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Review

Isozymes of plant hexokinase: Occurrence, properties and functions

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Abstract

Hexokinase (HK) occurs in all phyla, as an enzyme of the glycolytic pathway. Its importance in plant metabolism has emerged with compelling evidence that its preferential substrate, glucose, is both a nutrient and a signal molecule that controls development and expression of different classes of genes. A variety of plant tissues and organs have been shown to express multiple HK isoforms with different kinetic properties and subcellular localizations. Although plant HK is known to fulfill a catalytic function and act as a glucose sensor, the physiological relevance of plural isoforms and their contribution to either function are still poorly understood. We review here the current knowledge and hypotheses on the physiological roles of plant HK isoforms that have been identified and characterized. Recent findings provide hints on how the expression patterns, biochemical properties and subcellular localizations of HK isoforms may relate to their modes of action. Special attention is devoted to kinetic, mutant and transgenic data on HKs from *Arabidopsis thaliana* and the *Solanaceae* potato, tobacco, and tomato, as well as HK gene expression data from *Arabidopsis* public DNA microarray resources. Similarities and differences to known properties of animal and yeast HKs are also discussed as they may help to gain further insight into the functional adaptations of plant HKs.

Keywords: *Arabidopsis*; Carbon metabolism; *Cruciferae*; Glucose; Glycolysis; Hexokinase; Hexose sensing; Microarray; Sequence analysis; *Solanaceae*

Contents

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1. Hexokinase: an 80-year old enzyme still full of life

HK (EC 2.7.1.1, Supplementary Table 1) was first described in a seminal paper by Otto Meyerhof (Nobel Prize in Physiology or Medicine in 1922) in an extract from baker's yeast (*Saccharomyces cerevisiae*) (Meyerhof, 1927). However, its reaction was elucidated more than a decade later (Von Euler and Adler, 1935; Meyerhof, 1935; Colowick and Kalckar, 1943) as summarized below:

hexose + ATP → hexose-6-phosphate + ADP

The elucidation of the Embden–Meyerhof–Parnas pathway in the 1940s established HK as a glycolytic enzyme in yeast and animals (Plaxton, 1996). The interest for plant HK came with evidence for the presence of a functional glycolytic pathway in plants (Stumpf, 1952; Beevers and Gibbs, 1954). The concomitant detection of the oxidative pentose-phosphate pathway (OPPP) in plants (Axelrod et al., 1953) demonstrated another route for the metabolism of D-glucose-6-phosphate (G6P), a product of HK. The first isolation and characterization of a plant HK (Saltman, 1953) was therefore a significant milestone. Using wheat (*Triticum aestivum*) germ extracts, Saltman (1953) demonstrated the existence of HK in a higher plant, which had only been inferred until then (Saltman, 1953). Early studies on plant HKs were also instrumental in providing evidence for soluble and particulate forms of the enzyme (Millerd et al., 1951; Saltman, 1953). Recent developments have now provided strong experimental evidence that HK activity is associated with the mitochondrion and plastids (Giegé et al., 2003; Wiese et al., 1999). The interest in this 80-year old enzyme has notably been renewed with the discovery that HK fulfills catalytic and sensing functions in plants (Moore et al., 2003).

It is now well established that HK is present in virtually all living organisms as part of the ancillary glycolytic pathway (Cárdenas et al., 1998). In plants, the enzyme phosphorylates several hexoses including D-glucose (Glc), D-fructose (Fru), D-mannose (Man) and D-galactose (Gal). Plant HK is thus distinct from glucokinase (GK), fructokinase (FK), mannokinase (MK) and galactokinase (GalK) that can be highly specific to Glc, Fru, Man and Gal, respectively (Cárdenas et al., 1998). Moreover, available FK and GalK protein sequences share no obvious homology with those of HK (Dai et al., 2002). As for MK and GK, no molecular or biochemical evidence of their existence in plants has been provided yet. The so-called GKs isolated to date could phosphorylate Fru or Man in addition to Glc, although with sometimes extremely low efficiency (Turner et al., 1977; Doehlert, 1989; Martinez-Barajas and Randall, 1998). Therefore, it has been concluded that these GKs in fact belong to the HK family, and that no true GKs have been found yet in Eukaryotes (Cárdenas et al., 1998; Dai et al., 2002).

HK's specificity for several hexose substrates makes this enzyme a gateway to glycolysis for hexoses arising from D-sucrose (Suc) or transitory starch degradation. Moreover,

the main product of HK, G6P, not only feeds cytosolic and plastidic glycolysis but is also a precursor for the OPPP and for fatty acid, starch and cell wall polysaccharide biosyntheses in their respective cell compartments (Neuhaus and Emes, 2000; Seifert, 2004). In addition to its catalytic role, HK acts as a hexose sensor and mediates hexose responses in gene expression, germination, growth, vegetative and reproductive development, stress and senescence (Rolland et al., 2002). Targeted mutagenesis and HK mutants have been used to demonstrate that the Glc sensing function of HK is distinct and independent from its catalytic activity (Moore et al., 2003). The sensing and signaling function of plant HK has recently been reviewed as part of the signaling network that integrates environmental cues with those from sugars, hormones, nutrients and stresses to govern plant developmental programs (Rolland et al., 2006).

Despite the cardinal importance of HK in plant life, several aspects of its physiology and biochemistry are still obscure. In particular, HK has been observed in multiple isoforms in a wide variety of plant species and tissues (Renz et al., 1993; da-Silva et al., 2001; Cho et al., 2006). However, the physiological significance of this heterogeneity requires clarification. The aim of this review is to explore how subcellular localizations, kinetic and regulatory properties as well as gene expression patterns of plant HK isoforms may interrelate in their individual roles and modes of action. We also present the current knowledge on the catalytic function and implication of HK in hexose sensing and signaling. Research on yeast and animal HKs is also considered as it may provide a useful basis for comparison. By integrating physiological, biochemical and molecular data, this comprehensive review highlights the complexity of the roles and modes of action of plant HKs.

