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Research article

# Glucose-6-phosphate dehydrogenase plays a central role in the response of tomato (*Solanum lycopersicum*) plants to short and long-term drought

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ABSTRACT

The present study was undertaken to investigate the expression, occurrence and activity of glucose 6 phosphate dehydrogenase (G6PDH – EC 1.1.1.49), the key-enzyme of the Oxidative Pentose Phosphate Pathway (OPPP), in tomato plants (*Solanum lycopersicum cv. Red Setter*) exposed to short- and long-term drought stress.

For the first time, drought effects have been evaluated in plants under different growth conditions: in hydroponic laboratory system, and in greenhouse pots under controlled conditions; and in open field, in order to evaluate drought response in a representative agricultural environment.

Interestingly, changes observed appear strictly associated to the induction of well known stress response mechanisms, such as the increase of proline synthesis, accumulation of chaperone Hsp70, and ascorbate peroxidase.

Results show significant increase in total activity of G6PDH, and specifically in expression and occurrence of cytosolic isoform (cy-G6PDH) in plants grown in any cultivation system upon drought.

Intriguingly, the results clearly suggest that abscissic acid (ABA) pathway and signaling cascade (protein phosphatase 2C – *PP2C*) could be strictly related to increased G6PDH expression, occurrence and activities.

We hypothesized for G6PDH a specific role as one of the main reductants' suppliers to counteract the effects of drought stress, in the light of converging evidences given by young and adult tomato plants under stress of different duration and intensity.



## 1. Introduction

Glucose 6 phosphate dehydrogenase (G6PDH – EC 1.1.1.49) is well known as the first and rate-limiting enzyme of the Oxidative Pentose Phosphate Pathway (OPPP), catalyzing the oxidation of glucose-6-phosphate (G6P) to 6-phospho- δ -glucono-1,5-lactone, spontaneously converted – or by the action of lactonase (EC 3.1.1.31) - to 6 phospho-gluconic acid; together with following 6-phosphogluconic acid dehydrogenase (6PGDH - EC 1.1.1.44) to ribulose-5-phosphate, these reactions produce moieties of reducing equivalents as NADPH (Kletzien et al., 1994; Castiglia et al., 2015).

It is widely recognized that different intermediates of the OPPP are used for biosynthetic pathways (e.g. ribose-5P, erythrose 4P for nucleotides biosynthesis), while a considerable part of the reducing power produced in the OPPP is utilized for nitrogen assimilation in plants (Bowsher et al., 1992; Esposito et al., 2003, 2005) and algae (Huppe and Turpin, 1996; Esposito et al., 2006; Ferrara et al., 2013).

A primary role in the regulation of G6PDH (and therefore of the whole OPPP) is played by NADPH/$NADP^+$ ratio. In photosynthetic organisms, this ratio is low during active biosynthetic processes (Huppe and Turpin, 1996) and it is modulated by stress conditions (Nemoto and Sasakuma, 2000); when the ratio is high the G6PDH activity decreases (Esposito et al., 2005).

It has been previously demonstrated the presence in higher

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plants of at least three different G6PDH isoforms, playing different roles in plant metabolism (Kruger and von Schaewen, 2003). Two compartmented enzymes are found in the plastids: P1-G6PDH seems exclusively found in green tissues (Esposito et al., 2005) and it is similar to algal isoform (Esposito et al., 2006), while P2-G6PDH is predominant in roots and heterotrophic tissues (Esposito et al., 2001). It is however known that the major part of the activity can be ascribed to the cytosolic isoform (Cy-G6PDH).

The cytosolic isoforms support the major part of the G6PDH total activity in plant cells, contributing for 60–80% of total rate measured (Esposito et al., 2005). The expression of these isoforms is induced at transcriptional level by abscissic acid (ABA – Hou et al., 2006) and/or by a sugar-sensing mechanism (Lejay et al., 2008). Cy-G6PDH is tightly regulated at post-transcriptional level by various mechanisms such nitrogen levels (Esposito et al., 2001, 2003). Moreover, cy-G6PDH is generally insensitive to light effects (Fickenscher and Scheibe, 1986), which exert the main control on the activity of P1-G6PDH (Wenderoth et al., 1997).

In plants, in the last years several studies described the key functions of G6PDH in stress-response mechanisms. G6PDH plays an important role in maintaining the redox state of plant cell under nutrient deficiency (Esposito et al., 2003); upon salt stress the oxidative burst is counteracted, at least in part, by G6PDH, both by expression and activities of cytosolic and plastidial isoforms (Nemoto and Sasakuma, 2000; Cardi et al., 2015; Valderrama et al., 2006) utilizing possibly different regulation patterns.

As a major example, under salt stress condition cy-G6PDH in *Arabidopsis thaliana* undergoes to a specific regulatory mechanism induced by the phosphorylation of $Thr_{467}$ by Glycogen Synthase Kinase 3 (ASKα) (Dal Santo et al., 2012); and this mechanism is possibly linked to a sugar-sensing signal (Lejay et al., 2008).

Although a major involvement of G6PDH activity during the plant general response to abiotic stress has been widely proven, little is known about possible, specific relationships between this reaction and the response and tolerance to drought.

Drought stress represents a constant menace for the world agricultural system, because it poses one of the most important constraints to plant growth, and consequently to crop productivity, in many regions all over the world (Fita et al., 2015).

In response to drought conditions, plants activate different mechanisms to reduce injuries and limit effects on growth and development, resulting in the induction of the expression of many genes involved in different biological processes, such osmolyte synthesis and accumulation (Xing and Rajashekar, 2001; Burg and Ferraris, 2008), abscisic acid (ABA) synthesis and signaling (Mehrotra et al., 2014), protection from Reactive Oxygen Species (ROS) (Gill and Tuteja, 2010), aquaporins activation (Maurel and Chrispeels, 2001), transcription factors regulation (Janiak et al., 2015), maintenance of leaf greenness (Rolando et al., 2015) and many others.

G6PDH could play a primary role during stress response being responsive to ABA signaling pathway and favoring ROS scavenging functions. In fact, G6PDH promoter presents different ABA Responsive Elements (ABRE elements); thus, its expression is in part modulated by this phytohormone (Cardi et al., 2011). Moreover, during drought plant cells increase their request for reducing power in order to sustain the antioxidant defense system and counteract ROS accumulation and consequent damages (Gill and Tuteja, 2010). Therefore, the enhanced G6PDH activity would be able to provide NADPH for the antioxidant system(s) in order to remove ROS excess (Dal Santo et al., 2012).

