6PDH activity profile (closed circles) measured in fractions eluted from from a Blue-Sepharose column of the active fraction from ion-exchange chromatographyQ-Sepharose column.

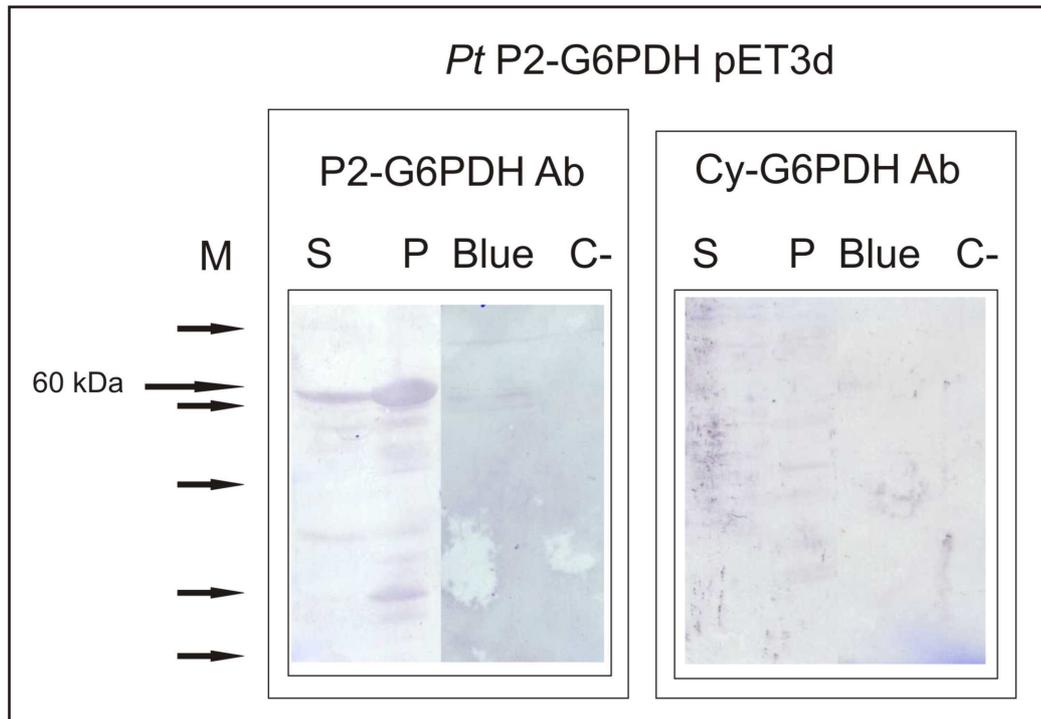
UV absorbance (280 nm) indicating total protein elution profile is indicated by the dashed line (- -), the dotted line (···) indicates the NaCl gradient. Other details in the text.

A Western analysis carried out using specific antibodies raised against potato cytosolic (Cy-G6PDH), chloroplastic (P1-G6PDH) and plastidic (P2-G6PDH) (Wendt *et al.*, 2000) confirmed that the purified protein G6PDH solely reacted versus potato P2-G6PDH antibodies (Fig. 33). No reaction was observed against Cy-G6PDH antibodies.

### 3.8.2 Kinetic properties of recombinant *PtP2-G6PDH* (pET3d)

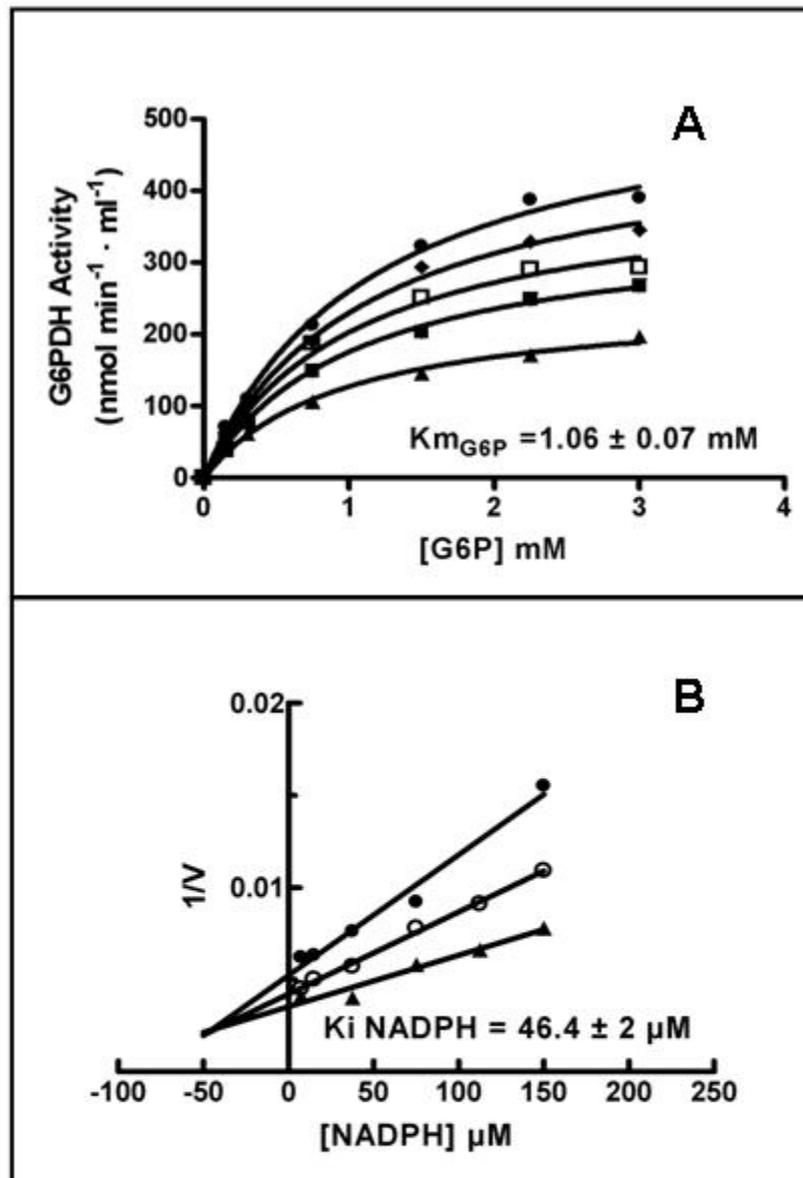
Recombinant *PtP2-G6PDH* (pET3d) from soluble fraction of bacteria lysates (Bl21-DE3 pET3d) showed a hyperbolic kinetics vs G6P, exhibiting a  $Km_{G6P}$  of 1.06mM (Fig. 34A). The apparent  $Km_{NADP^+}$  showed a similar hyperbolic kinetics with  $Km_{NADP^+} = 16\mu\text{M}$  (not shown).