2. Evidence for multiple HK isoforms with different biochemical properties and implications in primary metabolism

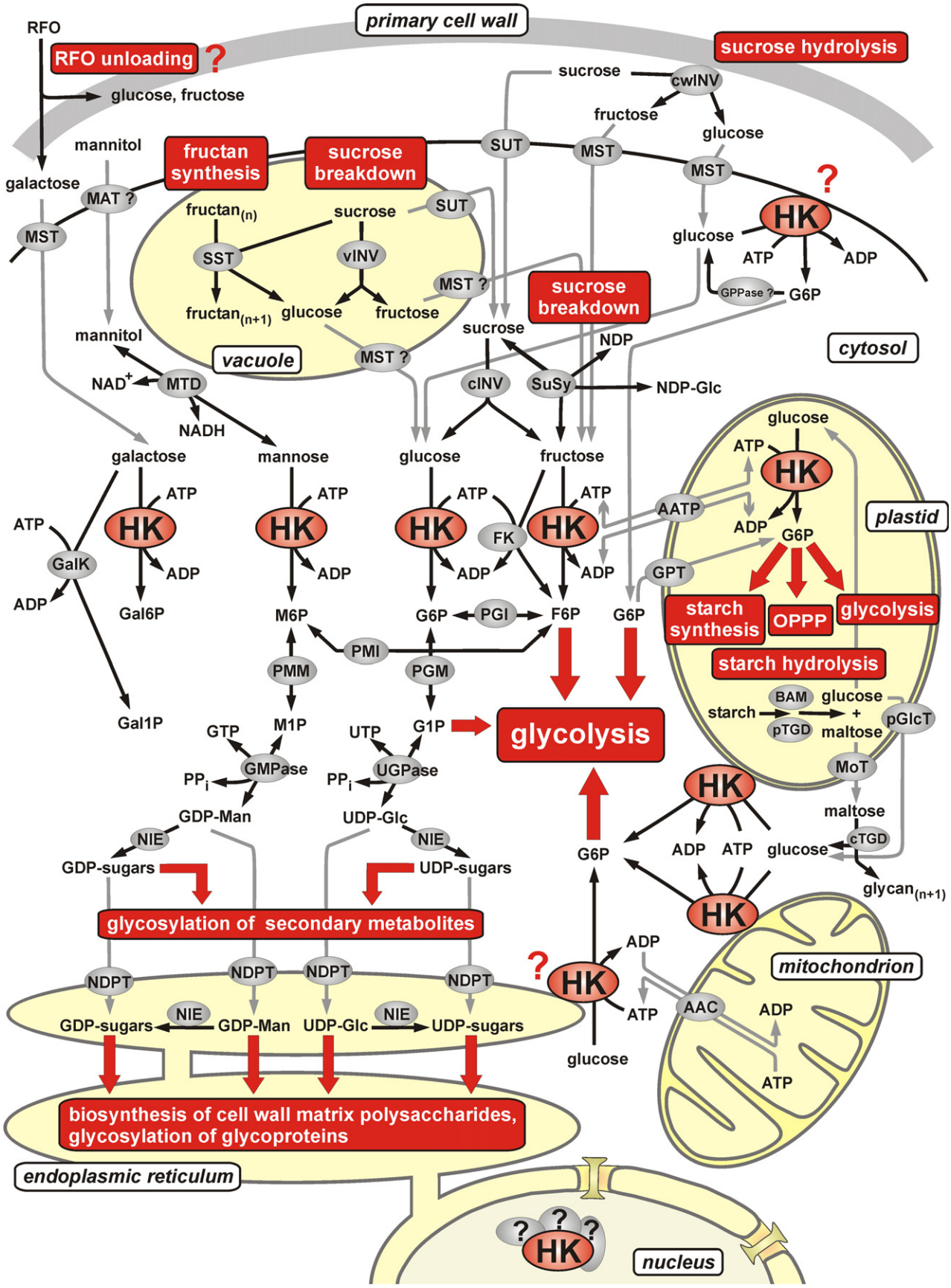
2.1. A variety of metabolic pathways feed the different HK isoforms

2.1.1. Suc catabolism

Due to its broad specificity to hexoses, HK uses substrates from multiple metabolic pathways including Suc catabolism (MSTs) (Fig. 1). In most plants, Suc is synthesized in source leaves and transported via the phloem to sink tissues, where it is stored or catabolized for starch synthesis or for heterotrophic metabolism and growth (Quick and Schaffer, 1996). Apoplastic unloading of Suc into sink cells is mediated by Suc transporters (SUTs). Alternatively, Suc may be hydrolyzed by invertase (INV) into Glc and Fru, which are subsequently taken up by monosaccharide transporters (MSTs) (Fig. 1) (Lalonde et al., 2004). There is convincing evidence also for endocytosis of apoplastic Suc and Glc to the vacuole (Etxeberria et al., 2005a; Etxeberria et al., 2005b). Within sink cells, Suc may be cleaved by INV and/or by Suc synthase (SuSy). INV exists as a vacuolar (vINV), a cytosolic (cINV) and a cell-wall bound (cwINV) isoforms (Roitsch and Gonzalez, 2004). INV activity is generally due mainly to cwINV and vINV whereas cINV activity is comparatively very low (Kim et al., 2000; Thévenot et al., 2005). SuSy is found in the cytosol, where it may be soluble, or attached to the actin cytoskeleton, or associated with the plasma membrane, the tonoplast or the Golgi membrane (Koch, 2004). This enzyme is also found within the mitochondrion where it possibly fulfills a non-catalytic function in signaling (Subbaiah et al., 2006). SuSy catalyses the conversion of Suc and UDP into Fru and UDP-D-glucose (UDP-Glc) according to a reversible reaction, but may function with other nucleoside-5′-diphosphates (NDPs) such as ADP (Baroja-Fernandez et al., 2003). Interestingly, INV and SuSy activities may mediate transitions through the prominent stages of sink organ development (Sturm and Tang, 1999; Koch, 2004; Roitsch and Gonzalez, 2004). It appears from observations made on root, tuber, fruit, and seed that Suc degradation by vINV and cwINV predominate at sink initiation and expansion. At this stage, cwINV activity and the resultant high hexose/Suc ratio are generally associated with high cell division rates (Weber et al., 1997; Koch, 2004). In the seed, for instance, evidence is based on mutant studies and histological mapping of hexose and Suc gradients (Weber et al., 2005). High cwINV activity also correlates with elevated expression levels of MSTs (Sherson et al., 2003; Weschke et al., 2003). Transition to the later storage and maturation phases is characterized by a switch from the INV− to the SuSy path of Suc cleavage (Weber et al., 1997; Sturm and Tang, 1999; Nguyen-Quoc and Foyer, 2001; Fernie et al., 2002b). The rise in SuSy activity coincides with a shift from cell division to cell differentiation and elongation, possibly due to a gradual decrease of the hexose/Suc ratio (Weber et al., 1997; Koch, 2004). These data suggest that the developmental stage of the organ may be of prime importance when considering the contribution of INV and SuSy activities to the supply of Glc and/or Fru to HK.

