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Research article

The effects of salt stress cause a diversion of basal metabolism in barley roots: Possible different roles for glucose-6-phosphate dehydrogenase isoforms

Manuela Cardi [a, 1], Daniela Castiglia [a, 1, 2], Myriam Ferrara [a, 1, 3], Gea Guerriero [a, 1, 4], Maurizio Chiurazzi [b], Sergio Esposito [a, *]

[a] Università di Napoli Federico II, Dipartimento di Biologia, Via Cinthia, 6, I-80126 Napoli, Italy
[b] Institute of Biosciences and BioResources – CNR, Via P. Castellino 111, I-80128 Napoli, Italy

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ABSTRACT

In this study the effects of salt stress and nitrogen assimilation have been investigated in roots of hydroponically-grown barley plants exposed to 150 mM NaCl, in presence or absence of ammonium as the sole nitrogen source.

Salt stress determines a diversion of root metabolism towards the synthesis of osmolytes, such as glycine betaine and proline, and increased levels of reduced glutathione. The metabolic changes triggered by salt stress result in a decrease in both activities and protein abundance of key enzymes, namely GOGAT and PEP carboxylase, and in a slight increase in HSP70. These variations would enhance the requirement for reductants supplied by the OPPP, consistently with the observed increase in total G6PDH activity.

The involvement and occurrence of the different G6PDH isoforms have been investigated, and the kinetic properties of partially purified cytosolic and plastidial G6PDHs determined. Bioinformatic analyses examining co-expression profiles of G6PDHs in *Arabidopsis* and barley corroborate the data presented.

Moreover, the gene coding for the root P2-G6PDH isoform was fully sequenced; the biochemical properties of the corresponding protein were examined experimentally.

The results are discussed in the light of the possible distinct roles and regulation of the different G6PDH isoforms during salt stress in barley roots.

1. Introduction

Soil salinity represents a major threat for crop yield: it exerts negative consequences on plant growth and triggers secondary stresses like oxidative, ionic and osmotic imbalances (Hayashi and Murata, 1998). These disorders may result from the negative consequences of salt stress on nutrient availability, competitive uptake, transport or partitioning within the plant. An excess of salts in the soil indeed negatively affects the water potential of plant cells, consequently causing a wide range of cellular and/or structural damages, like protein misfolding, generation of reactive oxygen species (ROS) and cell shrinkage (Huang et al., 1990; Huang and van Steveninck, 1990).

The ability of plants to survive and grow under saline conditions is known as salt tolerance, which is a multigenic trait that depends on many factors (Hare et al., 2002; Ashraf, 2004). The capability to withstand salinity implies a series of cellular mechanisms, as well as wide networks of biochemical processes, some of which have however not yet been thoroughly revealed (Ashraf and Harris, 2004).

* Corresponding author. Università di Napoli "Federico II", Dipartimento di Biologia, Complesso Universitario di Monte Sant'Angelo, Edificio 7, Via Cinthia, 6 I-80126 Napoli, Italy.
E-mail address: sergio.esposito@unina.it (S. Esposito).
[1] Joint first authors.
[2] Present address: CNR-IBBR (Istituto di Bioscienze e BioRisorse), Via Università, 133, I-80055 Portici, NA, Italy.
[3] Present address: Universitätsmedizin Greifswald, ZIK HIKE – Humoral Immune Reactions in Cardiovascular Diseases, Fleischmannstraße 42-44, 17489 Greifswald, Germany.
[4] Present address: Department Environment and Agro-biotechnologies (EVA), Centre de Recherche Public-Gabriel Lippmann, 41, Rue du Brill, L-4422 Belvaux, Luxembourg.

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In order to achieve salt tolerance, plants have to maintain growth and metabolic functions: among the different response mechanisms triggered upon salt stress, the most prominent one is the synthesis of compatible solutes (a.k.a osmolytes), which contribute to stabilize protein structures. These compounds are usually polyphenolics, quaternary ammonium compounds (e.g. glycine betaine) and aminoacids (notably proline), which lower the cytosolic osmotic potential, thus restoring the physiological cellular osmolarity.

Different studies have shed light on the regulation of osmolyte accumulation and several others in the literature have reported an improved salt tolerance in transgenic crop lines overexpressing enzymes involved in osmolyte synthesis (e.g. Gamboa et al., 2013).

It has been shown that specific genes, named WESR (Wheat Early Salt Responding genes, Nemoto et al., 1999), are involved in the response to salt stress in wheat; among these, a putative glucose-6-phosphate dehydrogenase (G6PDH; EC1.1.1.49) gene has been identified, hence suggesting that the early response to osmotic stress involves the oxidative pentose phosphate pathway (OPPP) (Nemoto and Sasakuma, 2000).

In recent years, functional, biochemical and physiological roles played by G6PDH in plant tissues have been investigated and the occurrence of different G6PDH isoforms has been extensively demonstrated (Kruger and von Schaewen, 2003).

Cytosolic isoforms (Cy-G6PDH), with kinetic and molecular properties similar to animal G6PDH and different organelle-localised enzymes (P1-G6PDH, located within chloroplasts, and P2-G6PDH, mainly detected in plastids of heterotrophic tissues) have been reported (Wendt et al., 2000; Esposito et al., 2001a). In particular, P2-G6PDH is expressed in nearly all plant organs (Wendt et al., 2000; Wakao and Benning, 2005) and it is responsible for the supply of reducing power during nitrogen assimilation (Bowsher et al., 1992; Knight et al., 2001; Esposito et al., 2003, 2005).