Curiously, G6PDH has been characterized in many model organisms such *Arabidopsis* (Wakao and Benning, 2005), barley (Cardi et al., 2013; Castiglia et al., 2015), tobacco (Scharte et al., 2009), wheat (Nemoto and Sasakuma, 2000), potato (Wendt et al., 2000) and others, but few information are known about tomato (*Solanum lycopersicum*), which represent the tenth horticultural crops cultivated worldwide, and the fourth vegetable in Italy (FAOSTAT, 2013). Most of the tomato varieties are sensitive to drought that halt the plant development, reduce fruit size and affect fruit quality properties (Nuruddin et al., 2003; Rai et al., 2013). Therefore, tomato is cultivated in Mediterranean environments using a consolidated irrigation schedule lasting for the whole growth season, to guarantee quality standard as well as sufficient yields. Tomato breeding objectives is actually focused on the development of drought-tolerant varieties, which could be able to grow under limited water supply. This is particularly urgent, considering the pressing need to cope with water scarcity, and the randomness of rains, as predicted by global climatic changes (Eckardt et al., 2009; Ripoll et al., 2014).

The aim of this paper is to elucidate the role(s) of G6PDH in response to drought stress in tomato plants. For the purpose, tomato plants were grown in different environments, from controlled laboratory hydroponics, to greenhouse pots, and finally in open field under common cultivation practices. Gene expression and enzymatic activity of G6PDH were examined to determine the involvement of this enzyme in drought stress response.

We hypothesized that up-regulation of G6PDH gene(s), and the activation of cytosolic G6PDH rate are required to respond to the oxidative stress condition induced by water deprivation.

This possible role(s) of G6PDH in the mechanisms of drought response in tomato is discussed.

## 2. Materials and methods

### 2.1. Plant materials, growth conditions and stress treatments

Plants of tomato, *Solanum lycopersicum*, L. 1753, cultivar Red Setter, were used in this study. Seeds were germinated in soil in a greenhouse.

For experiments in hydroponics, seedlings at two-leaves stage (25 days after sowing) were transferred in a hydroponic system, and grown in a 5 L solution containing $Mg(NO_3)_26H_2O$ (384 mg/L), $Ca(NO_3)_24H_2O$ (812.9 mg/L), $KNO_3$ (101.5 mg/L), $K_2SO_4$ (319.3 mg/L), $KH_2PO_4$ (204.8 mg/L), Hydromix (14.0 mg/L) for 3 weeks. Then plants were divided in three groups: “control” plants were kept in the same nutritive solution; “drought” plants grown in 15% PEG 8000 MW, (Sigma-Aldrich), added to the hydroponic solution; “salt “ stressed plants grown in the hydroponic solution supplemented with 150 mM NaCl. Leaves of tomato were collected from each group after 3 h, 6 h, 24 h, 48 h from stress imposition.

Plants in greenhouse were grown from seedlings at two-leaves stage transferred in 30 cm diameter soil-filled plastic pots, and irrigated regularly for 30 days. Then plants were divided in two groups: i) control group was kept in full irrigated regime; ii) drought group was deprived of water for 16 days; then leaves from control and drought groups were collected for further analyses.

Open field plants were grown starting from seedlings at two-leaves stage planted in a field at Acerra, NA (40°57′6″12 N; 14°22′37″56 E) during May–July 2015, and grown under full irrigation regime for 60 days. Then, plants were divided in two groups: i) control group with irrigated plants; ii) drought group totally deprived of water. Leaves from control and drought groups were collected after 30 days (48% less water than control); and 45 days (58% less water than control) from the start of water withholding.

### 2.2. Stomatal conductance measurements

Stomatal conductance was measured using the AP4 Porometer (Eijkelkamp – Giesbeek, The Netherlands), according to

Manufacturer's instructions. In brief, stomatal conductance (gs, mmol $H_2O$ $m^{-2}$ $s^{-1}$) was determined during the daylight; measurements were done on 3 control plants vs 3 drought-stressed plants, choosing one well-illuminated leaf for each plant.

### 2.3. RNA extraction and cDNA synthesis

Leaves from tomato plants grown under different experimental conditions were used for RNA extraction using TRizol reagent (Life Technologies, Carlsbad, CA, USA). RNA amount was measured by NanoDrop ND-1000 Spectrophotometer (NanoDropTechnologies), and RNA integrity was verified using denaturing formaldehyde gel. Dnase treatment and Reverse transcription was carried out using 1000 ng of RNA, utilizing Quantitech reverse transcription Kit (Qiagen, Hilden, Germany).

### 2.4. qRT-PCR

#### 2.4.1. Gene expression analysis was carried out by qRT-PCR

The *Solanum lycopersicum* genes analysed were: cytosolic G6PDH isoform (cy-G6PDH; Solyc02g093830); 9-cis-epoxycarotenoid dioxygenase (NCED Solyc07g056570), the first enzyme of abscissic acid synthesis pathway; protein phosphatase 2C-type (PP2C Solyc03g007230), the target of the ABA-ABA receptor (PYR-PYL/RCAR) complex; pyrroline-5-carboxylate synthetase (P5CS Solyc08g043170) the proline biosynthesis rate-limiting enzyme; ascorbate peroxidase (APX Solyc09g007270), one of the most important ROS scavenging enzyme in plant cell. A list of primers for each gene is given in Supplemental Table S1.

Triplicate quantitative assays were performed using an ABI 7900 HT (Applied Biosystems, Foster City, CA, USA) and Platinum SYBR Green qPCR SuperMix (Life Technologies, Carlsbad, CA, USA). Leaf samples of plant grown in control condition were used as calibrators; Elongation Factor EF1α served as endogenous reference gene (Nicot, 2005). Quantitation of gene expression was carried out using the $2^{-\Delta\Delta Ct}$ method as in Livak & Schmittgen (Livak and Schmittgen, 2001). For each sample, mRNA amount was calculated relatively to the calibrator sample for the same gene.

### 2.5. Proline content determination

Proline content was determined using the method of Claussen (Claussen, 2005). 250 mg of finely ground leaf tissue were suspended in 1.5 mL of 3% sulphosalicylic acid, and filtered through a layer of glass-fiber filter (Macherey-Nagel, Ø 55 mm, Germany). 1 mL of glacial acetic acid and 1 mL ninhydrin reagent (2.5 g ninhydrin/100 mL of a 6:3:1 solution of glacial acetic acid, distilled water and 85% ortho-phosphoric acid, respectively) were added to 1 mL of the clear filtrate. After incubation at 100 °C for 1 h, samples were read at an optical density measured at 546 nm.

### 2.6. Glucose-6-phosphate dehydrogenase assay

G6PDH was extracted from leaves by grounding 300 mg of leaf tissue, suspended in 600 μl of solution containing 50 mM Tris-HCl a pH 8.0, 5 mM $MgCl_2$, 4 mM EDTA, 10% glycerol, 15 μM $NADP^+$, 1 mL/30 gr Protease Inhibitor Cocktail (Sigma P9599). G6PDH activity was assayed according the method described by Castiglia et al. (2015), by monitoring $NADP^+$ reduction at 340 nm using a Cary 60 spectrophotometer (Agilent Technologies, USA). The assay mixture contained: 50 mM Tris–HCl pH 8.0, 50 mM $MgCl_2$, 1.5 mM $NADP^+$, 30 mM glucose-6P, and extract (10–100 μl). Activity was expressed as nmol reduced $NADP^+$ $min^{-1}$ $mg^{-1}$ protein.

