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Characterization of isoforms of hexose kinases in rice embryo

Lorenzo Guglielminetti[a,b], Pierdomenico Perata[c], Akiyoshi Morita[b], Elena Loreti[a], Junji Yamaguchi[b], Amedeo Alpi[a,*]

[a]*Dipartimento di Biologia delle Piante Agrarie, Sezione di Fisiologia Vegetale, Università degli Studi di Pisa, via Mariscoglio 34, 56124, Pisa, Italy*
[b]*Bioscience Center and Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan*
[c]*Dipartimento di Biologia e Patologia Vegetale, Università di Bari, Via Orabona 4, Bari, Italy*

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Abstract

Hexose kinases in rice embryos have been characterized. Six isoforms were detected: i.e. three glucokinases (GK1–3), two hexokinases (HK1 and HK2) and one fructokinase (FK1). Out of these, GK3, HK1 and HK2 were inhibited by mannoheptulose and glucosamine, known inhibitors of hexokinase activity. These inhibitors are also known to be modulators of sugar sensing processes. The results suggest that GK3, HK1 and HK2 may play a role in sensing the cellular sugar status in the rice embryo. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Sugars are an important source of energy and carbon skeletons for plant growth and development, but they also act as signaling molecules affecting developmental and metabolic processes. In fact, a variety of genes, whose translation products are involved in diverse metabolic pathways, are regulated depending on the availability of sugars in different living organisms ranging from bacteria to eukaryotic cells (Ronne, 1995; Thevelein & Hohmann, 1995). Understanding how cells sense sugars and how the signal is transduced to affect these processes is an important issue.

Hexose kinases, catalyzing the production of hexose-6-phosphate from hexoses such as glucose or fructose, are involved in the initial metabolic step of glycolysis in cells growing on free sugars, but experimental evidence suggests that they may also act as sugar sensors.

Analyses of several yeast mutants revealed that hexokinase PII encoded by *HXK2*, one of the three yeast hexose kinase genes, plays a major role as a glucose sensor, i.e., the entry of glucose into glycolysis mediated by the *HXK2* gene product is a key step in glucose sensing in yeast (Trumbly, 1992). In mammalian cells, glucokinase (hexokinase IV) has been implicated as a glucose sensor involved in insulin release in the pancreatic β-cell (Matschinsky, Liang, Kesavan & Wang, 1993; Grupe, Hultgren, Ryan, Ma, Bauer & Stewart, 1995).

In plants, one of the most studied physiological process concerning sugar regulation is the transcriptional control of photosynthetic genes (Sheen, 1990; Smeekens, 1998). It has been reported that repression of transcription of many photosynthetic genes is triggered by sugars that act as substrates for hexokinase, suggesting this enzyme as a putative sugar sensor in plants (Smeekens, 1998; Jang & Sheen, 1994; Graham, Demby, and Leaver, 1994; Koch, 1996). Mannoheptulose, a hexokinase inhibitor, causes the derepression of photosynthetic genes (Jang & Sheen, 1994). In addition, Jang, Leon, Zhou & Sheen, 1997 showed that the over-expression of hexokinase genes in transgenic *Arabidopsis* plants results in increased sugar sensitivity and that, on the contrary, the corre-

* Corresponding author. Tel.: +39-050-542898; fax: +39-050-540296.
E-mail address: aalpi@agr.unipi.it (A. Alpi).