The inhibition by NADPH was measured at different sub-saturating  $NADP^+$  concentrations of 15, 30, and 50 $\mu\text{M}$ . The Dixon plot analysis (not shown) allowed the estimation of different  $K_{iNADP^+}$  in the different nitrogen feeding conditions: in N-starved cells  $K_{iNADP^+}$  was 55 $\mu\text{M}$  (Fig. 34B).



**Figure 33:** Western blotting of bacteria BL21(DE3) lysate, transformed with *Pt*P2-G6PDH (pET3d) plasmid. Legend: M, Marker; S, surnatant; P, pellet, Blue, fraction purified from Blue-sepharose step; C-, negative control (bacteria transformed with an empty plasmid).

Western blotting was made utilising antibodies raised against potato plastidic (P2-G6PDH Ab) and cytosolic (Cy-G6PDH Ab) isoforms (Wendt *et al.*, 2000). Other details in the text.



**Figure 34:** Kinetic properties of PtP2-G6PDH(pET3d) purified from the supernatant of the *E. coli* BL21(DE3). A) Effect of varied G6P concentration. B) Inhibition constant for NADPH ( $K_{i \text{ NADPH}}$ ) calculated by the Dixon plot analysis.

The  $K_i$  was determined at 3mM G6P using non saturating NADP<sup>+</sup> concentrations: (●) 15μM NADP<sup>+</sup>; (○) 30μM NADP<sup>+</sup>; (▼) 50μM NADP<sup>+</sup>; regression lines show a  $r^2$  value at least of 0.96. The graphs are representative of at least three independent G6PDH purifications and kinetic parameters determinations. Other details in Materials and methods.

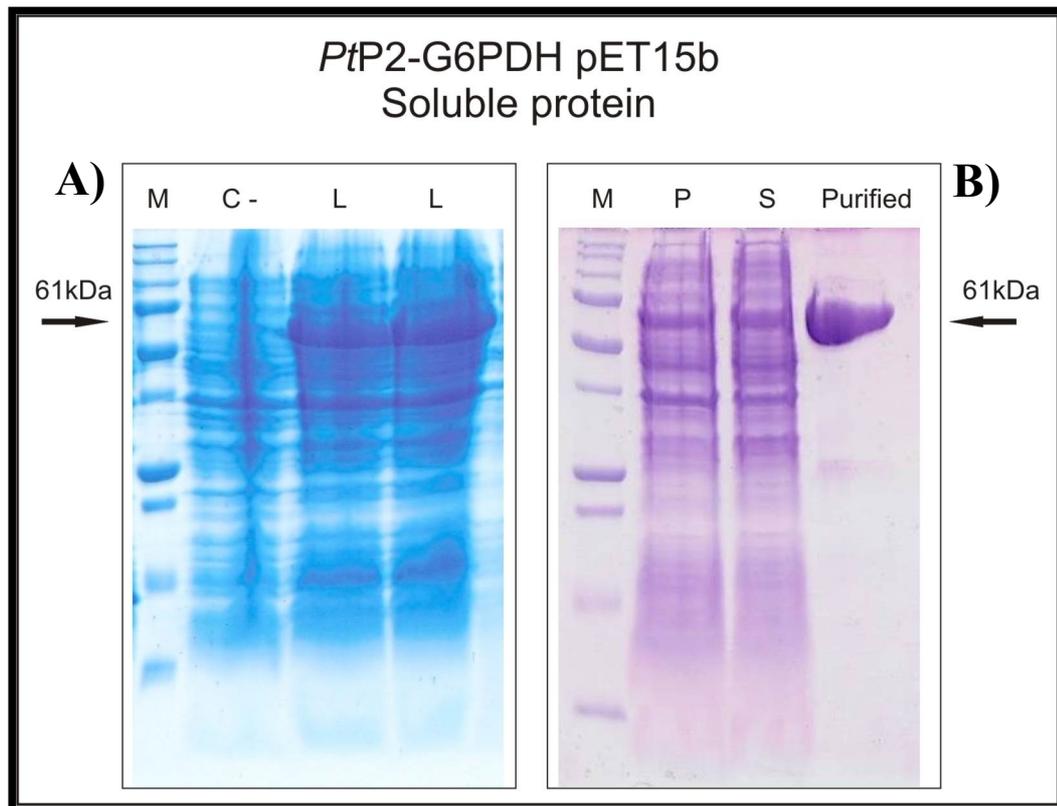
## **3.9 Purification of recombinant protein *PtP2-G6PDH* (pET15b)**

### **3.9.1 Purification of recombinant from soluble fraction**

Batch cultures of *E.coli* BL21(DE3) containing *PtP2-G6PDH* (pET15b) plasmid were grown and bacteria lysates were analysed for the presence of the recombinant protein. The recombinant *PtP2-G6PDH* protein accumulated both in the pellet and in the soluble fractions. The estimated molecular weight of the G6PDH monomers was 61 kDa (Figure 35).