### 2.7. Western blotting

For western blotting analysis, proteins were separated using electrophoresis SDS-PAGE. Then the polypeptides were transferred on a Hybond membrane (Ge Healthcare). The membrane was incubated with primary cy-G6PDH antibody from potato (Wendt et al., 2000; Castiglia et al., 2015) or cy-HSP70 antibody (Esposito et al., 2012). After incubation of the membrane with secondary antibodies, cross-reacting polypeptides were identified by enhanced chemio-luminescence using the ECL Prime kit (GeHealthcare).

### 2.8. Bioinformatics analyses

Tomato G6PDH sequences were found using solgenomics database at (https://solgenomics.net). Other G6PDH sequences from higher plants were found using TAIR database for *Arabidopsis thaliana* (https://www.arabidopsis.org) and EnsemblPlants database (http://plants.ensembl.org/index.html) for the other organisms; a complete list of the sequences utilized in this work is given in Table S2. Alignment and phylogenetic analyses were conducted using the software *MEGA* version 6 (Tamura et al., 2013). Sequences alignment was achieved using the MUSCLE algorithm. Phylogenetic tree was constructed using the maximum likelihood method with the substitution JTT model, gamma distributed. The test of phylogeny was performed using the bootstrap method with a number of bootstrap replication equal to 100.

### 2.9. Statistical analyses

Each experiment was made in at least three replicates. Values were expressed as mean ± standard error (SE). The statistical significance of qRT-PCR, G6PDH activity assays and proline content in different treatments was evaluated through Student's *t*-test ($p \leq 0.05$).

## 3. Results

### 3.1. Water deficit in tomato plants: biochemical and molecular response

Three different sets of experiments were designed to study the response of tomato (*Solanum lycopersicum* cv. Red Setter) plants to water limitations for different time lengths related to the growth and development stage of the plants. To describe a general pattern of response of tomato plants to water deficit, short-, –medium-, and long-term severe drought stress conditions were imposed to plants grown in hydroponic cultures, in greenhouse in pots, and in open field, respectively. The effects of the water shortage on morpho-physiological, biochemical and molecular parameters were evaluated.

Hydroponic grown plants showed severe damage after 2 days of drought imposed by 15% PEG, with a massive loss of leaves and evident chlorosis (Fig. 1A).

Plants grown in the greenhouse after 16 days of water withdrawal presented chlorotic leaves, and evident injury, but substantially were of the same height of control plants (Fig. 1B). Finally, 45 days of drought severely harmed the field-grown plants (Fig. 1C); but, despite of the evident stressful status (wilting, leaf curling, halt in plant growth), drought stressed plants were able to recover from the stress after re-watering (data not shown).

The ability of gas exchange, in plants subjected to drought stress, was monitored by evaluating stomatal conductance under different experimental conditions. As shown in Table 1, drought severely reduces the transpiration ability. Shock stress conditions imposed

to hydroponic grown plants resulted in 70% decrease in stomatal conductance within 3 h after 15% PEG exposure, with a further decrease to 10% of the control conditions after 48 h of stress. Greenhouse grown plants diminished their stomatal conductance at less than 8% of control conditions – after 16 days in absence of irrigation – to values that indicate the complete stomata closure.

In field grown plants the difference between control and drought plants is less marked but the stomatal conductance values reduced less than 50% after 30 days (less than 43% after 45 days).

Other well-known metabolic alterations, induced by drought stress in plants, include the accumulation of proline and abscissic acid (ABA).

In order to verify possible changes in osmoregulation mechanisms, proline content was measured in leaves. As shown in Fig. 2A, in the hydroponic grown PEG-stressed plants proline levels doubled after 6 h; enduring the stress conditions, proline concentrations increased up to 11 times after 24 h, and 27 times at 48 h.

**Table 1**
Stomatal conductance (gs) in leaves of tomato plants grown under different conditions. Values represent average measurements ± SD, n ≥ 3. Drought was imposed by adding PEG 15% in hydroponic cultures, or by withholding irrigation in greenhouse or field grown plants.

| Growth conditions | | Stomatal conductance, gs (mmol $s^{-1}$ $m^{-2}$) | | |
|---|---|---|---|---|
| | | Control | Drought | % |
| Hydroponic | 0 | 111 ± 5.57 | 117.7 ± 15 | 6.01 |
| | 3h | 118.7 ± 10.6 | 32.8 ± 11.6** | −72.36 |
| | 6h | 124 ± 3.61 | 40.2 ± 10.9** | −67.58 |
| | 24h | 108.3 ± 3.21 | 17.9 ± 5.4** | −83.51 |
| | 48h | 127 ± 8.54 | 12.7 ± 1.2** | −90.03 |
| Greenhouse | 16d | 222.3 ± 30.7 | 17.2 ± 2.31** | −92.28 |
| Field | 30d | 419.5 ± 107.5 | 204.5 ± 55.3* | −51.25 |
| | 45d | 242.2 ± 61.8 | 138.7 ± 69* | −42.72 |

(*) and (**), indicate significantly different values in drought stress compared to control plants at $p \leq 0.05$, $p \leq 0.0005$, respectively.
%: Percentage of stomatal conductance between control/stressed plants.