2.1.2. Competition for Fru between HK and FK

The Fru produced by INV and/or SuSy activities may serve as substrate for HK or, alternatively, for FK (Fig. 1). Numerous plant sources have been shown to express between one and three FK isoforms (Pego and Smeekens, 2000). Study of potato (*Solanum tuberosum*) tuber early development has shown high cwINV and HK activities in the mitotically active, subapical region of the non-tuberizing stolon (Appeldoorn et al., 2002). Later, during tuberization, most hexose-phosphorylating activity was due to FK, which coincided with the predominant SuSy path of Suc cleavage (Renz et al., 1993; Appeldoorn et al., 2002; Davies et al., 2005). Therefore, the relative levels of HK and FK activities correlated with a possible carbon flow via the INV and SuSy routes, respectively, at


primary cell wall
RFO
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glucose, fructose
sucrose hydrolysis
sucrose
cwINV
fructose
glucose
mannitol
fructan synthesis
sucrose breakdown
SUT
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sucrose breakdown
NDP
cytosol
mannitol
NAD+
MTD
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glucose
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HK
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GPPase ?
G6P
galactose
mannose
glucose
fructose
ATP
HK
ADP
GalK
Gal6P
FK
AATP
plastid
M6P
G6P
PGI
F6P
G6P
GPT
starch synthesis
OPPP
glycolysis
starch hydrolysis
PMI
PMM
PGM
BAM
starch
glucose + maltose
pTGD
pGlcT
MoT
Gal1P
GTP
M1P
UTP
G1P
glycolysis
PPi
GMPase
PPi
UGPase
maltose
cTGD
GDP-Man
UDP-Glc
NIE
NIE
GDP-sugars
UDP-sugars
glycan(n+1)
glycosylation of secondary metabolites
G6P
ADP
ATP
glucose
NDPT
NDPT
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HK
ADP
mitochondrion
NIE
GDP-sugars
GDP-Man
UDP-Glc
NIE
UDP-sugars
ATP
AAC
ADP
glucose
ATP
biosynthesis of cell wall matrix polysaccharides, glycosylation of glycoproteins
endoplasmic reticulum
? ? ?
HK
nucleus

different time points in tuber development. Results from transgenic experiments have also supported the view that Fru produced by SuSy during tuberization may not feed the HK reaction for further metabolism. Tuber yield was dramatically altered in potato plants with decreased SuSy activity (Zrenner et al., 1995), whereas those carrying HK antisense constructs exhibited little difference with the wild-types (Veramendi et al., 1999; Veramendi et al., 2002). Transgenic tomato (*Solanum lycopersicum*) studies have shown a similar SuSy/FK combination in Suc partitioning in seed, root, stem and reproductive tissues (Odanaka et al., 2002; German et al., 2003). Coordinated regulation of FK and SuSy activities have been reported elsewhere (Huber and Akazawa, 1986; Hill et al., 2003), suggesting a widespread occurrence of the SuSy/FK pathway of Suc degradation in plants (Pego and Smeekens, 2000; Davies et al., 2005). Given the poor efficiency of HK and high efficiency of FK with Fru (Renz and Stitt, 1993; da-Silva et al., 2001; Menu et al., 2001; Claeyssen et al., 2006), the above data suggest that Fru could be consumed almost entirely by FK in situations where the SuSy/FK and INV/HK routes may coexist.

2.1.3. Impact of substrate cycles on carbon flow through HK

There is evidence for substrate (futile) cycles that involve Suc synthesis and degradation. Suc synthesis occurs via SuSy, Suc-phosphate synthase (SPS) and Suc-phosphate phosphatase, whereas its degradation is mediated by SuSy (reverse reaction), cINV and vINV. These reactions are believed to govern fruit sugar content and composition (Nguyen-Quoc and Foyer, 2001). In other systems, Suc cycling has been proposed to buffer variations in metabolite concentrations or adjust metabolism to Suc supply, consuming between 5% and 80% of the ATP generated by the cell (Dieuaide-Noubhani et al., 1995; Fernie et al., 2002a; Rontein et al., 2002). Interestingly, increasing Suc degradation activity in potato tubers by transforming plants with a yeast INV, a bacterial Suc phosphorylase or a bacterial xylose isomerase, led to increases in Suc cycling and glycolytic activity (Trethewey et al., 1999; Fernie et al., 2002a; Urbanczyk-Wochniak et al., 2003). It was concluded that glycolytic flux may be controlled, at least in part, by processes like Suc cycling that increase the ATP demand of the cell (Fernie et al., 2002a; Urbanczyk-Wochniak et al., 2003). In other terms, Suc cycling may contribute to modulation of carbon flow through HK and glycolysis by way of ATP dissipation, although other unknown factors seem to be involved (Junker et al., 2006).

An additional substrate cycle, involving Glc and Glc-P, i.e. either G6P or D-glucose-1-phosphate (G1P), has been described in maize (*Zea mays*) root tips (Fig. 1) (Alonso et al., 2005). The high rate of Glc-P to Glc turnover was shown to consume up to 40% of the ATP generated, whereas Suc cycling used at most 6% of the ATP produced in the roots (Alonso et al., 2005). Several Glc-P phosphatases have been characterized in animals, yeast or bacteria, including D-glucose-6-phosphatase, D-glucose-1-phosphatase and 2-deoxy-D-glucose-6-phosphate phosphatase (Lee et al., 2003; Mithieux et al., 2004; Tsujimoto et al., 2000). However, sequence homology or biochemical evidence for the presence of these enzymes in plants is lacking (Claeyssen and Rivoal, unpublished observations). *In vitro* phosphatase activities with G1P and G6P have been reported, but seemed to be due to low substrate specificity of acid phosphatases (Duff et al., 1989; Bozzo et al., 2004; Yoneyama et al., 2004). Whether these activities have physiological relevance to Glc/Glc-P cycling remains to be established. Ultimately, newly synthesized Glc may re-enter the hexose-phosphate (hexose-P) pool by way of the HK reaction, together with consumption of a new ATP molecule (Fig. 1). Although its role and regulation remain to be clarified, the Glc/Glc-P cycle may directly implicate HK in modulation of the energy status of the cell. This novel substrate cycle has also been ascribed the hypothetical role of raising glycolytic rate through increased energy demand, by analogy to Suc cycling (Alonso et al., 2005).