### **3.9.2 Measurement of mid-point redox potential on *PtP2-G6PDH* (pET15b)**

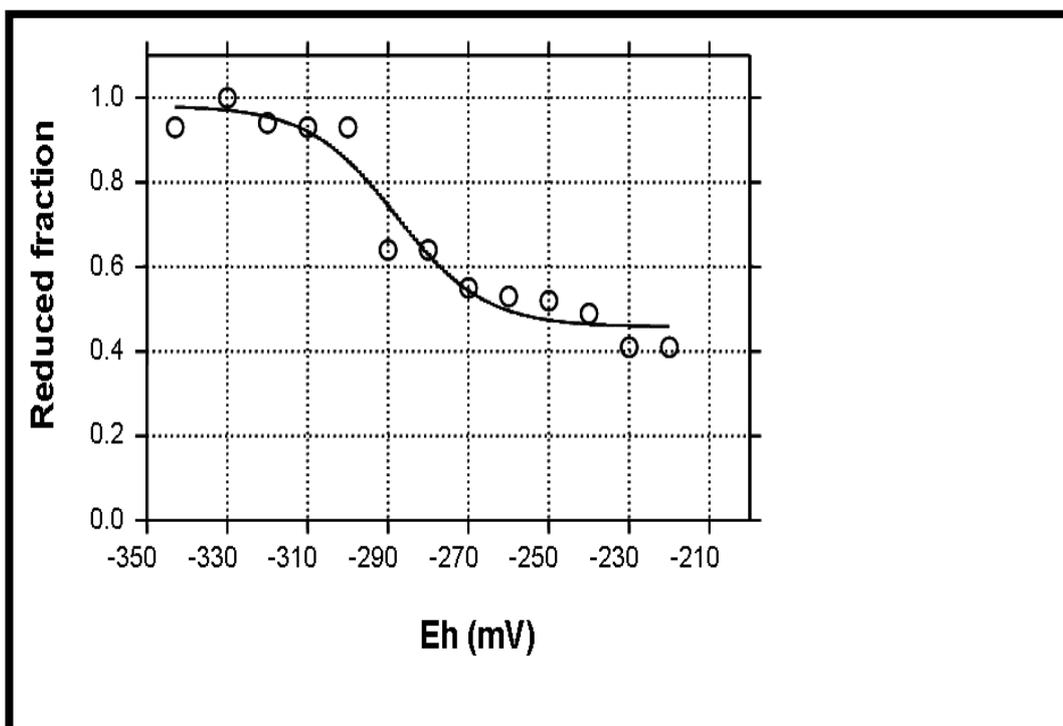
The pure protein was used to measure the redox midpoint potential ( $E_m$ ) by measuring the fluorescence of mBBr at different DTT<sub>ox</sub>/DTT<sub>red</sub> buffer. Titration gave an excellent fit to the Nerst equation for two-electron redox couple corresponding to a single regulatory disulfide. At pH 7 an  $E_m$  value of  $-288,2 \pm 4,052$  mV ( $R^2= 0,9511$ ) was found.



**Figure 35:** A) SDS-PAGE of production test of bacteria transformed with *PtP2-G6PDH* (pET15b) plasmid. B) SDS-PAGE of samples from *E.coli* BL21(DE3) lysate, transformed with *PtP2-G6PDH* (pET15) plasmid. The molecular weight of the overexpressed G6PDH protein, calculated with the relative mobility factor method, is indicated.

Legend: M, marker; C- negative control (bacteria transformed with an empty plasmid); L, different transformed colonies; P, pellet; S, supernatant; Pur, purified protein.

The molecular weight of the overexpressed G6PDH protein, calculated with the relative mobility factor method, is indicated.



**Figure 36:** Redox titration of *PtP2-G6PDH*. The percentage of oxidation was determined by measuring G6PDH fluorescence (closed circles) Interpolation curve (full line) was obtained by non-linear regression of the data using a Nernst equation for 2-electrons exchanged ( $n = 2$ ) and one redox component.  $E_m$  *PtP2G6PDH* =  $-288,2 \pm 4,052$  mV ( $R^2 = 0,9511$ ).

## CHAPTER 4

### DISCUSSION

The oxidative pentose phosphate pathway (OPPP) plays a primary role in cell metabolism. In plants, the main functions of the OPPP are the production of reducing power (NADPH) for amino acid and fatty acid synthesis, and the synthesis of precursors of nucleotides and aromatic amino acids, namely erythrose-4-phosphate and ribose-5-phosphate (Dennis *et al.*, 1997). A major role of G6PDH under abiotic (Bredemeijer and Esselink, 1995; Nemoto and Sasakuma, 2000; Wang *et al.*, 2008) and biotic (Sindelar *et al.*, 1999) stress has been proposed.

The existence of different cytosolic and plastidic OPPP has been suggested in plant cells, both in photosynthetic and heterotrophic tissues (Kruger and von Schaewen, 2003) even if some enzymes of the pathway can be missing in the cytosol (Schnarrenberger *et al.*, 1995; Debnam and Emes, 1999).

Glucose-6P dehydrogenase represents the key enzyme of the OPPP, and together with 6P-gluconate dehydrogenase it characterizes the first and most important phase of the pathway, representing a NADPH-producing system which contributes to regulate the redox poise of the cell.

G6PDH is known as a homotetramer of 50-60 kDa subunits, the occurrence of different G6PDH isoforms has been demonstrated in many plant tissues (Schnarrenberger *et al.*, 1973; Nishimura and Beevers, 1981; Wendt *et al.*, 2000; Kruger and von Schaewen, 2003; Wakao and Benning, 2005) and confirmed in barley roots (Esposito *et al.*, 2001a, 2001b), where the two enzymes can be separated by anion-exchange chromatography, and distinguished by differences in their main kinetic properties (Esposito *et al.*, 2001b, 2005).

The different plant G6PDH isoforms are subjected to distinct regulations: chloroplastic P1-G6PDH isoform is under light control and is susceptible to the NADPH/NADP<sup>+</sup> ratio (Lendzian, 1980; Fickenscher and Scheibe, 1986) and controlled by thioredoxins (Nèe *et al.*, 2009); this effect is mediated by two

cysteine residues present in the first half of the deduced amino acid sequence (Wenderoth *et al.*, 1997).

The existence of a different plastidic (P2-G6PDH) isoform has been proven (Wendt *et al.*, 2000; Esposito *et al.*, 2001a); this enzyme, expressed in most plant tissues (Wendt *et al.*, 2000; Wakao and Benning, 2005), plays a pivotal role during nitrogen assimilation in non-photosynthetic tissues (Esposito *et al.*, 2001b, Esposito *et al.*, 2003, Wang *et al.*, 2008), and fatty acid synthesis (Hutchings *et al.*, 2005). Interestingly, P2-G6PDH is less susceptible to NADPH with respect to the P1-G6PDH isoform, even if it presents the regulating cysteines are in the same positions as in the chloroplastic isoform (Wendt *et al.*, 2000).

The cytosolic isoform is involved in the basal metabolism of plant cell (Esposito *et al.*, 2005), and appears to undergo to a sugar sensing regulation (Haushild and von Schaewen, 2003). A role of cy-G6PDH has been proposed in plants subjected to salt stress (Nemoto and Sasakuma, 2002).

The phylogenetic tree generated by the comparison of 72 sequences from various organisms, available on web database, present three main branches: the first groups all the Cy-G6PDHs from Planta (monocotyledons and dicotyledons), green algae, Rhodophyta, Briophyta, Fungi and Metazoa; the second branch puts together G6PDHs from diatoms, Bacteria, Cyanobacteria; the third arranges the plant plastidic isoforms, then subdivided in P1-G6PDH and P2-G6PDH.