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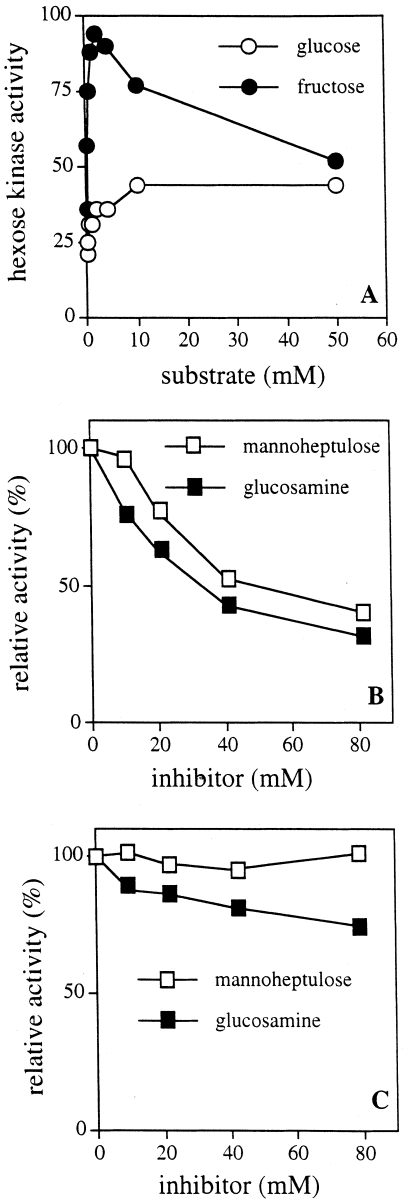

Fig. 1. *Enzyme properties of hexose kinases from rice embryos.* Panel A: hexose kinase activities (mU/mg protein) as a function of substrate concentration. Panels B and C: inhibitory effects of glucosamine and mannoheptulose on hexose kinase activities. Glucose 4 mM (Panel B) and fructose 2 mM (Panel C) were used as the substrate. Relative activity is expressed as: no inhibition (without inhibitors) = 100%. Data are means ($n = 2$). Variation was lower than 20% of the reported data.

sponding antisense plants are hyposensitive to sugars.

An alternative plant model system used to study the modulation of gene expression by carbohydrates is the germinating cereal seed. In this system, transcription of α-amylase genes (mainly the *RAmy3D* gene in rice) is controlled by the cellular sugar status (Hwang, Karren, Thomas, Chen & Rodriguez, 1998; Toyofuku, Umemura & Yamaguchi, 1998; Lu, Lim & Yu, 1998). Furthermore, transient expression experiments using the *RAmy3D* gene promoter indicate a possible role of hexose kinases in the sugar-sensing mechanism triggering repression of the *RAmy3D* gene (Umemura, Perata, Futsuhara & Yamaguchi, 1998). Indeed, an inhibitor of hexose kinase (glucosamine) causes the derepression of the *RAmy3D* gene (Umemura et al., 1998).

Overall, these results imply that hexose kinases may play a role as a sugar sensor in higher plants as well. In yeast and human, however, specific hexose kinase isoforms play a critical role in sensing the cellular sugar status.

In this study, we have characterized hexose kinase isoforms in rice embryos. Our aim was to test the sensitivity of each single isoform to mannoheptulose and/or glucosamine, the two hexokinase inhibitors able to derepress sugar-repressible genes in different plant systems.

2. Results

2.1. Hexose kinases in rice embryos

Total hexose kinase activity was measured in extracts from 5-day-old rice embryos in the presence of increasing substrate concentration. Total hexose kinases (glucose as substrate) increases to a plateau (Fig. 1A). When fructose was used as the substrate, the activity dramatically increased up to 2 mM, but a negative effect of higher substrate concentration was observed (Fig. 1A). The inhibitory effect of mannoheptulose and glucosamine on the hexose kinase activity was studied using either 4 mM glucose or 2 mM fructose. Both inhibitors were effective when glucose was used as the substrate (Fig. 1B). When fructose was used as the substrate, mannoheptulose did not affect the activity (Fig. 1C), while a minor effect was observed with glucosamine.