2.1.4. Starch degradation

A major source of Glc for HK may arise with degradation of transitory starch in leaves or storage starch in seeds, roots, tubers, and fruit. In photosynthesizing leaves, starch accumulates in chloroplasts at daytime, when photoassimilate supply exceeds the demand for Suc. This transitory starch is remobilized at night for continued synthesis and

Fig. 1. Schematic representation of the metabolic pathways that involve hexokinase (HK) in plant carbon metabolism. Metabolic pathways relating to HK activity are indicated in red. Black arrows illustrate reactions while translocation of intermediates is in grey. Question marks indicate HK localization, proteins or transporters for which experimental evidence is lacking. Transport mechanisms (by facilitation or H^+-coupled cotransport) and tissue- and organelle-specificities of SUTs and MSTs (Lalonde et al., 2004) are not detailed. NIE specificities are reviewed in Seifert (2004). *Metabolic pathways and intermediates*: F6P, D-fructose-6-phosphate; G1P, D-glucose-1-phosphate; G6P, D-glucose-6-phosphate; Gal1P, D-galactose-1-phosphate; Gal6P, D-galactose-6-phosphate; GDP-Man, GDP-D-mannose; M1P, D-mannose-1-phosphate; M6P, D-mannose-6-phosphate; NDP, nucleoside-5′-diphosphate; NDP-Glc, NDP-D-glucose; OPPP, oxidative pentose-phosphate pathway; PP_i, pyrophosphate; RFOs, raffinose-family oligosaccharides; UDP-Glc, UDP-D-glucose. *Enzymes and transporters*: AAC, mitochondrial ADP/ATP carrier; AATP, plastidic ATP/ADP translocator; BAM: β-amylase, cTGD, cytosolic transglucosidase; FK, fructokinase; FTF, fructosyltransferase; GalK, galactokinase; GMPase, GDP-D-mannose pyrophosphorylase; GPPase?, putative D-glucose-phosphate phosphatase; GPT, D-glucose-6-phosphate/P_i translocator; HK, hexokinase; MAT?, putative mannitol transporter; MST, monosaccharide transporter; MST?, putative monosaccharide transporter of the tonoplast; cINV, cwINV and vINV, cytosolic, cell-wall and vacuolar isoforms of invertase, respectively; MoT, chloroplastic maltose transporter; MTD, mannitol dehydrogenase; NDPT, nucleoside-5′-diphosphate sugar transporter; NIE, nucleoside-5′-diphosphate sugar interconverting enzyme; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase; pGlcT, plastidic D-glucose translocator; PMI, phosphomannose isomerase; PMM, phosphomannomutase; pTGD, plastidic transglucosidase; SuSy, sucrose synthase; SUT, sucrose transporter; UGPase, UDP-D-glucose pyrophosphorylase. EC numbers of cited enzymes are available in Supplementary Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

export of Suc to heterotrophic plant parts, and for the immediate metabolic requirements of the leaf cell (Preiss and Sivak, 1996). Breakdown of transitory starch has been most extensively characterized in *Arabidopsis* and potato leaves, based on mutant and transgenic studies (Zeeman et al., 2004; Smith et al., 2005). Attack of the starch granule in the chloroplast yields linear α-1,4-glucans that are degraded mainly by β-amylase (BAM) into maltose and, to a lesser extent, maltotriose (Fig. 1) (Zeeman et al., 2004). Maltotriose may be degraded into Glc by plastidic transglucosidase (pTGD) (Critchley et al., 2001), and feed a stromal HK (Fig. 1) (Ritte and Raschke, 2003). This is very likely for chloroplasts of the moss *Physcomitrella patens* where stromal HK accounts for 80% of total HK activity (Olsson et al., 2003). In higher plants, however, stromal NtHxk2 of tobacco (*Nicotiana tabacum*) and its two candidate orthologs in *Arabidopsis*, At1g47840 and At4g37840, are expressed at low levels, if at all, in leaves (Zeeman et al., 2004; Giese et al., 2005). Stromal HK may rather be recruited in starch sheath chloroplasts or in non-green plastids, to feed glycolysis or the OPPP with G6P (Giese et al., 2005). Alternatively, Glc may leave the chloroplast by way of a plastidic Glc translocator (pGlcT, Fig. 1), moving along a gradient of concentration across the chloroplast envelope (Häusler et al., 1998; Weber et al., 2000). Indeed, stromal levels of Glc may be relatively high, e.g. 14 mM in isolated spinach (*Spinacia oleracea*) chloroplasts (Stitt and Heldt, 1981), assuming a chloroplastic volume of 25 μL/mg chlorophyll (Gerhardt et al., 1987). Cytosolic Glc levels appear lower, e.g. 0.4 mM in potato leaves (Leidreiter et al., 1995). Wiese et al. (1999) have suggested that an HK inserted in the chloroplastic outer envelope membrane could be particularly suited to maintain a gradient of Glc by phosphorylating Glc during export from the plastid. In accordance with a predominant β-amylolytic pathway, starch catabolites are exported to the cytosol at night almost exclusively as maltose and Glc in several species (Servaites and Geiger, 2002; Weise et al., 2004). Export of maltose to the cytosol occurs via a specific maltose transporter of the chloroplast envelope (MoT, Fig. 1) (Niittylä et al., 2004). In *Arabidopsis* leaves, maltose is then cleaved by cytosolic transglucosidase (cTGD, Fig. 1), which transfers one glucosyl residue onto an unknown heteroglycan acceptor and releases the second Glc (Chia et al., 2004; Lu and Sharkey, 2004). Therefore, it appears that transitory starch degradation yields substantial amounts of cytosolic Glc, which may lead to a major carbon flow through the cytosolic HK reaction. Interestingly, antisense repression of cytosolic HK resulted in higher Glc and lower Suc levels in transgenic potato leaves compared to wild-types at the end of the night (Veramendi et al., 1999). These data may then point to HK as an obligate step in commitment of starch catabolites to Suc synthesis and glycolysis in the leaf cell at night (Fig. 1).

Moreover, mutant and gene expression profiling studies suggest a common pathway of starch breakdown in other *Arabidopsis* plant organs that store it transiently (Smith et al., 2005). However, this pathway is likely to differ from that occurring in storage organs. In cereal endosperm, starch breakdown takes place in nonliving tissue, in which no intra- or intercellular compartmentation remains. Linear α-1,4-glucans are probably degraded via α- and β-amylases and α-glucosidase to maltose and Glc, which can enter the embryo (Smith et al., 2005). The high energy and carbon requirements for biosyntheses during germination may thus rely heavily on HK for energization of starch-derived Glc within the cereal embryo. As for starch-storing legume seeds and potato tuber, the pathway of starch breakdown has not yet been elucidated. Therefore, the degree of implication of HK is uncertain for those systems (Smith et al., 2005).