The possible scenario of the evolution of the different G6PDH isoforms in planta comprehends the presence of cytosolic isoforms deriving from the bacterial G6PDH: it appears that the bacterial isoform is the ancestor of all the other G6PDHs present in living organisms; Cy-G6PDHs of fungi, diatoms, algae and plants seem to derive from the same bacterial enzyme, showing no regulatory cysteine.

Another prokaryotic branch, comprising the cyanobacteria, originated the redox regulated G6PDHs; this group evolved possibly under the requirement to

avoid a futile pentose phosphate cycle of reduction/oxidation in the light. When the symbiosis of cyanobacteria originated the chloroplasts in green algae, the cyanobacterial G6PDH (encoded by plastidic DNA) was substituted by a nuclear encoded enzyme of the host G6PDH, which acquired a plastidic transit peptide, as demonstrated for a number of chloroplastic proteins.

This isoform originally did not present any regulatory cysteine, but photosynthetic activity, producing high reductants levels in the light, forced this protein to acquire a redox regulation. This redox control was acquired with the insertion of two regulatory cysteines in the N-terminal end of the sequence (Wendt *et al.*, 1999). From this chloroplastic isoform derived the P1 and P2-G6PDH of higher plants, differentiating on their distinct tolerance towards reductants and NADPH (see below).

The bioinformatic data suggest that in unicellular eukariotic organisms, such as green algae, it is present one plastidic and one cytosolic isoform. These findings are confirmed by published studies on G6PDH purified from algal cultures and recombinant algal enzymes (Jin *et al.*, 1998; Honjoh *et al.*, 2003, 2007; Esposito *et al.*, 2006). It is therefore possible that the distinction into P1- and P2-G6PDH has occurred during the re-differentiation of non-photosynthetic cells in higher photosynthetic organisms. The lost of photosynthetic pathway in these cells possibly induced a minor sensitivity of P2-G6PDHs versus NADPH with respect to chloroplastic P1-G6PDHs.

Moreover, it should be noted that the similarity among higher plants cytosolic, plastidic and chloroplastic isoforms is higher than that observed among compartmented isoforms and cyanobacterial enzymes, even if all these last enzymes present a redox control by cysteines regulation.

In diatoms, where the chloroplast has originated from a secondary endosymbiotic event, possibly from an ancestor similar to a red alga, the chloroplastic enzyme was lost and not replaced anymore: therefore diatoms possess only a Cy-G6PDH (Michels *et al.*, 2005).

Even if the different G6PDH isoforms have been purified and characterized

from algae (Esposito *et al.*, 2006) and plant tissues, including leaves (Schnarrenberger *et al.*, 1973; Scheibe *et al.*, 1989), roots (Hong e Copeland, 1991; Esposito *et al.*, 2001a) and cultured cells (Krook *et al.*, 1998), the purification of G6PDH from plant cells usually produced low yields of enzyme activity (Srivastava and Anderson, 1983), possibly given by an intrinsic instability of the protein, or at least of the active tetramer.

Given the number of papers published about G6PDH in photosynthetic organisms, only some dealt with green algae (Hipkin and Cannons 1985; Huppe *et al.*, 1994; Huppe and Turpin, 1996; Jin *et al.*, 1998; Esposito *et al.*, 2006).

In green algae, both cytosolic and plastidic G6PDH have been described (Klein *et al.*, 1986; Honjoh *et al.*, 2003; 2007). A single G6PDH isoform was purified from *Chlorella sorokiniana*, it specifically reacts versus P2-G6PDH antibodies (Esposito *et al.*, 2006). The chloroplastic algal enzyme exhibits high  $K_{iNADPH}$ , 102 $\mu$ M in *Chlamydomonas* (Klein, 1986), 90 $\mu$ M in *C. sorokiniana* (Esposito *et al.*, 2006). From an evolutionary point of view, it is interesting to note that all algal chloroplastic G6PDHs so far described (Klein, 1986; Jin *et al.*, 1998; Esposito *et al.*, 2006) present high tolerance at elevated NADPH, similar to the P2-G6PDH (expressed in non photosynthetic tissues) and curiously different with respect to the higher plant chloroplastic P1-G6PDH (see below).

This lower sensitivity to NADPH may allow this enzyme to operate under physiological conditions previously presumed to be inhibitory.

This tolerance to the NADPH suggests that under high NADPH/NADP<sup>+</sup> ratios the enzyme would be able to produce reducing power for assimilatory processes, Previous findings suggest a NADPH/NADP<sup>+</sup> ratio between 2 and 6 in *Selenastrum minutum* (Vanlerberghe *et al.*, 1992), and 3 in *Chlamydomonas reinhardtii* (Forti *et al.*, 2003) in the light.

Under similar conditions, *Chlorella sorokiniana* G6PDH would retain a considerable part of activity for a fast and efficient nitrogen assimilation during starvation; upon nitrate feeding, some post-translational changes in the

enzyme structure may occur, lowering the  $K_{i\text{NADPH}}$  (Esposito *et al.*, 2006).

These changes in  $K_{i\text{NADPH}}$  could be ascribed to post-translational modifications of the enzyme: it has been proposed that chloroplastic (and possibly plastidic) G6PDH undergoes phosphorylation in potato (Hauschild and von Schaewen, 2003).

The biochemical characterization of higher plants G6PDHs allowed a classification of the isoforms in two main groups, the cytosolic G6PDH (Cy-G6PDH) and the plastidic enzymes.

The Cy-G6PDH accounts for about 80-95% of the total extractable activity in plants (Debnam and Emes, 1999; Esposito *et al.*, 2001a): a great part of this activity appears to be implicated in basal metabolism of plant cell.

The cytosolic isoform appears insensitive to reductants (Fickenscher e Scheibe, 1986; Wenderoth *et al.*, 1997; Esposito *et al.*, 2001a). In barley, the cytosolic isoform exhibited a molecular weight of 57.5 kDa, with its maximum activity at pH 7.90; this isoform is not inhibited by DTT (Esposito *et al.*, 2001), in accord with other higher plants Cy-G6PDHs (Graeve *et al.*, 1994; Fickenscher and Scheibe, 1986).