2.2. Isoforms of hexose kinases in rice embryos

Fig. 2 shows the zymogram (activity staining) of rice hexose kinase isoforms separated by isoelectric focusing (IEF). Six bands of activity were observed in the presence of both glucose (4 mM) and fructose (2 mM) (closed arrowheads in Fig. 2A, lane G + F). Five or three bands were detected when only glucose or fructose, respectively, were used as the substrate (Fig. 2A, lane G and F). These results suggest the presence of three distinct categories of rice hexose kinases; three glucose-specific forms (designated glucokinase; GK1, GK2 and GK3), one fructose-specific form (fructokinase; FK1) and two non-specific hexokinase forms (hexokinase; HK1 and HK2). The isoforms have the following apparent p*I* values: GK1 = 5.7; GK2 =

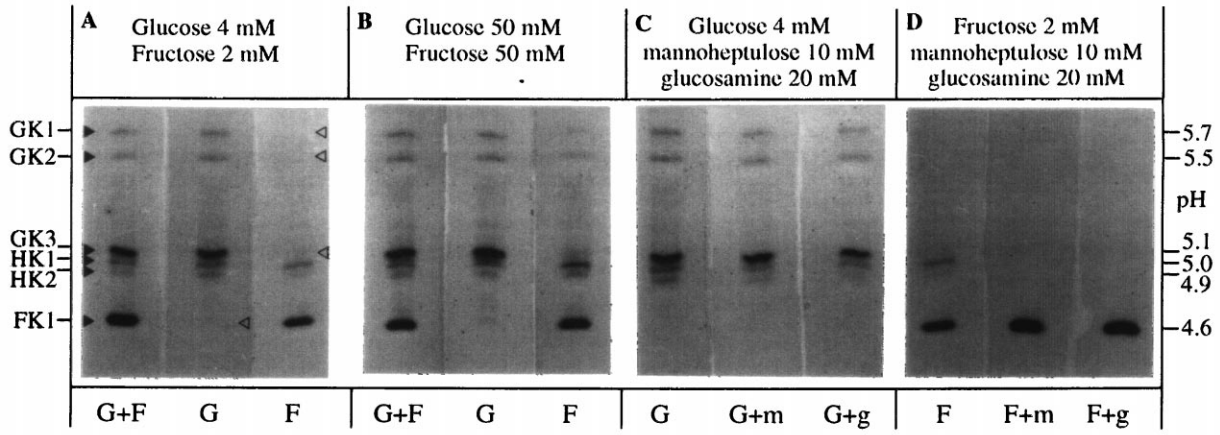

Fig. 2. *Pattern of hexose kinase isoforms in the presence of various substrates and inhibitors*. The procedures for sample preparation and activity staining after IEF are described in Sections 2.1 and 2.2. The concentrations of substrates and inhibitors are indicated in the upper part of the figure and the substrate (and inhibitor) used in each lane is indicated in the lower part of the figure; G, glucose; F, fructose; m, mannoheptulose; g, glucosamine. The pI value of each isoform is given on the right side. In panel A, as a reference, each isoform band is indicated by closed arrowhead (lane G + F) and bands disappearing depending on the substrate used are indicated by open arrowheads (lane G and F).

5.5; GK3 = 5.1; FK1 = 4.6; HK1 = 5.0; and HK2 = 4.9. A similar pattern was also observed when a higher concentration of the substrate was used (50 mM in Fig. 2B). Weakly stained bands corresponding to GK1, GK2 and GK3 were detected when fructose was used as the substrate (lane F in Fig. 2B). We also examined the inhibitory effect of glucosamine and mannoheptulose on activities of the isoforms. As shown in Fig. 2C, both inhibitors weakly affect the two hexokinases (HK1 and HK2) when glucose was used as the substrate (Fig. 2C, lanes G + m and G + g compared with G). When fructose was used as the substrate (Fig. 2D, lane F), FK1 was not affected by the inhibitors, while the HK1 and HK2 bands disappeared when using either glucosamine (lane F + g) or mannoheptulose (lane F + m).

2.3. Enzymatic properties of the hexose kinase isoforms

We attempted to separate the hexose kinase isoforms from crude extracts of rice by using a preparative liquid IEF system.