2.1.5. Catabolism of polyols and raffinose-family oligosaccharides

A number of plants transport and store soluble sugars such as polyols (e.g. mannitol, sorbitol), raffinose-family oligosaccharides (RFOs), or a combination of those, in parallel with Suc (Turgeon, 1996). In celery, equal amounts of mannitol and Suc are translocated to sink tissues (Davis and Loescher, 1990). Mannitol is synthesized in the cytosol of leaf cells, from photosynthetic triose-phosphates via D-fructose-6-phosphate (F6P), D-mannose-6-phosphate (M6P), and D-mannitol-1-phosphate (Rumpho et al., 1983; Loescher et al., 1992). In sink tissues, mannitol is stored in the vacuole or oxidized directly to Man by mannitol dehydrogenase (MTD) (Stoop and Pharr, 1992). Man may be readily phosphorylated by HK into M6P, the latter entering glycolysis after isomerization to F6P by phosphomannose isomerase (PMI) (Fig. 1) (Fujiki et al., 2001).

RFOs are of widespread occurrence in seeds, where they may serve as desiccation tolerance agents or as carbohydrate reserves for energy supply to the new seedling during germination (Keller and Pharr, 1996). Their degradation possibly involves INV, α-galactosidase and α-glucosidase (Cook et al., 2004). The released Glc, Fru and Gal may subsequently feed the HK reaction or, alternatively, Gal may be phosphorylated into D-galactose-1-phosphate (Gal1P) by GalK. Accordingly, in germinating *Vicia faba* seeds, a steady decrease in raffinose and stachyose levels was correlated to an increase in GalK activity. The latter was sufficiently high to phosphorylate all the Gal released from RFO breakdown and correspondingly, no free Gal was detected (Dey, 1983). Therefore, HK and GalK may compete for Gal during germination of legume seeds. As mentioned above, some plants use RFOs as long-distance carbon allocation forms, together with Suc (Turgeon, 1996). Eventually, their degradation in sink tissues will generate Glc, Fru and Gal and, again, HK and GalK may compete for Gal (Fig. 1). This may be the case for *Arabidopsis*, which has been shown to transport small amounts of raffinose (Haritatos et al., 2000) and to express a GalK in all organs investigated (Kaplan et al., 1997).

2.1.6. Fructan synthesis yields Glc

Fructans, rather than starch, are used as reserve carbohydrates by up to 15% of higher plants. Fructans are soluble Fru oligomers and polymers that are stored in the vacuole of both photosynthetic and storage cells (Vijn and Smeekens, 1999). They accumulate when photoassimilate production exceeds demand, or upon cold or drought stress (Ritsema and Smeekens, 2003). Several specific fructosyltransferases (FTFs) catalyze fructan synthesis in the vacuole, with Suc as the sole precursor (Vijn and Smeekens, 1999). Consequently, fructan accumulation releases substantial amounts of Glc, which may exit the vacuole for subsequent phosphorylation by HK (Fig. 1). Also, fructan degradation produces large amounts of Fru, which may serve as substrate for cytosolic HK and FK.

2.2. The different HKs feed various metabolic pathways

2.2.1. HK feeds the glycolytic pathway

HK is a glycolytic enzyme and hence, contributes to breakdown of carbohydrates to fuel respiration and provide carbon intermediates to numerous anabolic pathways (Fig. 1). The plant glycolytic pathway is compartmentalized (Plaxton, 1996). Therefore, the fate of glycolytic intermediates or the end product pyruvate depends on the cell compartment where they occur. A complete, functional glycolytic pathway, present on the cytosolic face of the outer mitochondrial membrane, has been proposed to provide pyruvate directly to the mitochondrion as respiratory substrate (Giegé et al., 2003). By the same token, mitochondrion-associated HK may gain preferential access to mitochondrial ATP, which is translocated to the cytosol via an ADP/ATP carrier (AAC) (Fig. 1) (Galina and da-Silva, 2000; Spagnoletta et al., 2002). In nonphotosynthetic plastids of castor (*Ricinus communis*) oilseeds, a complete or partial glycolytic pathway may provide pyruvate to the lipid biosynthetic pathway (Neuhaus and Emes, 2000). Although compartmentalized, the cytosolic and plastidic glycolytic pathways are interconnected via highly selective metabolite transporters in the plastidic inner envelope membrane (Plaxton, 1996). Cytosolic pyruvate may thus be imported by a pyruvate carrier into the nonphotosynthetic plastid where it may serve as a precursor for fatty acid synthesis (Eastmond and Rawsthorne, 2000; Neuhaus and Emes, 2000). Similarly, G6P, phospho*enol*pyruvate (PEP) and triose-phosphates produced by cytosolic glycolysis, may enter the plastid via specific transporters to feed glycolysis and subsequent fatty acid biosynthesis (Neuhaus and Emes, 2000).

2.2.2. HK feeds starch biosynthesis and the OPPP

Apart from its contribution to glycolysis-derived carbon supply, HK may play a pivotal role in providing G6P to several pathways (Fig. 1). Thus, cytosolic G6P may enter the nonphotosynthetic plastid via a G6P/inorganic phosphate (P_i) translocator (GPT) to feed starch biosynthesis (Neuhaus and Emes, 2000). Alternatively, HK-derived G6P may feed the OPPP by way of its first enzyme, G6P dehydrogenase (G6PDH). The OPPP provides carbon for synthesis of nucleotides, aromatic amino acids and phenylpropanoids, and reductant for nitrogen assimilation and fatty acid synthesis in the plastid (Neuhaus and Emes, 2000). In rapeseed (*Brassica napus*) embryo, for example, the OPPP may produce up to 22% of the reductant needed for plastidic fatty acid synthesis (Schwender et al., 2003). At least part of the OPPP occurs in both the cytosol and the (non)green plastid, as evidenced by coexistent cytosolic, plastidic and chloroplastic G6PDH activities (Kruger and von Schaewen, 2003). Therefore, both cytosolic and stromal HKs may feed G6P to the OPPP and hence, contribute to OPPP-mediated supply of carbon and reductant for plastid metabolism. Several of the plastidic processes cited above rely heavily on ATP supply (Neuhaus and Emes, 2000). In chloroplasts, ATP is produced by photosynthesis. In nonphotosynthetic plastids, a partial glycolytic pathway may allow synthesis of ATP from G6P. However, some of the ATP needed may have to be imported from the cytosol via an ATP/ADP translocator (AATP) located in the inner envelope membrane (Fig. 1) (Neuhaus et al., 1997; Neuhaus and Emes, 2000). In the latter case, the glycolytic or respiratory origin of ATP has not been determined.