The plastidic G6PDH enzymes can be divided into two subgroups, with chloroplastic isoforms (P1-G6PDH) distinguishable from the plastidic enzymes (P2-G6PDH). This dissimilarity is based on the evidence that chloroplastic isoforms, expressed solely in green tissues, are strictly regulated by reductants such as DTT *in vitro*; this redox sensitivity is given *in vivo* by thioredoxins, which look as the main regulators of chloroplastic OPPP. The plastidic P2-G6PDH, on the contrary, are expressed in most of the plant tissues (Wendt *et al.*, 2000; Wakao and Benning, 2005), and show a less sensitive, even if clearly evident, redox regulation; the possibly actions of thioredoxins on P2-G6PDH have not investigated yet, primarily for the intrinsic high instability of both enzyme purified from plants (Esposito *et al.*, 2001a) and recombinant bacteria (Wendt *et al.*, 2000) studied so far. Most important, the inhibition by NADPH

(the main regulator of G6PDH activity) is much higher in P1-G6PDH (e.g.  $K_{i_{\text{NADPH}}} < 10 \mu\text{M}$ ) than in P2-G6PDH (e.g.  $K_{i_{\text{NADPH}}} > 50 \mu\text{M}$ ). This description fits with the most of G6PDH purified from various plant tissues. The only known exception is given in *Arabidopsis*, where *AtG6PDH1* (P1-type) shows a  $K_{i_{\text{NADPH}}} = 30\text{--}70 \mu\text{M}$ , while *AtG6PDH2* and *AtG6PDH 3* (P2-type) exhibit  $K_{i_{\text{NADPH}}} = 2.5 \mu\text{M}$  and  $22 \mu\text{M}$ , respectively (Wakao and Benning, 2005).

The plastidic P2-G6PDH isoform represents 15~20% of the total G6PDH activity in plant tissues (Hong and Copeland, 1991; Bredemeijer and Esselink, 1995; Debnam and Emes, 1999; Esposito *et al.*, 2001a), and it is strictly related to nitrogen metabolism (Bowsher *et al.*, 1989, 1992; Esposito *et al.*, 2003); usually higher plants P2-G6PDHs exhibit higher  $K_{m_{\text{G6P}}}$  with respect to the cytosolic isoforms (Wright *et al.*, 1997; Esposito *et al.*, 2001a; 2001b; 2003; Wakao and Benning, 2005).

Barley plastidic P2-G6PDH exhibited a molecular weight of 52kDa, and optimum pH at 8.50; DTT caused a 50 % inhibition of this isoform; the inhibition could be partially recovered by oxidizing agents (such as sodium tetrathionate) (Esposito *et al.*, 2001a). Barley P2-G6PDH isoform exhibit  $K_{m_{\text{G6P}}} \sim 1\text{mM}$ ,  $K_{m_{\text{NADP}^+}} \sim 9.4 \mu\text{M}$ , and  $K_{i_{\text{NADPH}}}$  is  $59 \mu\text{M}$  (Esposito *et al.*, 2003), a value similar to that found in potato ( $70 \mu\text{M}$ ) (Wendt *et al.*, 2000); on the other hand its properties appear different from those measured for both P2-G6PDHs described in *Arabidopsis thaliana* (Wakao and Benning, 2005).

In the experiments described here, barley (*Hordeum vulgare* cv.Nure) recombinant P2-G6PDH was overexpressed and purified from two different plasmids, pET3d and pET15b. The clone length encoded for an enzyme devoid of the first 99 aminoacids; because the prediction software suggested plastidic transit peptide of 38 aminoacids, the cloned sequence would encode for a truncated protein. Once cloned in both plasmids (pET3d and pET15b), the coding *HvP2-G6PDH* fragments were sequenced confirming that they were in frame and no mutation occurred in the cloning process (not shown).

Although well expressed, most of the recombinant protein *HvP2-G6PDH*

(pET3d) accumulated in the pellet of bacteria lysates. Therefore, even if the soluble enzyme was purified using conventional biochemical strategies, at the same time a protocol of denaturation/refolding of the insoluble protein from the inclusion bodies was adopted, in the aim to increase the yield of the enzyme.

The soluble protein was purified by anion exchange chromatography followed by blue agarose chromatography; even if the starting amount of soluble protein in the lysates was low, the purified enzyme from this purification strategy was enough to carry on measurements of the main properties of the recombinant *HvP2-G6PDH*. Anyway, the protein was unstable in the medium period, losing 90% of the activity within 5 days at 4°C.

The procedure utilised to recover the recombinant protein present in the inclusion bodies was modified from protocols optimised for human G6PDH (Wang *et al.*, 2009). The bulk of recombinant *HvP2-G6PDH* present in the pellet of bacteria lysates was completely denatured in guanidine-HCl overnight, and then enzyme was allowed to refold in Arg/DTT/NADP<sup>+</sup> buffer. This process took several days (usually 5 to 10 d) before an acceptable refolding of the enzyme for the determination of kinetic properties was achieved.

Both soluble enzyme and refolded protein showed similar  $K_{m_{G6P}}$ , 0.94 - 0.86mM; most important the inhibition constants ( $K_{i_{NADPH}}$ ) measured were 75-80µM, very similar to values observed for the plastidic G6PDH enzyme purified from barley roots (Esposito *et al.*, 2003, 2005) and potato P2-G6PDH (Wendt *et al.*, 2000).

Similarly, the reactivity vs antisera raised against the different recombinant potato G6PDH isoforms (Wendt *et al.*, 2000) confirmed that the overexpressed protein reacted only vs P2-G6DPH antibodies and not versus Cy-antibodies.

In order to obtain a better yield, and possibly an increased percentage of the recombinant protein in the soluble fraction of lysates, the *HvP2-G6DPH* was cloned in a his-tag expression vector (pET15b). This time the quantity of

recombinant protein present in the supernatant of the bacteria lysates was sensibly higher, encouraging the purification of the enzyme from the soluble fraction. Therefore the *HvP2-G6PDH* was purified using the inorganic metal affinity chromatography (IMAC), utilising HiTrap columns, having Nickel (Ni) as binding metal for the his-tag. The good yield of the purified protein unfortunately caused a sensitive loss of enzyme activity, even if the residual activity recovered was enough for the determination of kinetic and immunological properties of the recombinant protein.

To ensure that the loss of enzyme activity was not specifically attributable to  $\text{Ni}^{++}$  ions, a further control was made purifying the protein using a Talon – Cobalt-linked resin, but even if this column gave a good yield of the protein, it resulted in a complete loss of enzymatic activity (not shown). These data support the hypothesis that some metals strongly inhibit G6PDH activity, and this is particularly unlikely when his-tagged proteins have to be purified.