Overall, these data suggest that the OPPP is involved in the response to abiotic stress in plant cells by generating reductants, such as NADPH. In plants, this reducing power is usually used during the reactions of nitrite reductase (NIR) (Bowsher et al., 1989) and glutamate synthase (GOGAT) (Bowsher et al., 1992; Esposito et al., 2003, 2005). Consequently stress conditions would cause at least a partial diversion of reductants produced via G6PDH from nitrogen metabolism to ROS scavenging (Liu et al., 2007).

Although many studies have been published on the effects of salt stress in crops, only a handful have analysed the effects on roots, by investigating the changes in primary metabolism. Therefore more efforts should be devoted to understanding the dynamics of primary metabolism and the partitioning of reductants upon salinity in roots.

The goal of the present paper is to fill this gap by investigating the effects of both salinity and ammonium supply in the roots of barley, a major cereal crop. In particular the envisaged strategy aims at unveiling the details of diversion of root primary metabolism upon salt stress by i) quantifying metabolite changes, namely aminoacids and related compounds; ii) measuring activities (and where possible, abundance) of some key enzymes involved in primary metabolism (e.g. PEP carboxylase, GOGAT isoforms); iii) discussing the role of G6PDH isoforms in salt stress response and nitrogen assimilation in the roots, by determining the kinetic and molecular properties of the different enzymes.

2. Materials and methods

2.1. Cultivation of plants

Seeds of barley (*Hordeum vulgare* L., var. Alfeo or Nure), were supplied by the Istituto Sperimentale per la Cerealicoltura (Fiorenzuola D'Arda – Italy). Seeds were germinated for 3–5 days in the dark on moistened paper, then seedlings were hydroponically grown in darkened plastic bottles (500 ml) at 20 °C, 60–80 % relative humidity, under 16h-light/8h-dark regime, with approximately 180 μmol photons m^{-2} s^{-1}. The composition of the medium (modified Hoagland solution), continuously bubbled with air, has been previously described (Rigano et al., 1996a,b); nitrogen in the form of 10 mM $(NH_4)_2PO_4$ was supplied after 7 days of hydroculture. For the salt stress experiments, 0.15 M NaCl was added to the medium on the 7th day of hydroculture. Nutrient solutions were controlled for pH and daily adjusted to maintain nitrogen concentrations.

2.2. Metabolite content determination

The total content of extractable aminoacids was determined by HPLC using a C18 Reverse phase column, as previously reported (Rigano et al., 1996b; Esposito et al., 1998). Cysteine and reduced glutathione were determined by HPLC, as previously described (Carfagna et al., 2011a,b). Glycine betaine was quantified according to Carillo et al. (2008).

Proline content was determined using the method described by Shetty et al. (2002) with few modifications. Approximately 100 mg of plant material were frozen in liquid nitrogen and proline was quickly extracted in 3% sulfosalicylic acid solution. After a centrifugation at 13,000 rpm for 10 min at 4 °C, 1 ml of the supernatant fraction was mixed with 1 ml of glacial acetic acid and 1 ml of 2.5% acid ninhydrin acid solution. The samples were then transferred to a water bath at 100 °C for 1 h and the reaction was stopped on ice. After 15 min, 2 ml of toluene were added to each sample. After a vigorous shaking, the upper coloured phase was withdrawn and the absorbance was read at 520 nm, using toluene as a blank. Proline content was expressed as μg per gram of FW, using a calibration curve with different concentrations of free proline in 3% sulfosalicylic acid solution.

2.3. Extraction and assay of glucose-6-phosphate dehydrogenase

Barley plants were collected, roots (5–10 g FW) were separated, blotted, weighed and immediately frozen in liquid nitrogen. All the subsequent steps were carried out at 4 °C. Cold extraction buffer A [50 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 4 mM EDTA, 15 μM $NADP^+$, 10% glycerol and Protease Inhibitor Cocktail (Sigma P9599) 100 μl/100 ml] was added (2:1 w/v) and tissues were ground in a mortar. The slurry was squeezed through four layers of muslin and centrifuged for 10 min at 13,000 rpm at 4 °C. The clear supernatant obtained was referred to as crude extract and assayed for total activity.

G6PDH activity was measured in triplicate against a control (minus G6P) as previously described (Cardi et al., 2013) in a Beckman DU65 spectrophotometer equipped with a kinetic soft pack module.

2.4. Purification and separation of G6PDH isoforms

Barley roots (25–50 g FW) were sampled and processed as described above Cold extraction buffer A was added (1:1 w/v) and the roots were ground in a mortar at 4 °C. The slurry was squeezed through four layers of muslin and centrifuged for 10 min at 13,000 rpm at 4 °C. The crude extract was brought to 70% $(NH_4)_2SO_4$ for 30 min and centrifuged as above. The pellet was dissolved in 5–10 ml of cold buffer A and desalted on a Sephadex G25 column. The sample was applied to a Q-Sepharose column (20 cm long, 1.27 cm i.d.) previously equilibrated with buffer A. After washing the column with buffer A, proteins were eluted by applying a 0–400 mM KCl linear gradient at 1 ml min^{-1}. 3 ml-fractions were collected and assayed for G6PDH activity. The active fractions were pooled, concentrated overnight with 70% $(NH_4)_2SO_4$