2.2.3. HK contributes to the production of NDP-sugars for various pathways

HK may contribute to the supply of NDP-sugars for synthesis of non-cellulosic cell wall polysaccharides, and glycosylation of glycoproteins and small lipophilic molecules (Fig. 1). In support of that view, NDP-sugar levels were diminished in maize root tips following inhibition of a mitochondrial HK (Galina and da-Silva, 2000). HK-derived G6P may be isomerized by phosphoglucomutase (PGM) to G1P, which can in turn be transformed into UDP-Glc by UDP-D-glucose pyrophosphorylase (UGPase). F6P may also feed the UDP-Glc pool after isomerization to G6P by phosphoglucose isomerase (PGI). Similarly, M6P may be isomerized by phosphomannomutase (PMM) to D-mannose-1-phosphate (M1P). The latter is then transformed into GDP-D-mannose (GDP-Man) by GDP-Man pyrophosphorylase (GMPase). GDP-Man is the precursor of L-ascorbate, which has major implications in cell wall synthesis, reactive O_2 species detoxification and, possibly, cell division (Conklin, 2001). Furthermore, UDP-Glc and GDP-Man are the two initial substrates of specific NDP-sugar interconverting enzymes (NIEs) that produce various UDP- and GDP-sugars (Seifert, 2004). Those most likely enter the endomembrane system via specific NDP-sugar transporters (NDPTs) to be used by glycosyltransferases involved in synthesis of glycoproteins and cell wall polysaccharides, e.g. pectins and hemicelluloses (Keegstra and Raikhel, 2001). Alternatively, the NDP-sugars may serve as donor substrates for glycosyltransferases that catalyze glycosylation of all hormones but ethylene, and secondary metabolites like phenylpropanoids or flavonoids (Bowles et al., 2006). These glyco-

Table 1
Kinetic constants of plant hexokinases for their substrates

Substrate	K_m (mM)	References
D-Glc	0.02–0.13	[a, b, c, d, e, f, g, h, i, j, k, l, m]
D-Fru	1.5–30	[a, b, c, d, f, g, h, i, j, k, l, m]
D-Man	0.02–0.5	[a, b, c, f, g, i, j, k, l]
ATP	0.05–0.59	[b, d, e, h, j, k, l]
UTP	1.3–4.5	[d, h, l]
GTP	0.75–2.2	[d, h, l]
CTP	1–3.2	[d, h, l]
TTP	0.23	[l]

References used are as follows: [a] Turner et al., 1977; [b] Turner and Copeland, 1981; [c] Tanner et al., 1983; [d] Renz and Stitt, 1993; [e] Galina et al., 1995; [f] Veramendi et al., 1999; [g] Wiese et al., 1999; [h] da-Silva et al., 2001; [i] Menu et al., 2001; [j] Dai et al., 2002; [k] Giese et al., 2005; [l] Claeyssen et al., 2006; [m] Kandel-Kfir et al., 2006.

syltransferases may be cytosolic or associated with the cytosolic face of compartment membranes (Fig. 1) (Bowles et al., 2006). Another class of glycosyltransferases, localized in the chloroplast envelope membranes, is implicated in synthesis of glycolipids that compose chloroplastic and extraplastidic membranes (Benning and Ohta, 2005). The data presented above suggest that the HK reaction is involved in numerous major aspects of plant physiology by feeding G6P to a variety of metabolic pathways.

2.3. HK isoforms have different biochemical properties that suggest specific roles

2.3.1. Physico-chemical properties

Studies of HK at the protein level have unraveled the complexity of multiple HK isoforms in various plant organs and tissues. Although few of those have been assigned a coding gene, they could be distinguished on the basis of their subcellular localizations, and chromatographic and kinetic properties (Miernyk and Dennis, 1983; Doehlert, 1989; Schnarrenberger, 1990; Renz et al., 1993; Renz and Stitt, 1993; da-Silva et al., 2001). The subcellular localizations of HK isoforms will be discussed in a later section. As for their chromatographic properties, native plant HKs have been assigned molecular masses ranging from 38 to 68 kDa (Higgins and Easterby, 1974; Miernyk and Dennis, 1983; Doehlert, 1989; Renz et al., 1993; Yamamoto et al., 2000). This would correspond to monomers since HK cDNAs encode peptides with molecular masses of 54 kDa (Veramendi et al., 2002; Giese et al., 2005; Claeyssen et al., 2006; Kandel-Kfir et al., 2006). The deviation of M_r data from the value of 54 kDa may then suggest proteolytic degradation or behavior different from that of globular proteins in size exclusion chromatography.

Regarding their kinetic behavior, HKs from a given plant tissue may differ in their affinities for their preferential substrate, Glc (Doehlert, 1989; Renz and Stitt, 1993; da-Silva et al., 2001). The K_m for Glc is generally low and may vary between 20 and 130 μM (Table 1). Affinity for Man is comparable, with a K_m ranging from 20 to 500 μM. By contrast, the K_m for Fru is always in the millimolar range (Table 1). HKs are known to display Michaelis-Menten kinetics with these sugar substrates (Higgins and Easterby, 1974; Doehlert, 1989; Schnarrenberger, 1990; Renz and Stitt, 1993; da-Silva et al., 2001; Menu et al., 2001; Claeyssen et al., 2006). Glc is probably not limiting in leaves, where its cytosolic levels have been estimated at 100-400 μM, in tobacco and other dicotyledons (Leidreiter et al., 1995; Moore et al., 1997). In potato tuber, cytosolic Glc concentrations can reach 30 mM while those of Man and Fru are respectively more than 3 orders of magnitude lower and not detected (Farré et al., 2001). Consequently, HK is likely to phosphorylate Glc almost exclusively in this heterotrophic organ. As for nucleoside triphosphates, in most cases HK has highest affinity for ATP, with a K_m between 50 and 590 μM (Table 1). Cytosolic concentrations above 200 μM have been measured for ATP in potato tuber (Farré et al., 2001), suggesting that ATP is not limiting in heterotrophic cells. By contrast, the K_m(UTP) of HK (Table 1) was much higher than the estimate of UTP levels in the cytosol of potato tuber cells (Farré et al., 2001). Therefore, it seems doubtful that UTP plays a significant role in hexose phosphorylation by HK in heterotrophic cells. Besides substrate affinity, HK sensitivity to pH changes may vary with the plant species or among isoforms of a given tissue. HKs may display broad or narrow response curves to pH, the latter with a pH optimum between 8 and 8.7 (Doehlert, 1989; Renz and Stitt, 1993; Veramendi et al., 1999; Dai et al., 2002; Claeyssen et al., 2006). Selective expression of HK isoforms with contrasting kinetic properties may be a means for the cell to finely tune hexose catabolism depending on substrate and energy levels, or following pH changes. In support of that view, a rise in HK activity was correlated to the requirement for carbon flow through the Glc pool at specific stages of potato tuber development (Renz et al., 1993). In particular, the kinetic properties of the different HK isoforms appeared of prime importance for their regulation *in vivo*, in relation to the physiological status of the tuber (Renz and Stitt, 1993). Additional regulatory properties, such as sensitivity to inhibition by ADP and G6P, are considered below as they may confer unique features to plant HKs.