It should be underlined that previous attempts to purify potato recombinant his-tagged potato P2-G6PDH failed, and crude bacteria lysates were used for measure of the properties of the enzyme (Wendt *et al.*, 2000). On the other side it should be noted that evidence has been given of a 5-fold activation of both *Chlorella vulgaris* C-27 G6PDHs (cytosolic and chloroplastic) by  $\text{Cd}^{++}$ , and it has been proposed that this effect was due to the binding of the metal ions to free sulfhydryl groups on the enzyme (Honjoh *et al.*, 2003).

The purified *HvP2-G6PDH* (pET15b) protein was analysed by Mass Spectrometry, and the sequencing of digested peptides confirmed unequivocally that the recombinant protein obtained has the identical amino acid sequence encoded in *HvP2-G6PDH* sequence present in the database for both Nure and Alfeo cv of *Hordeum vulgare*.

Therefore, even given the low yield of recombinant *HvP2-G6PDH* (pET15b), the purification protocols here utilised can be considered successful, being able to obtain an active enzyme suitable for a precise determination of kinetic

properties of the enzyme.

Also in this case a strategy was utilised to purify the his-tagged enzyme by IMAC chromatography.

This time the procedure was designed to isolate bacterial inclusion bodies, and rupturing them by sonication in Guanidine-HCl. Then different chromatography protocols using a his-trap column (Ni) were tested, e.g. partial refolding as described for *Hv*P2-G6PDH (pET3d) enzyme, on column refolding, direct purification on the column both using an imidazole continuous gradient, or a step gradient. The latter procedure gave the best results; even in this case the purified *Hv*P2-G6PDH (pET15b) from inclusion bodies specifically reacts against potato P2-G6PDH antibodies.

The availability of the complete *Populus trichocarpa* genome (Tuskan *et al.*, 2006) is a great opportunity to clone and characterize the P2-G6PDH from tree plants.

Therefore specific primers were designed to clone the *Pt*P2-G6PDH in the aim to obtain a further recombinant enzyme to be described. The primers were designed at 66aa from their beginning of the initial methionine, again giving an enzyme devoid of the transit peptide and of the initial aa sequence.

Initially the *Pt*P2-G6PDH was cloned in pET3d plasmid, and then in pET15b plasmid (his-tag). Once cloned in both plasmids (pET3d and pET15b), the coding *Pt*P2-G6PDH fragments were sequenced confirming that they were in frame and no mutation occurred in the cloning process (not shown).

The protein was highly expressed, and differently with respect to barley protein, *Pt*P2-G6PDH (pET3d) was present in a good percentage in the soluble fraction of bacteria lysates. Therefore this soluble enzyme was purified using anion exchange chromatography followed by blue-sepharose chromatography; the yield of this purification strategy was acceptable and the purified enzyme was used for the measure of the main properties of the recombinant *Pt*P2-G6PDH. This purified protein was stable for 7d, losing 20% of the activity

when stored at 4°C.

*PtP2-G6PDH* showed a  $Km_{G6P}$  of 1mM, and the  $Ki_{NADPH}$  measured was 55 $\mu$ M, similar to values measured for the plastidic G6PDH enzyme purified from barley roots (Esposito *et al.*, 2003, 2005) and potato P2-G6PDH (Wendt *et al.*, 2000). It obviously should be noted the similarities between the properties of the recombinant *PtP2-G6PDH* and recombinant *HvP2-G6PDH* previously described.

Again, the reactivity vs antisera raised versus the different recombinant potato G6PDH isoforms (Wendt *et al.*, 2000) confirmed that the overexpressed *PtP2-G6PDH* protein reacted only vs P2-G6DPH antibodies.

*PtP2-G6PDH* was cloned in pET15b expression vector (his-tag).

Again the protein was well expressed and highly present in the surnatant of bacteria lysates. The protein was purified using IMAC chromatography; the yield was high and the purified enzyme was very stable for over one month at 4°C, and used for the measure of the redox potential.

The redox midpoint potential ( $E_m$ ) of *PtP2-G6PDH* (pET15b) was of -288 mV. Titration gave an excellent fit with different DTT red/ox buffers to the Nernst equation for a two-electron redox couple corresponding to a single regulatory disulfide. This  $E_m$  value is lower than that found for *AtP1-G6PDH* (-330 mV; Née *et al.*, 2009). This suggest that *PtP2-G6PDH* present the regulatory cysteines exhibiting a potential sensibly less negative than f- and m-type Trxs (-351 and -358 mV ; Née *et al.*, 2009) and therefore the enzyme can be easily reduced, but less favourably oxidized, by these thioredoxins.

In recent years an increasing role in response to stress has been given to the OPPP (Popova *et al.*, 1996; Shetty *et al.*, 2002; Van Heerden, 2003; Hou *et al.*, 2007), and namely to the reaction of G6PDH as one of the main redox poise regulating reactions in the plant cell (Jin *et al.*, 1998).

Among stresses, nitrogen starvation has been extensively linked on the activities and the occurrence of the different plant G6PDHs. The presence of the different G6PDH isoforms in plant cells was correlated with the furnishing

of reductants for nitrite reductase (Bowsher *et al.*, 2007); an electron shuttling from NADPH produced by G6PDH and nitrite reductase has been proposed (Oji *et al.*, 1985; Jin *et al.*, 1998).

Previous data indicate that plastidic OPPP in the roots produces reductants for nitrogen assimilation (Emes and Fowler, 1979; Bowsher *et al.*, 1992; Esposito *et al.*, 2003). The increase in total G6PDH activity in the roots under nitrogen has been investigated (Esposito *et al.*, 2001b; 2003; 2005), suggesting that the appearance of P2-G6PDH isoform is depending on nitrate or ammonium feeding: in barley roots grown without nitrogen the P2-G6PDH protein is not detectable, whereas in N-fed roots, P2-G6PDH represents 25 % of the total activity (Esposito *et al.*, 2005).

In the leaves from plants grown without nitrogen, cytosolic and P2-G6PDH were detectable, while P1-G6PDH band was faint; upon nitrogen nutrition, the chloroplastic isoform increased together with Fd-GOGAT (Esposito *et al.*, 2005).

Salinity represents another abiotic stress inducing changes in OPPP and namely in activities of the various G6PDHs (Wang *et al.*, 2003).