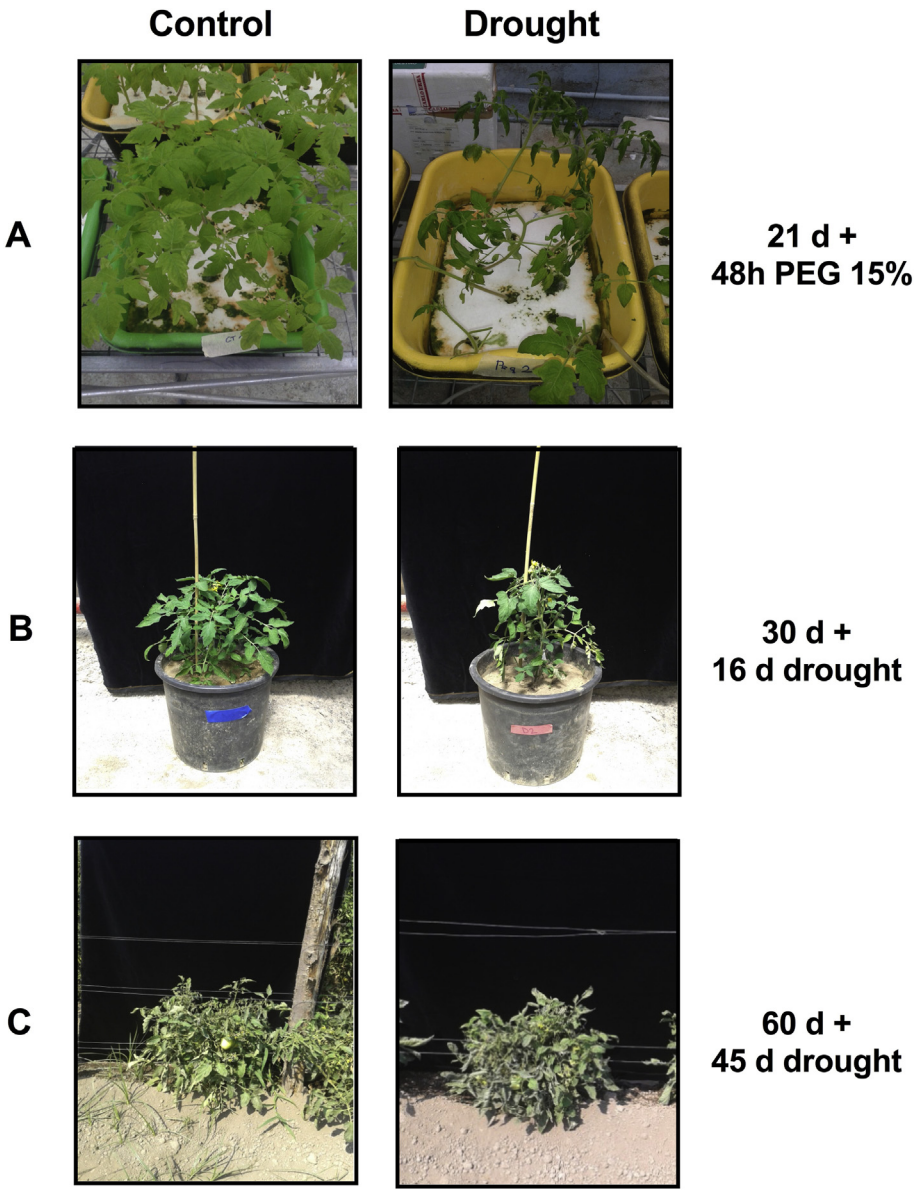


**Fig. 1.** Effects of drought on tomato plants grown under different experimental conditions; drought was induced by A) 15% PEG in hydroponic grown plants after 21days of regular growth (no PEG); and by lack of watering for B) 16 days in potted soil adult plants in greenhouse after 30 days of regular growth (irrigation); and C) 45 days in field-grown plants after 60 days of regular growth (irrigation).

Similarly, in greenhouse plants (Fig. 2B) proline levels increased after 16d in absence of irrigation up to 16 times.

The accumulation of stress-related proteins was investigated by measuring the levels of cytosolic HSP70 (Cardi et al., 2015). Western blotting analyses show an increase of Cy-HSP70 proteins both in hydroponic and greenhouse plants under drought (Fig. 2C), thus confirming the achieved stress condition status.

To investigate the correlation between metabolite accumulation and transcription of the gene encoding the proline biosynthesis rate-limiting enzyme, the expression of the tomato Pyrroline-5-carboxylate synthetase (*P5CS*, Solyc08g043170) gene was evaluated by qPCR. Both in hydroponics and in greenhouse plants, a significant up-regulation of this gene was observed after 48 h and at 16 days under drought, respectively (Table 2), as for the proline content.

In order to verify the ABA involvement in tomato response to stress conditions imposed, expression levels of *Solanum lycopersicum NCED* gene Solyc07g056570, encoding for 9-cis-epoxycarotenoid dioxygenase, and *PP2C* gene Solyc03g007230, encoding for a Protein Phosphatase 2C-type, were evaluated in leaves by qRT-PCR.

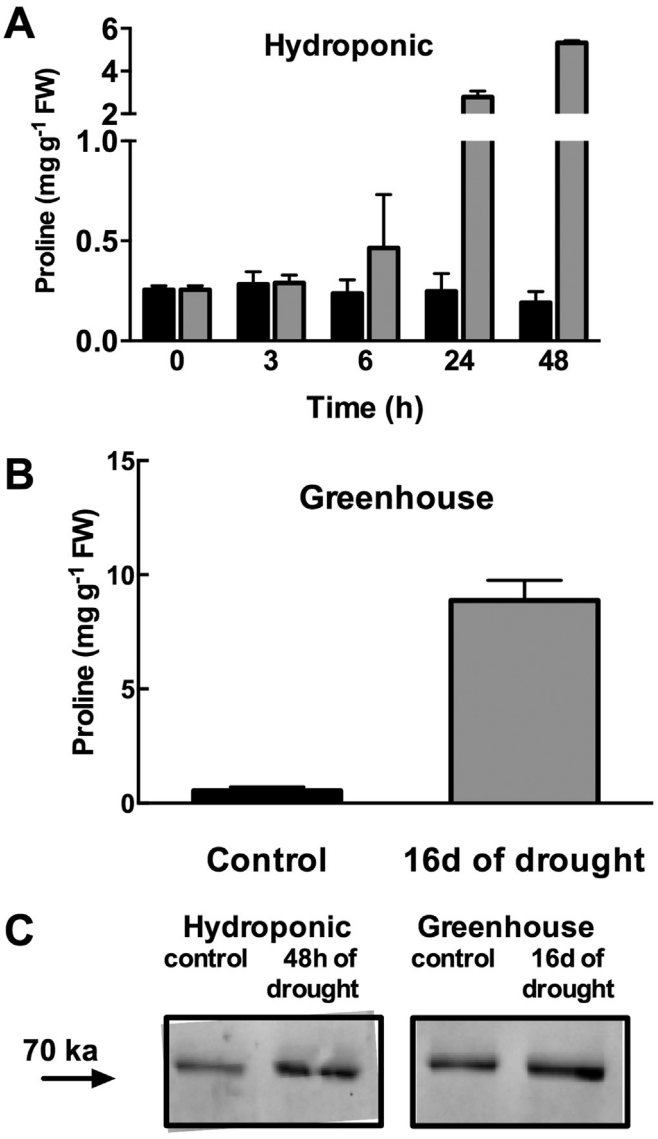


**Fig. 2.** Biochemical responses to drought stress in tomato. Changes in the levels of proline in young plants grown in A) hydroponics upon PEG-induced water stress; and in B) potted soil adult plants after 16 days of drought. C) Occurrence of Hsp70 in the plants subjected to water deficit as evidenced by Western blotting using anti bovine Hsp 70 antibodies. Levels in stressed plants are in grey bars; controls in black bars.