When glucose (4 mM) was used as the substrate (Fig. 3A), hexokinase activity was detectable in fractions 13–19 with a peak between fractions 14 and 16. When the glucose concentration was increased up to 50 mM, a higher activity was observed in fractions 15 and 16. Isoform analysis in IEF gels allowed us to identify HK2 in fraction 13, a mixture of HK1 and HK2, partially contaminated by GK3, in fraction 14, GK3 in fractions 15 and 16, GK2 in fraction 18 and GK1 in fraction 19 (see Fig. 3A, activity staining). Enzyme kinetic analysis (Fig. 3A, upper panel) revealed a low K_m value for glucose in fractions 13 and 14 (mainly HK1 and HK2), as well as in fractions 18 (predominantly GK2) and 19 (predominantly GK1), while a higher K_m value was estimated for activities present in fractions 15 and 16 (enriched in GK3).

When fructose was used as the substrate, activity was detected in two different peaks (Fig. 3B), the first corresponding to fraction 10 and the second to fraction 14. Isoform analysis allowed the identification of FK1 in fraction 10 and a mixture of HK1 and HK2 in fraction 14 (see Fig. 3B, activity staining). These activity peaks, however, showed a distinct affinity towards fructose: a reduced activity when the substrate concentration was increased from 2 to 50 mM in fraction 10 (FK1), while an enhanced activity was observed in fraction 14 (HK1 and HK2), indicating substrate inhibition of FK1. A low K_m value for fructose was determined for FK1, while higher K_m values were found for the mixture of HK1 and HK2.

We also examined the inhibitory effect of mannoheptulose (m) and glucosamine (g) on the active fractions. An inhibitory effect was detected in fraction 14 (HK1 and HK2) by both inhibitors when fructose was used as the substrate, while no inhibition was observed in fraction 10 (FK1) (see upper panel in Fig. 3B). When glucose was used as the substrate (upper panel in Fig. 3A), a weak inhibitory effect was observed in fractions 18 and 19 (GK1 and GK2), and activity was inhibited by about 50% in fractions 13–16 (HK1, HK2 and GK3).