In wheat (*Triticum aestivum*) salt stress induced an increase in the transcription of five early salt-stress responding genes (WESR: Wheat Early Salt Responding; WESR1–5) (Nemoto and Saskuma, 2000). These transcripts were analyzed for their temporal accumulation of mRNA during salt stress, osmotic stress and abscisic acid (ABA) treatment. All genes showed transient stimulation by 0.15M NaCl treatment. Notably WESR5 has been identified as a putative G6PDH encoding gene.

WESR5 clones obtained codify for proteins with deduced MW of 57.7 – 58.3kDa and they did not contain any plastid targeting sequences, so the analysis suggested that the early response to the osmotic stress would involve the cytosolic oxidative pentose phosphate pathway (Nemoto and Sasakuma 2000, 2002).

G6PDH transcription shows a rapid induction within first 2 hours of exposure

to NaCl and it reaches the highest level after 12 hours. During the following hours the expression of G6PDH in the initial response to NaCl stress (Nemoto and Sasakuma, 2000).

Moreover, specific increase in G6PDH content in particular leaf tissues (epidermis and mesophyll) has been demonstrated in Olive trees (Valderrama *et al.*, 2006) and reed (Wang *et al.*, 2008) under oxidative stress induced by salinity.

In barley the addition of NaCl caused a two-fold increase of G6PDH activity in the roots, regardless of nitrogen supply (Esposito S., personal communication). It is thus evident the salt stress induced increase in G6PDH activity lies outside the normal regulatory mechanisms present in physiological conditions. Although the role of OPPP in the response and/or tolerance to drought, salinity and nutrient starvation in plants has been widely accepted in the last years (Valderrama *et al.*, 2006; Esposito *et al.*, 2001b; 2005), the possibility of a direct effect of ABA on the expression of this enzyme opens new perspectives in the study of the regulation of the adaptation of whole plant to abiotic stresses.

Abscissic acid is a hormone playing a central function in cellular reactions such as stomatal aperture, but plays an important role in development, growth, seed germination and gene expression. At the same time it has a pivotal role in the adaptive response to the changing environmental conditions: ABA regulates the response to different environmental stresses such as salinity, cold and drought and is an integral part of stress signal transduction (Finkelstein *et al.*, 2002; Zhu, 2002). Application of ABA to the barley seedlings caused risible effects in the first 24h, but after 48h, visible growths in leaf and root lengths were observed. These effects could be linked to the ABA-induced restriction of ethylene production (Sharp, 2002).

Abiotic (namely salt) stress can be subdivided in an osmotic component and an ionic component. The osmotic component is strictly related to drought, and it is possibly ABA insensitive. In contrast, the ionic component is ABA sensitive

(Zhu, 2002). In the experiments presented here, ABA was supplied to the roots of barley plants in order to define the role(s) of the different G6PDH isoforms. First of all, it is worth mentioning that the cytosolic G6PDH isoform(s) accounts for 78 to 92% of total measurable activity in plant tissues (Debnam and Emes, 1999; Esposito *et al.*, 2001b; 2005), whereas the plastidial activity, which probably represents the rest of G6PDH activity is undetectable upon nitrogen starvation, but induced both by ammonium (Esposito *et al.*, 2001b) and nitrate (Esposito *et al.*, 2005).

Therefore, total enzymatic activity, protein occurrence and transcript levels of the Cy- and P2-G6PDHs have been investigated in the ABA treatment, keeping in mind that ABA plays pivotal roles in various aspects of plant growth and development as well.

It has been proven that ABA levels affect directly several basic-metabolism enzymes. In addition, a central role has been described for glucose-6P dehydrogenases both in ABA signalling pathway and salt stress response (Kempa *et al.*, 2008). Thus we are anticipating that G6PDH regulation by this plant hormone might be crucial upon stress conditions. The *in silico* analysis of the P2-G6PDH of rice (*Oryza sativa*) revealed the presence of an ABRE element (Hou *et al.*, 2006; Lenka *et al.*, 2009). As barley and rice P2-G6PDH sequences show a high similarity, they both likely share an ABRE element, indicating that the barley enzyme might be regulated by abscisic acid as well (Hou *et al.*, 2006; Lenka *et al.*, 2009). In our experiments a rapid increase was measured in both leaves and roots in G6PDH activity confirm previous results in higher plants upon nitrogen starvation (Esposito *et al.*, 2001b; 2005), under biotic (Sindelar *et al.*, 1999) and abiotic (Bredemeijer and Esselink, 1995) stresses.

The abundance of G6PDH proteins was investigated by Western blotting using antibodies able to discriminate the different isoforms (Wendt *et al.*, 2000; Esposito *et al.*, 2005): in the leaves no apparent change in the occurrence of cyt-G6PDH and a decrease in P1-G6PDH were observed, but a 2 fold increase

in P2-G6PDH protein was measured. It should be noted that nitrogen starvation and nitrogen supply did not change the levels of P2-G6PDH isoform in leaves (Esposito *et al.*, 2005). These results suggest also in green tissues the involvement of plastidial (and possibly chloroplastic) isoforms in the changes in G6PDH activity observed in crude leaf extracts.

Cy-G6PDH protein content slightly increased following ABA treatment; this pattern is similar to that observed for Cy-G6PDH in barley roots after nitrogen supply (Esposito *et al.*, 1998). A similar up and down accumulation related to ABA levels was observed in non-dormant *Arabidopsis* with respect to dormant seeds for two isoforms of cytosolic glyceraldehyde-3-P dehydrogenase (Chibani *et al.*, 2006).

On the other side, supply of exogenous ABA increased progressively the root P2-G6PDH signal in 12h, mimicking that observed for total activity. This increase of P2-G6PDH protein content in roots following ABA supply strongly supports the involvement of this isoform in the response to abiotic stress in plants.

To understand the role of ABA on the transcriptional regulation of the different G6PDH isoforms, semi-quantitative RT-PCRs were performed on roots and leaves from barley plants experiencing an ABA treatment.

The Cy-G6PDH and P2-G6PDH encoding genes were amplified from *Hordeum vulgare* root and leaf cDNAs with specific pair of primers. Even though P1-G6PDH is present in leaves, it cannot be detected in our RT-PCR measurements due to primers' design, as they were conceived not to be able to anneal with the P1-G6PDH. About the P1- RT-PCR, unfortunately in barley this protein has not been sequenced yet. Therefore we have no specific primers designed for barley P1-G6PDH.

Semi-quantitative RT-PCR analysis indicates that Cy- and P2-G6PDHs are expressed both in roots and leaves.