Results show a 3-fold change increase of *NCED* transcript in hydroponic grown plants after 3 h of exposure to PEG15%; this increase remained stable up to 24 h (Fig. S1). After 48 h the levels of *NCED* transcript further increased up to 12-fold the level present in control not stressed plants (Table 2 and Fig. S1). Similarly, in plants grown in soil a 1,8-fold change *NCED* increase was observed in leaves of 16d drought-stressed plants (Table 2).

Correspondingly, PP2C expression shows a 300-fold change increase after 48 h in plants treated with PEG15% and a 60-fold change after 16 days in absence of irrigation in greenhouse plants (Table 2). To verify the activation, in such conditions, of the ROS scavenging system, an expression analysis by RT-PCR of ascorbate peroxidase gene (*APX*) from *Solanum lycopersicum* (Solyc09g007270) was carried out on leaves. Results show a significant 243-fold increase after 48 h PEG treatment; similarly, up-regulation (14-fold increase) of APX was observed in soil potted plants, after 16 days of drought (Table 2).

### 3.2. Occurrence of different G6PDH isoforms in Solanum lycopersicum

To characterize the *Solanum lycopersicum* G6PDH response under drought, a bioinformatics approach was carried out to identify the various isoforms of G6PDH in tomato. Different putative G6PDH-encoding genes were found by *Solanum lycopersicum* genome scanning using Solanacee Genomics Network (SGN) database. Five genes coding for putative G6PDH proteins were identified (Solyc01g100950, Solyc01g100960, Solyc02g093830, Solyc05g015950, Solyc07g045540). To assign *Solanum lycopersicum* -G6PDHs to the isoform sub-families, a comparison of the amino acidic sequence of the five genes was performed versus 35 known G6PDH protein sequences from different higher plants, e.g. *Arabidopsis thaliana, Oryza sativa, Hordeum vulgare, Populus trichocarpa, Solanum tuberosum, Prunus persica, Vitis vinifera, Zea mays*. Thus, after a model-selection analysis, an un-rooted tree was constructed using maximum likelihood method, in order to investigate the phylogenetic relationship (Fig. 3).

The results confirmed the partition of the different plant-G6PDHs into three main branches, including inactive-G6PDHs (P0-G6PDH, Meyer et al., 2011), cytosolic-G6PDHs and compartmented-G6PDHs; this latter branch splits in two more forks, including P1-G6PDH and P2-G6PDH. Particularly, Solyc01g100950 and Solyc01g100960 clustered within the inactive-G6PDH group (P0-G6PDH); Solyc02g0939830 placed in the cytosolic-G6PDH group; Solyc0501950 and Solyc07g04540 clustered in P2-G6PDH and P1-G6PDH groups, respectively. Like all the compartmented-G6PDHs, the last two genes present a putative plastidial transit-peptide. As expected, the tomato- and potato-G6PDHs are grouped nearby, with a bootstrap 100, to indicate the similarity between the two *Solanacee*.

### 3.3. The involvement of G6PDH in drought stress response

An in-deep characterization of the effect of different water deficit conditions on tomato cy-G6PDH accumulation and activity was performed. Different approaches (gene expression analysis, total enzymatic activity and immunoblotting) were utilized in the aim to determine the possible, central roles of G6PDH in stress-related functions associated with tolerance mechanisms.

As determined by qRT-PCR, the expression Cy-G6PDH gene (Solyc02g093830) increased up to 2 times after 3 h under PEG, and

**Table 2**
Changes in the expression of different genes (as fold-change with respect to control) in leaves of tomato plants grown in hydroponic (48 h, PEG15%) and in greenhouse conditions (16 days water withholding) under drought, using qRT-PCR. For each sample, mRNA amount was calculated relatively to the calibrator sample for the same gene.

| Gene | Locus | Relative expression | |
|---|---|---|---|
| | | Drought 48 h PEG 15% | Drought 16d No watering |
| Cy-G6PDH | Solyc02g093830 | 4.87 | 1.8 |
| NCED | Solyc07g056570 | 12.99 | 1.85 |
| P5CS | Solyc08g043170 | 2.74 | 1.68 |
| PP2C | Solyc03g007230 | 327.76 | 68.66 |
| APX | Solyc09g007270 | 242.71 | 13.8 |

this increase remained stable for 24 h; a further 4-fold raise was observed after 48 h of stress imposition (Fig. 4A). A similar 60% increase was observed upon prolonged 16d drought stress in soil-grown tomato plants (Fig. 4B).

Similarly, the enzymatic rate showed a similar trend, with a 1.4 times increase at 3 h, unchanging up to 24; then, a 2-fold increase was observed after 48 h, up to 6.73 U/mg of protein in plants (Fig. 5A).

The expression profile of cy-G6PDH was comparable with *NCED,* P5CS and PP2C gene expression under drought stress (Table 2). Likewise, under greenhouse conditions, plants grown without irrigation for 16 days showed a 1.8 times increase in expression of cy-G6PDH (Fig. 4B), together with a 4.2 times increase of total G6PDH activity (Fig. 5B).

A similar increase in both in cy-G6PDH gene expression and G6PDH enzymatic activity was evidenced in field-grown plants as well. Plants grown for 30 days and 45 days without irrigation showed an increase of G6PDH total activity, about 1.67 and 1.32 times, respectively (Fig. 5D).

Finally, the enzymatic activity and expression changes were associated to protein accumulation revealed by Western Blotting analysis using polyclonal antibodies against cytosolic G6PDH of *Solanum tuberosum*. Our results indicated an evident increase of cy-G6PDH protein under drought conditions (Fig. 5C); this increase was verified using as housekeeping RubisCO large subunit (not shown).

## 4. Discussion

Nowadays the most important challenge of the global agriculture is the providing of the sustenance for the continuously increasing world population. This is made more difficult as result of the climatic changes, inducing erratic abiotic stress conditions. Particularly, drought stress is the most limiting factor for plant growth, development, and productivity (Reynolds and Tuberosa, 2008). In the next-coming years, several factors could worsen the situation, as result of reductions in rainfalls all over the world; subsequently, loss of arable land by soil erosion will reduce the availability of fields for agriculture and crop production (Krannich et al., 2015). Therefore, drought stress remains an important problem, given the possible (and forecasted) variability and unpredictable patterns of rainfalls in the near future. This is particularly true in the Mediterranean area, where limited extensions of fields are cultivated, often with crops of high quality and commercial value, as tomato traditional varieties. All these considerations underline the need of selecting new crops with increased drought tolerance, together high-yield features, as a strategy to ensure sustainability (Boyer, 2010).

The purpose of this work was to study the involvement of G6PDH in plant response to different drought stress conditions. The approach was based on the strategy of studying the effects of experimental and field stress conditions in an important crop as tomato. First, hydroponic-grown plants were exposed to PEG to simulate abrupt drought conditions, for an accurate analysis of short-term response to stress. Second, using greenhouse-grown plants, tomato were grown in soil pots and drought-stressed, to investigate the effects at adult stage of severe drought conditions. Finally, plants were grown in open field in absence of any irrigation up to 45 days, for a better understanding of the behavior under common agricultural conditions.