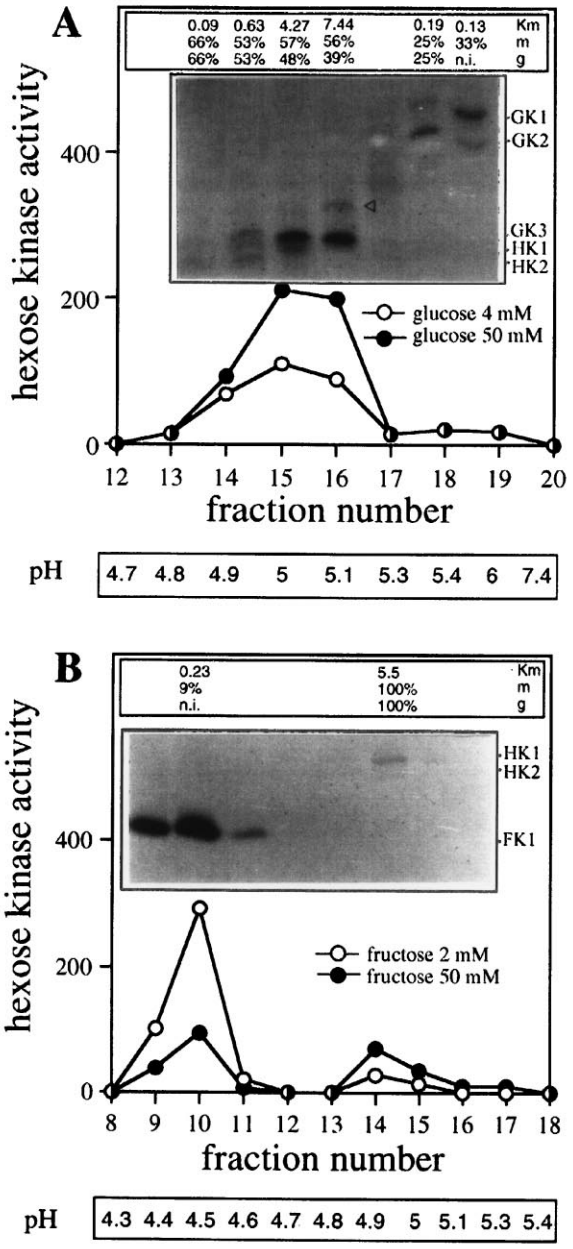

Fig. 3. *Enzymic properties of hexose kinase isoforms from rice embryos*. In panels A and B: activity pattern of hexose kinase in each fraction after preparative IEF (pH 4–6.5), using glucose (A) and fructose (B) as the substrate. Hexose kinase activity: mU/fraction. Zymogram in panels A and B: activity staining of hexose kinases in the active fractions using glucose (A) and fructose (B) as the substrate. The marked band in fraction 16 of zymogram in panel A is due to non-specific staining since staining was also observed without substrate addition (data not shown). Insert in panels A and B: K_m values (mM) for glucose (A) and fructose (B) in each active fraction. Percentage inhibition of hexose kinase activities by 10 mM mannoheptulose (m) or 20 mM glucosamine (g) is also reported using 4 mM glucose (A) or 2 mM fructose (B) as the substrate. n.i.: no inhibition. pH value of each fraction is also given.

3. Discussion

3.1. Hexose kinase isoforms in plants

In this study, rice embryo hexose kinases have been characterized with respect to their isoform pattern and their sensitivity to mannoheptulose and glucosamine. Six isoforms were identified: three glucokinases (GK1, GK2 and GK3), two hexokinases (HK1 and HK2), and one fructokinase (FK1) (Fig. 2). GK1 and GK2 have low apparent K_m values (less than 0.2 mM), while GK3 has a higher K_m value (4–7.5 mM) for glucose (Fig. 3A). HK1 and HK2 have low K_m values (0.1–0.6 mM) for glucose but high (about 5 mM) for fructose. FK1 has a low K_m value (about 0.2 mM) for fructose (Fig. 3B).

Several plant hexose kinases have previously been characterized (Schnarrenberger, 1990; Renz & Stitt, 1993; Martinez-Barajas & Randall, 1998). Schnarrenberger (1990) analyzed hexose kinases partially purified from spinach leaves, resolving two hexokinases (I and II) and two fructokinases (I and II). Renz & Stitt (1993) identified three hexokinases (HK1–3) and three fructokinases (FK1–3) from potato tubers. Plant hexokinases (also including glucokinases) usually have low K_m values for glucose and high values (millimolar range) for fructose. Rice GK1 and GK2 appear to resemble potato HK2, having low K_m values for glucose, but very high K_m values or no affinity at all for fructose. Rice HK1 and HK2 resemble somehow spinach hexokinase I and II and potato HK1 and HK3, in having low K_m values for glucose, but relatively high K_m values for fructose. On the contrary, rice GK3 is unique in showing high K_m value for glucose and no affinity for fructose. Plant fructokinases, including rice FK1, are highly specific for fructose, with low K_m values in the micromolar range and substrate inhibition in the millimolar range (Kanayama et al., 1998).

3.2. Hexose kinase isoform(s) as a sugar sensor

Several lines of evidence suggest a possible role of hexose kinases as sugar sensors in plants (Smeekens, 1998; Jang & Sheen, 1994; Graham et al., 1994; Koch, 1996; Jang et al., 1997; Perata, Matsukura, Vernieri & Yamaguchi, 1997). In yeast and mammalian cells, certain hexose kinases have a central role in the early stage of sugar perception (Trumbly, 1992; Matschinsky et al., 1993). However, little is known about hexose kinase isoforms as putative sugar sensor(s) in plant cells.

Jang & Sheen (1994) succeeded in uncoupling photosynthetic genes from sugars repression by the use of 10 mM mannoheptulose.