2.3.2. HK sensitivity to ADP inhibition

HK isoforms from different plant species and tissues (Table 2) are sensitive, to varying degrees, to inhibition by ADP (K_i of 0.03–1 mM). Furthermore, in maize roots, an HK bound to mitochondria or microsomal membranes was highly sensitive to inhibition by ADP whereas cytosolic HK was not (Table 2). ADP competed with neither hexose nor ATP, suggesting a distinct binding site for ADP (Galina et al., 1995). As for soluble HKs partially purified from wheat germ and potato tuber, ADP was non-competitive inhibitor to Glc but acted competitively to ATP with a small non-competitive component (Higgins and Easterby, 1974; Renz and Stitt, 1993). These data may reflect differences between soluble and non-cytosolic HKs regarding the regulatory mechanisms underlying their inhi-

Table 2
Effectors of hexokinase activity

Effector	Species, tissue, HK isoform	K_i or % inhibition with D-Glc as substrate	References
ADP[a]			
	T. aestivum, wheat germ HK	$K_i = 1$ mM	[a]
	P. sativum, seed, HK I and II	50% at ~1 mM	[b, c]
	Z. mays, kernel, HK1 and HK2	45% at 1 mM	[d]
	Z. mays, root, non-cytosolic HK	$K_i = 0.03$ mM	[f, g, i]
	cytosolic HK	No effect	[f, g, i]
	S. lycopersicum, recombinant LeHxk1 to LeHxk4	~50% at 5 mM	[j, k]
	S. tuberosum, tuber, HK1	$K_i = 0.04$ mM	[e]
	HK2	$K_i = 0.11$ mM	[e]
	S. chacoense, recombinant ScHK2	60% at 0.1 mM	[l]
G6P			
	P. sativum, seed, HK I	No effect	[b]
	Z. mays, kernel, HK1 and HK2	Weak inhibition	[d]
	Z. mays, root, non-cytosolic HK	No effect	[f]
	cytosolic HK	No effect	[f]
	S. lycopersicum, recombinant LeHxk2	20% at 5 mM (pH 7)	[j]
	S. tuberosum, tuber, HK1	$K_i = 4$ mM at pH 7	[e]
	HK2	No effect	[e]
	S. chacoense, recombinant ScHK2	No effect (pH 7, pH 8)	[l]
Glucosamine or *N*-acetylglucosamine[b]			
	Z. mays, root, non-cytosolic HK	$K_i = 0.8$ mM	[i]
	cytosolic HK	$K_i = 80$ mM	[i]
	S. lycopersicum, recombinant LeHxk2	20% at 50 mM	[j, k]
	LeHxk3 and LeHxk4	20% at 15 mM	[j, k]
	S. chacoense, recombinant ScHK2	50% at 5 mM	[l]
	S. oleracea, leaf, SoHxK1	$K_i = 50$ mM	[m]
Mannoheptulose[b]			
	Z. mays, root, non-cytosolic HK	$K_i = 0.5$ mM	[h, i]
	cytosolic HK	$K_i = 20$ mM	[h, i]
	S. lycopersicum, recombinant LeHxk2	20% at 50 mM	[j]
D-Trehalose-6-phosphate			
	S. lycopersicum, recombinant LeHxk1 to LeHxk4	No effect	[k]
	S. chacoense, recombinant ScHK2	No effect	[l]
	S. oleracea, leaf, SoHxK1	No effect	[m]
	A. thaliana, leaf, AtHXK1 and AtHXK2	No effect	[n]

HK isoforms were named as in the articles cited. References are as follows: [a] Higgins and Easterby, 1974; [b] Turner et al., 1977; [c] Turner and Copeland, 1981; [d] Doehlert, 1989; [e] Renz and Stitt, 1993; [f] Galina et al., 1995; [g] Galina et al., 1999; [h] Galina and da-Silva, 2000; [i] da-Silva et al., 2001. Note: non-cytosolic HK is associated with mitochondrial, Golgi or microsomal fractions; [j] Menu et al., 2001; [k] Kandel-Kfir et al., 2006; [l] Claeyssen et al., 2006; [m] Wiese et al., 1999; [n] Eastmond et al., 2002.

[a] Possible chelation effects of ADP were taken in account in [a], [c], [d], [e], [f], [g], [i] and [l] to ensure that HK activity inhibition was due to ADP and not to Mg^{2+} chelation.

[b] Glucosamine, *N*-acetylglucosamine and mannoheptulose have been used to demonstrate the implication of *Arabidopsis* HK in sugar sensing (Jang and Sheen, 1994; Jang et al., 1997).

bition by ADP (Galina et al., 1995). Their contrasting sensitivities may have important repercussions on carbohydrate metabolism under energy-limited conditions such as hypoxia or anoxia. During a switch from normoxia to anoxia, there is a rise in ADP levels, e.g. from 30 to 170 μM in maize roots (Hooks et al., 1994), which may inhibit non-cytosolic HK significantly (Galina et al., 1995). Nevertheless, the physiological significance of such preferential inhibition under anoxia remains to be clarified. In addition to ADP, mannoheptulose and glucosamine inhibited non-cytosolic HK activity to a far greater extent than cytosolic HK activity (Table 2). These effects were interpreted as evidence for a role of non-cytosolic HK in hexose and energy charge sensing, whereas cytosolic HK would carry out a catalytic function in glycolysis (Galina and da-Silva, 2000; da-Silva et al., 2001). Although direct evidence is needed, these findings raise the interesting possibility that the regulatory properties and subcellular localizations of HK isoforms may be integrated in their individual roles.