G6PDH enzymatic activity increased in plants under drought in any condition tested. This increase has been found in the first phase of drought, upon few hours of low water potential conditions imposed by PEG, as well as under prolonged deprivation of water, in greenhouse and field-grown plants. Therefore, activity assays indicated that G6PDH could play a pivotal role counteracting the drought-induced oxidative stress. Actually, NADPH is the main reductant requested in the ascorbate-glutathione cycle (Leterrier et al., 2007) and G6PDH represents one of the major sources of NADPH reduction in the plant cell. Furthermore, drought induced a significant increase of ascorbate peroxidase expression upon short and medium-long severe drought. It is known that ascorbate peroxidase activity, which reduces $H_2O_2$ to $H_2O$, is essential in plants to maintain adequate photosynthetic rates upon abiotic stress, particularly under drought (Shigeoka, 2002; Krannich et al., 2015).

Likely, G6PDH total activity increase shown in this study is ascribed for the most part to the cytosolic isoform (Esposito et al., 2001). This activity is involved firstly in the preservation of the basal cellular metabolism (Esposito et al., 2005), but a major role for cy-G6PDH has already been demonstrated upon salt stress (Nemoto and Sasakuma, 2000; Dal Santo et al., 2012; Cardi et al., 2015). To investigate the role of cy-G6PDH also in drought stress response, changes in the expression of *Solanum lycopersicum* cy-G6PDH gene was investigated by qRT-PCR. This gene was identified using a bioinformatics approach, which has clearly shown that the Solyc02g093830 gene belongs to dicotyledons cy-G6PDH subfamily. Moreover, an increase of cy-G6PDH expression was observed in plants subjected to drought stress in any condition tested. An enhanced expression of cy-G6PDH is quickly promoted in the first stage of deprivation of water to respond to drought. This hypothesis is supported by the higher levels of cy-G6PDH expression observed under prolonged drought conditions (both in greenhouse and open field plants). This would support the need by the leaf cell to maintain a continuous providing of NADPH under water deprivation. It should be underlined that the changes in gene expression were associated to G6PDH enzyme accumulation and activity under drought.

Although cy-G6PDH is differentially expressed in first phase of water deficit, its increase remained constant during the prolonged drought. On the other side, the raise of G6PDH activity showed higher fold-increase during the extended drought, as observed in

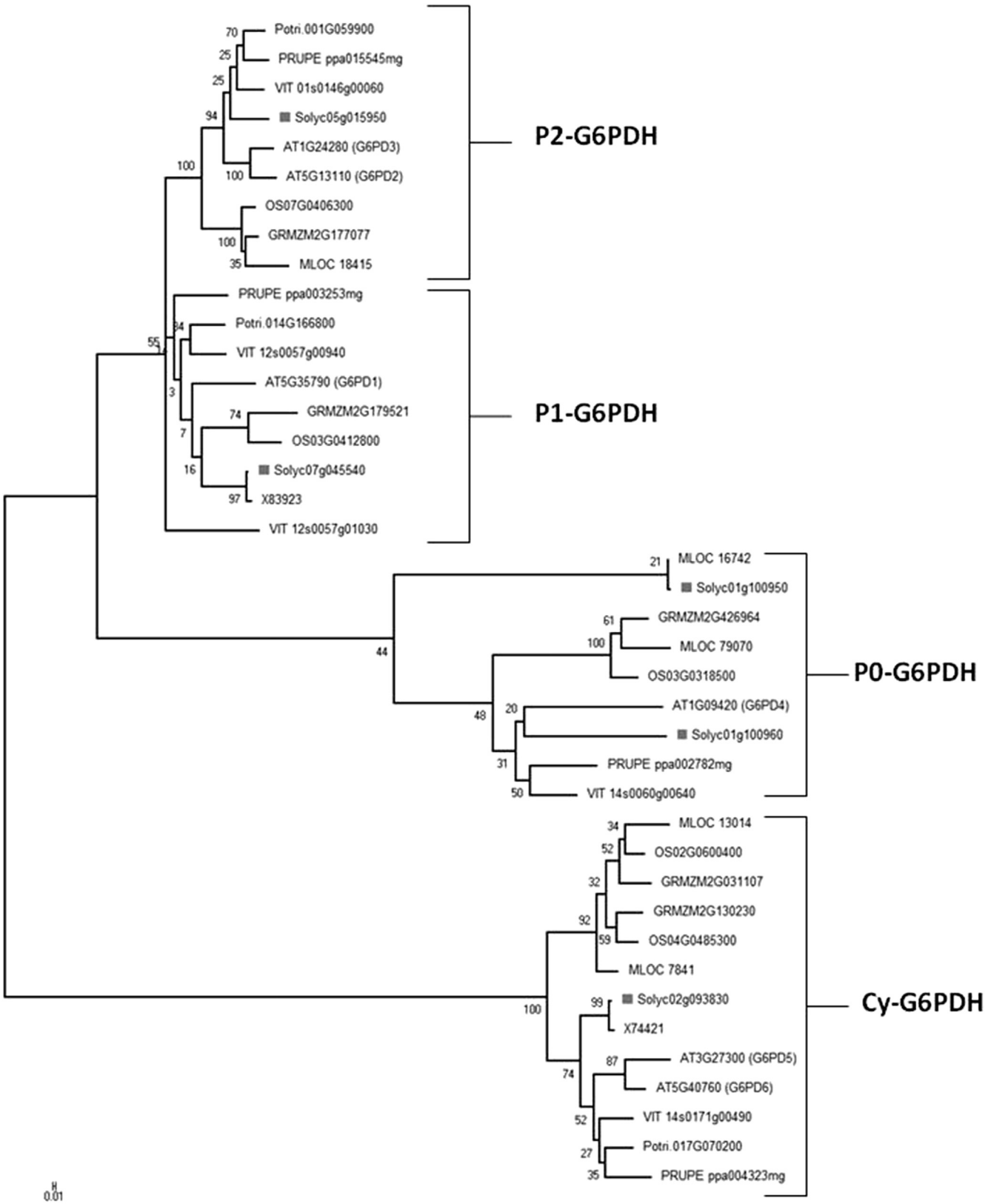


**Fig. 3.** Un-rooted phylogenetic tree constructed using maximum likelihood method, for the relationships among 40 different G6PDH isoforms from higher plants; 13 sequences are Cy-G6PDH; 9 sequences are P0-G6PDH; 9 sequences are P1-G6PDH and 9 sequences are P2-G6PDH. Grey square-boxes highlight *Solanum lycopersicum* G6PDHs. Legend for plant species: AT, *Arabidopsis thaliana*; GRMZM, *Zea mais*; MLOC, *Hordeum vulgare*; PoTri, *Populus trichocarpa*; PROPE, Prunus persica; Solyc, *Solanum lycopersicum*; OS, *Oryza sativa*; VIT, *Vitis vinifera*; X, *Solanum tuberosum*. A complete list of sequences used for tree construction is reported in Supplemental Table S2.