In rice embryo, transcription of the *RAmy3D* gene is

strictly under sugar control, and sugar-repression of this gene does likely involve a hexose kinase-mediated signaling process (Umemura et al., 1998). Umemura et al. (1998) also reported that another inhibitor of hexose kinase activity, glucosamine, used at 20 mM concentration, is able to relieve the *RAmy3D* promoter activity from repression by endogenous sugars. Assuming that the glucosamine effect is due to in vivo inhibition of specific isoform(s) of hexose kinase triggering sugar repression in rice embryo, the respective isoform(s) might be a putative sugar sensor. One can hypothesize that rice HK1, HK2 and GK3 isoforms, which are sensitive to inhibition by mannoheptulose and glucosamine (Fig. 3), when used at concentrations close to those able to uncouple sugar-repressed genes from the effects of various carbohydrates, are involved in the sugar-sensing process in rice embryos. However, while mannoheptulose is able to derepress photosynthetic genes in vivo (Jang et al., 1997), in the rice embryos system, glucosamine, but not mannoheptulose, is effective in derepressing the *Ramy3D* gene (Umemura et al., 1998). On the contrary, in vitro, both inhibitors effect the same rice hexose kinase isoforms (Fig. 3). It is possible that, in the rice system, mannoheptulose uptake into the embryos is impaired. Further experiments will be needed to clarify the role of hexose kinases in sugar sensing.

4. Experimental

4.1. Plant materials

Rice seeds (*Oryza sativa* L. cv. Nipponbare) were surface-sterilized with a 3% NaClO solution containing 0.1% Tween 20 for 30 min, washed 10 times with sterile distilled water (DW), and sown with sterile DW at 25°C in the dark. After 5 days, embryos were dissected from the endosperm. The plumule and root were also removed.

Embryos as described above were extracted in 50 mM Tris–HCl buffer (pH 7.6, containing 10 mM DTT and 10% glycerol). The extract was centrifuged at 15,000 *g* for 15 min, and the resulting supernatant was desalted on a Sephadex G25 column equilibrated with 20 mM Tris–HCl buffer (pH 7.6, containing 10 mM DTT).

4.2. Hexose kinase assay and activity staining

Hexose kinase activity was measured according to the method described previously (Guglielminetti, Perata & Alpi, 1995). When used, glucosamine and mannoheptulose were added together with the substrates.

Samples were loaded on an IEF ampholine gel (pH range: 4–6.5). Preliminary experiments revealed that all the hexose kinase isoforms had isoelectric points ranging from pH 4.5 to 6. After electrophoresis, the gel was incubated in the following staining mixture: 50 mM Hepes–KOH buffer (pH 7.5), containing 2 mM $MgCl_2$, 1 mM EDTA, 15 mM KCl, 2 mM ATP, 0.75 mM NADP, 2 U/ml G6PDH (glucose-6-phosphate dehydrogenase), 0.25 mg/ml MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide), 0.075 mg/ml PMS (phenazine methosulfate). When fructose was used as the substrate, 2 U/ml PGI (phosphoglucose isomerase) were added to the staining mixture. Concentrations of glucose, fructose, mannoheptulose, or glucosamine used in the mixture were as reported in the figures. A concentrated (4%) solution of melted agarose (type I-A: Low EEO) was added to the mixture, when the temperature of the agarose was lower than 55°C, to a final concentration of 1%. After adding the staining mixture, gels were incubated at 37°C for 30 min in the dark. An acetic acid solution (1%) was used to stop the reaction. Each activity staining was also conducted without substrates to evaluate unspecific staining.

4.3. Preparative isoelectric focusing

The hexose kinase isoforms were separated by preparative IEF methodology. Rice embryos were extracted and the extract dialyzed according to the procedures described above. The crude extract (60 ml) was concentrated to 3 ml using a Centripep-10 (Amicon) at 3000 *g*, 4°C and loaded into a 50 ml Rotofor cell (Bio-Rad). Prefocusing was performed as follows: the cell contained 2.5 ml ampholite (40%, pH 4–6, LKB), 8.6 ml glycerol and 36.9 ml DW (45 min at 3000 V, 150 mA, and 12 W, 4°C, using 0.1 M H_3PO_4 as the anode solution, and 0.1 M NaOH as the cathode solution). The concentrated extract was loaded at the estimated pH 5 loading position. Isoelectric focusing (4 h) was performed under the same running conditions of prefocusing. Twenty fractions of 2.5 ml were collected.

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