The transcript level of Cy-G6PDH in both roots and leaves did not appreciably change after ABA supply, in accord with the role in basic metabolism and cell

growth proposed for the Cy-G6PDH in roots (Kruger and von Schaewen, 2003; Esposito *et al.*, 2003; 2005). This is also in accordance with previous observations that no apparent change in Cy-G6PDH transcript could be observed in wheat roots during the first 24h upon exogenous ABA or mannitol supply, in contrast to the two-fold increase upon salt stress (Nemoto and Sasakuma, 2000). It is noteworthy that contrasting evidence has been observed previously. Indeed, transcript levels of Cy-G6PDH are unchanged upon ABA stress in rice (Hou *et al.*, 2006), whereas they were down-regulated in *Arabidopsis thaliana* (Wang *et al.*, 2008), and up-regulated in wheat upon salt stress (Nemoto and Sasakuma, 2000).

P2-G6PDH transcripts are detectable throughout the plant, mainly in stem and roots of *N. tabacum* (Knight *et al.*, 2001) and *A. thaliana* (Wakao and Benning, 2005). In *S. tuberosum*, P2-G6PDH transcripts are prominent in roots and growing tissues, but in leaves both P1- and P2- isoforms could be detected (Wendt *et al.*, 2000).

In our experiments P2-G6PDH transcript levels, increased by 40 % in roots and 66% in leaves within 3 h after ABA supply, suggesting that the progressive increase in total G6PDH activity measured in the first 12h could be due at least in part to an increase in transcription and synthesis of P2-G6PDH protein induced by the hormone in the roots.

In order to study in more detail the regulation of G6PDH genes in the different plant organs and under stress conditions (see after), we took advantage of the barley affymetrix arrays results available in the genevestigator website (<https://www.genevestigator.com>). The genevestigator database contains quality-controlled and well-annotated microarray experiments of barley (and rice as well) (Zimmermann *et al.*, 2008). Contig 24228 corresponds to a plastidial barley G6PDH (P2-type), whereas contigs 3308 and 3309 likely correspond to two different Cy-G6PDH (similar to Cy-G6PDH from *Triticum aestivum* and *Oryza sativa* respectively).

Contig 3308 is highly expressed in in seed pericarp and cell cultures, whereas

contig 3309 is expressed in shoots and leaves of barley.

This likely indicates that the barley cy-G6PDH detected in our experiments is the sole isoform present in the two tissues examined (roots and leaves). It should be noted that the two different cytosolic isoforms described in *Arabidopsis thaliana*, AtG6PDH5 and AtG6PDH6 (Wakao and Benning, 2005), are similarly differentially distributed, the former being expressed mainly in roots, and the latter constitutively expressed in leaves (Wakao and Benning, 2005; Wakao *et al.*, 2008).

Concerning contig 24228, a relatively high expression is detected in shoots (leaf and flag leaf) compared to the other organs. Besides, Knight and colleagues found that the P2-G6PDH was expressed in all tissues, including roots, leaves, stem and flowers of *N. tabacum* (Knight *et al.*, 2001). This result is in accordance with the result of the semi-quantitative RT-PCR in barley leaves and roots presented.

To get a better view of the expression and regulation of the G6PDHs in other stress situations, we used the microarray data available using Genevestigator. Regarding environmental constraints, the contig 3308 is strongly up-regulated by biotic stress, in particular during the infection by *Blumeria graminis* and slightly up-regulated by drought (not shown). In contrast, the contig 3309 is not regulated in any stress conditions tested except for a slight down-regulation during malting and drought stress. The P2-G6PDH (contig 24228) is heavily down-regulated by drought stress and up-regulated during malting. The latter process is a consequence of gibberellic acid (GA) release - an antagonist of ABA. In the experiments shown here, the P2-G6PDH isoform is up-regulated by ABA treatment in mature barley plants, This apparent discrepancy could be explained by the existence of several (but not yet identified in barley) P2-G6PDH isoforms with distinct expression pattern, as in *A. thaliana* or *P. trichocarpa*. Alternatively, it could be the same isoform which is differently regulated in germinating seeds compared to leaves.



## CHAPTER 5

### CONCLUSIONS

The recombinant P2-G6PDH of *Hordeum vulgare* and *Populus trichocarpa* were cloned and expressed in two different expression vectors. The enzyme coding sequence was inserted in both pET3d and pET15 plasmids, in order to obtain a pure and stable protein. After different attempts, the enzyme was purified from both soluble fraction and inclusion bodies of bacteria overexpressing the protein.

The overexpressed protein was analysed by MS, confirming that the sequence was correctly inserted and producing an active P2-G6PDH enzyme.

The main kinetic parameters measured for both *Hv*P2-G6DPH and *Pt*P2G6PDH are in accord with the values known for the native and recombinant P2-type G6PDH studied so far, and particularly for the barley protein purified from roots. The western blotting confirmed these proteins were recognized only by antibodies raised versus recombinant potato P2-G6PDH but not Cy-G6PDH antisera. The recombinant protein is moderately sensitive to reductants (DTT); moreover the *Populus trichocarpa* enzyme present a redox potential (-280 mV) favourable to the control on the behalf of thioredoxins *m* and *f*.

Moreovoer it has been demonstrated that ABA supply causes an increase in total G6PDH activity both in barley roots and leaves with the plastidial P2-isoform playing a specific role in ABA-treated barley plants. In roots, as the P1-G6PDH protein is not detected and as the abundance of Cy-G6PDH is not varying significantly, the G6PDH activity increase upon ABA supply is most likely attributed to the plastidial P2-G6PDH whose protein content is gradually increased from 3 to 12h of ABA treatment. This increase in protein level can be correlated with the transcript levels, which increase 3h-9h after treatment. These results suggest that a considerable part of the response to ABA effect in roots involves the plastidial P2-G6PDH.

In leaves, the Cy-G6PDH transcript levels did not change in response to ABA treatment. However, at the protein level, the abundance of P1-G6PDH and Cy-G6PDH proteins was not influenced by ABA whereas the P2-G6PDH protein increased within 6h, decreasing gradually afterwards. Thus, the two-fold increase in total G6PDH activities observed at 6 h is likely related to an increase in P2-G6PDH activity in leaves.

This data supports the following hypothesis: ABA supply causes an increase in total G6PDH activity both in roots and in leaves, this increase can to a large extent be attributed to an increase in P2-G6PDH transcript and protein, suggesting a direct effect of ABA on P2-G6PDH gene, possibly due to the presence of an ABRE element on the gene promoter.

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