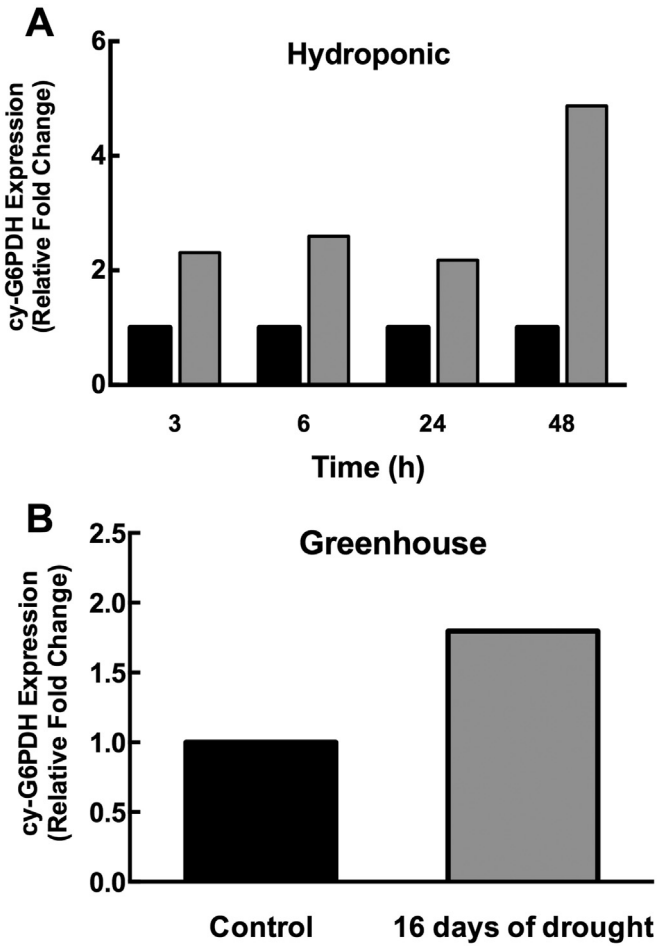


**Fig. 4.** Changes in the expression of cy-G6PDH in leaves of *Solanum lycopersicum*, A: hydroponic; B, greenhouse, measured by qRT-PCR. Variations are indicated as fold-change in stressed plants (grey bars) with respect to control (black bars). For each sample, mRNA level was calculated relatively to the calibrator sample for the same gene. Other details in the text.

greenhouse-grown plants. Probably, an activation of enzymatic activity may be hypothesized in the protracted stress conditions, in addition to the transcriptional up-regulation.

A consistent accumulation of cy-G6PDH protein levels was observed upon drought stress, under different experimental growing conditions, while no major changes where observed in the occurrence of the plastidial isoform, suggesting a direct connection between the drought stress condition, and the accumulation of cy-G6PDH. Nevertheless, the possible role of plastidial G6PDH isoform upon drought should be better investigated in the future, given previous evidence of its increase in ABA-supplied barley roots (Cardi et al., 2011).

Increase in proline content (Ashraf and Foolad, 2007), *P5CS* gene expression (Sharma et al., 2011), HSP70 protein levels (Sruthy et al., 2015) and ABA synthesis confirm that under the stress condition imposed, tomato plants activate a whole array of responses to reduce drought effects and sustain growth under unfavorable conditions.

As far as ABA synthesis activation upon drought stress signal, it is well known the overexpression of *NCED* and *PP2C* genes leads to higher levels of ABA, a reduction of the transpiration rate in leaves, and thus enhancing drought tolerance (Thompson et al., 2000; Singh et al., 2015). An increase in the expression of *NCED* gene was observed in our experiments, in accordance with cy-G6PDH increased expression and activity. At this regard, it is worth to point out that G6PDH promoter presents ABA Responsive Elements (ABRE elements) (Nemoto and Sasakuma, 2000; Cardi et al., 2011).

The activation of the ABA transduction pathway was further verified using the stomatal conductance analysis, thus confirming that one of the primary responses of the plants under drought is an ABA-induced stomatal closure in order to reduce the water loss (Shinozaki and Yamaguchi-Shinozaki, 2007; Banik et al., 2016).

Therefore, drought signal(s), whose occurrence is demonstrated by increased proline content and HSP70 levels, is warned by ABA, and induces, among the various responses, the expression of cy-G6PDH, to support the increased request of NADPH under stress. Intriguingly, the expression of cy-G6PDH is promoted since the first phase of stress. Afterwards, prolonged deprivation of water maintains high levels of G6PDH activity. Data shown demonstrated that cy-G6PDH is up-regulated in drought condition together with other drought-related genes, such as the enzyme of the ABA-pathway *NCED*, the ABA transduction factor *PP2C*, the *P5CS* enzyme involved in proline synthesis, and ascorbate peroxidase, involved in ROS detoxification.

These results strongly supports the notion of the involvement of G6PDH, namely the cytosolic isoform, in drought response. Specifically, drought induces ABA synthesis and signaling, specifically activating ABA responsive genes (e.g. possessing ABRE elements). Among these, cy-G6PDH is strongly and specifically induced to satisfy the increased reductants' request provoked by the increase of scavenging systems (e.g., APX) to control and pace the ROS increment, and modulate the response to drought in plant cells (Fig. 6).

In conclusion, the involvement of G6PDH during drought stress in tomato has been demonstrated. More generally, a central role of G6PDH activity in the mechanisms triggered during oxidative stress induced by water limitation has been defined in different growth conditions, stress intensity and duration. Results obtained from plants grown under highly controlled experimental conditions showed to be more consistent and reliable.

As expected, open field cultivation, under natural environmental conditions, showed results less marked, but absolutely linear and consistent with the effects observed in laboratory/greenhouse-grown plants, probably due to adaptive mechanisms induced by prolonged stress conditions.

To our knowledge for the first time has been unequivocally proven the predicted role of G6PDH in the response to drought stress in higher plants, namely in the widely cultivated tomato crop.

Intriguingly, most of these drought responses are similar to salt stress effects in different plants (Nemoto and Sasakuma, 2000; Dal Santo et al., 2012; Cardi et al., 2015). This would design a complex pattern of involvement, in abiotic stress response, of G6PDH isoforms in plant cells. It could be postulated the kind and intensity of responses could be modulated by different signaling pathways, often strictly connected and/or converging, such as ROS levels, ABA signal, reductants' availability.

Further studies are required to elucidate both the complete pathway(s) of signaling, from detection of stress to the increase in G6PDH activity, both the mechanisms providing a possible feedback modulation of reductants' delivering by this pivotal enzyme of cell metabolism.

## Contribution

**SL** Projected the whole research project and made the experiments, wrote and amended the manuscript.

**RN** Grew tomato plants, made the experiments.

**ADL** Made enzymatic assays and the western blottings.

**ML** Made enzymatic assays and the western blottings.

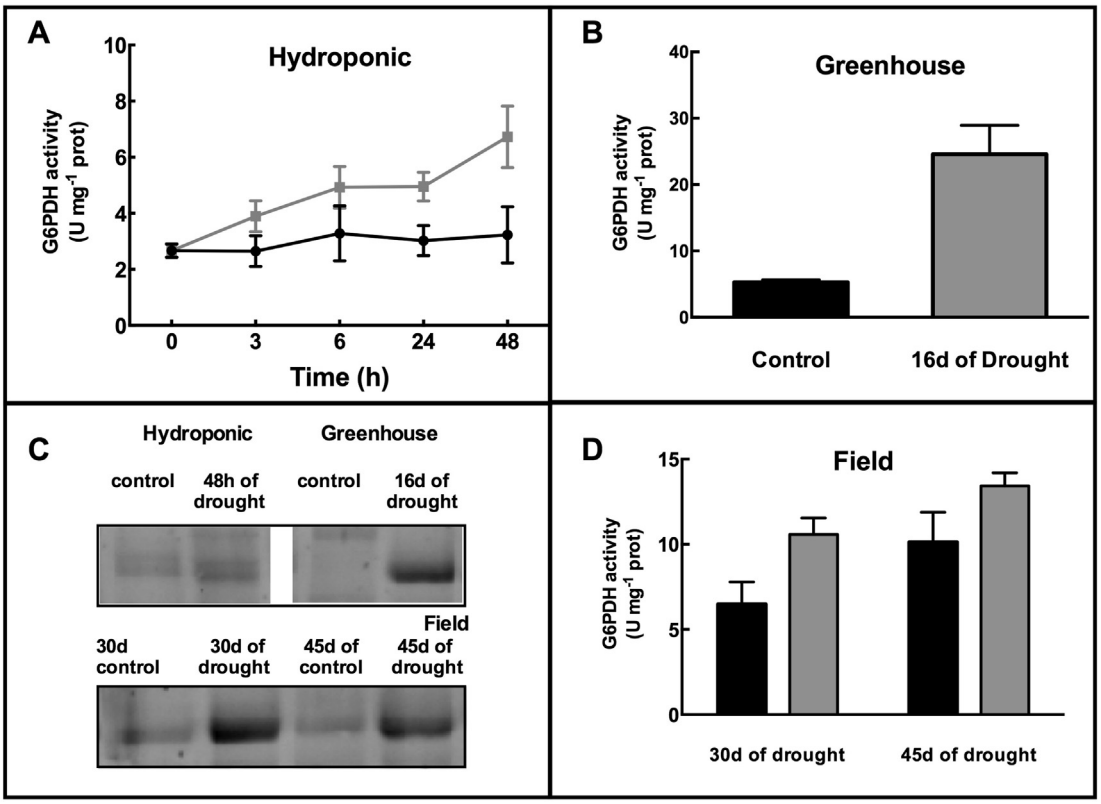


**Fig. 5.** Effects of drought stress on G6PDH protein levels and enzymatic activity in tomato plants. Changes in the activity of G6PDH in A) young plants grown in hydroponics upon PEG-induced water stress; B) in potted soil adult plants after 16 days of drought; D) in field grown plants after 30 and 45 days of irrigation withholding; C) accumulation of cytosolic G6PDH revealed by Western blotting using anti potato cy-G6PDH antibodies. Levels in stressed plants are in grey bars; controls in black bars.

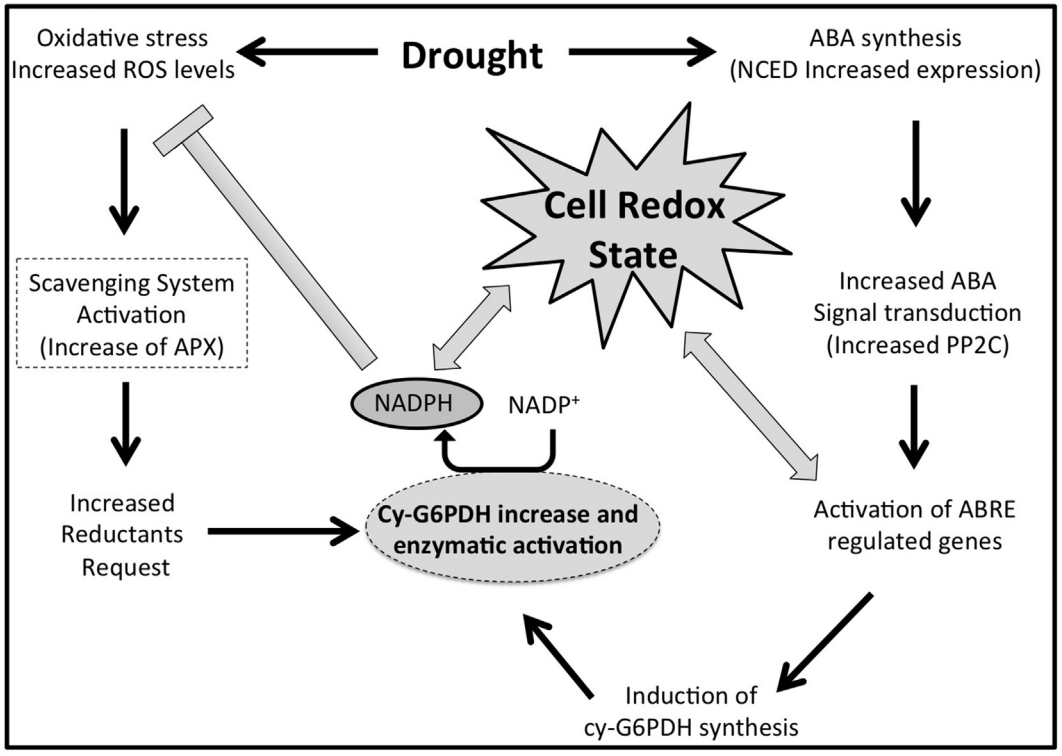


**Fig. 6.** Scheme of the proposed interactions among drought stress, ABA signaling pathway, and induction of expression, and activation, of cy-G6PDH in tomato plants in order to finish reductants to counteract the oxidative stress. Other details in the text.

**SG** Projected the whole research project, and the experiments, wrote and amended the manuscript.

**SE** Coordinated the experiments, projected the whole research project, wrote and amended the manuscript. Corresponding author.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.04.013.

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