2.3.3. Disparate sensitivities of HK isoforms to G6P inhibition

Plant HKs differ from mammalian HKs with respect to their sensitivities to G6P. Mammalian HKs I, II, and III are highly sensitive to inhibition by G6P (Wilson, 2003). This contrasts with the low sensitivity ($K_i = 4$ mM) or absence thereof, to near-physiological G6P levels for plant HKs (Table 2). Cytosolic concentrations of G6P have indeed been estimated at 6 mM in spinach leaves and

0.3 mM in potato tuber (Winter et al., 1994; Farré et al., 2001). Furthermore, sensitivity to G6P seems to vary between HK isoforms (Table 2). In potato tuber, activity of HK1 was inhibited by G6P whereas HK2 was insensitive (Renz and Stitt, 1993). When inhibition by G6P was observed in tomato and potato tuber, it was pH-sensitive (Table 2). The physiological significance of *in vivo* regulation of certain HK isoforms by G6P remains elusive (Renz and Stitt, 1993). In human (*Homo sapiens*) brain cells, G6P controls the binding of HK I to mitochondria, thereby regulating the relative levels of soluble and membrane-bound forms (Cárdenas et al., 1998). In plants, G6P could solubilize mitochondrion-associated HK from castor bean endosperm, but not those from pea (*Pisum sativum*) stems and leaves (Miernyk and Dennis, 1983; Dry et al., 1983; Tanner et al., 1983). Altogether, these results suggest major differences between plant and mammalian HKs, and among plant HKs, with respect to G6P-mediated regulation.

2.3.4. Plant HKs are insensitive to D-trehalose-6-phosphate

Regulation of plant HKs may also differ substantially from that of yeast HKs, especially with respect to sensitivity to D-trehalose-6-phosphate (T6P), an intermediate of D-trehalose biosynthesis. In the yeast *S. cerevisiae*, mutant studies have implicated T6P in the control of glycolytic flux via inhibition of the most active HK isoform, HK PII (Thevelein and Hohmann, 1995). In plants, T6P has been involved in regulation of major processes such as photosynthesis, carbohydrate utilization for growth, starch synthesis and seed maturation (Paul et al., 2001; Schluepmann et al., 2003; Kolbe et al., 2005; Gomez et al., 2006). By analogy to the yeast model, T6P has been proposed to control plant glycolysis through inhibition of HK (Paul et al., 2001; Schluepmann et al., 2003). However, to date, experimental evidence for inhibition of HK activity by T6P is still lacking (Table 2). Nonetheless, one cannot rule out the possibility that plants contain T6P-sensitive HKs until all members of their gene family have been characterized. The findings presented above suggest that kinetic and regulatory properties and subcellular localizations of HK isoforms may be of prime importance in their individual roles in plants. These observations also apply to mammalian HKs (Wilson, 2003). However, the peculiarity of plant HKs with respect to inhibition by G6P and T6P suggests that their modes of action may be unique among Eukaryotes.

3. On-line molecular data raise intriguing questions about the roles and modes of action of HK isoforms

3.1. HK distribution at the organismic level demonstrates a complex regulation and suggests that HK isoforms have tissue- and stress-specific roles

3.1.1. The Arabidopsis genome expresses six HK genes

The existence of multiple HK isoforms in plants raises questions about their specificities to certain tissues, and their individual roles in cells where they occur. These questions led us to examine expression profiles of HK genes from *Arabidopsis*, for which DNA microarray data are publicly accessible. BLAST searches of the *Arabidopsis* genome (The Arabidopsis Initiative, 2000) were carried out, using *At4g29130* and *At2g19860* nucleotide sequences, and amino acid sequences of their respective protein products AtHXK1 and AtHXK2 (The Institute for Genomic Research website, http://www.tigr.org/tdb/e2k1/ath1/). Four uncharacterized HK-like expressed sequence tags (ESTs) were identified: *At1g47840*, *At1g50460*, *At3g20040* and *At4g37840*. Multiple alignment of the six HK sequences illustrates that they have 66 to 96% sequence homology (Supplementary Fig. 1). These data support the view that the various HK isoforms of one organism have significant sequence homology. A UniProt entry, named T2E6.5 (Q9FZG4), is also available at the Expert Protein Analysis System (ExPASy) website (http://ca.expasy.org/) (Gasteiger et al., 2003). However, this genomic sequence has no corresponding EST. It encompasses *At1g47840* and contains additional regions homologous to HK sequences. These regions are apparently not expressed, based on EST search in the *Arabidopsis* Tiling Array Transcriptome (http://signal.salk.edu/) (Yamada et al., 2003). It is possible that these regions of T2E6.5 have arisen from a partial duplication of an HK sequence.

3.1.2. The expression patterns of the six HK genes of Arabidopsis are not correlated at the entire plant level

The expression profiles of *AtHXK1*, *AtHXK2*, *At1g47840*, *At1g50460*, *At3g20040* and *At4g37840* (Fig. 2) were obtained from two data sets available on-line: the AtGenExpress Consortium Tissue and Stress series (Botany Array Resource, http://bbc.botany.utoronto.ca/) (Toufighi et al., 2005). The six HK genes did not seem to be subject to co-regulation at the whole plant level or in response to abiotic stresses (Fig. 2A and B). At best, *At4g37840* and *AtHXK1* exhibited a moderate correlation in expression patterns in the entire plant, with a Pearson's correlation coefficient of 0.67 (Expression Angler tool, http://bbc.botany.utoronto.ca/). A perfect correlation between two genes would lead to a coefficient of 1. Correlation coefficients were lower for all other HK sequences in the Tissue and Stress data sets (data not shown).

3.1.3. Some HK isoforms may have tissue-specific roles in plants

At1g47840 was highly expressed in root and seed (Fig. 2A), which may reflect a specific role of its protein product in these systems. In the seed, *At1g47840* expression peaked during heart to late torpedo stages (Fig. 2A, seed stages 4–7 described by Schmid et al., 2005). This corresponded to the end of embryo morphogenesis, the first phase of *Arabidopsis* seed development, characterized by cell divisions. The second phase, embryo maturation, consists of cell expansion and accumulation of storage proteins and lipids in the embryo. Transient starch synthesis and

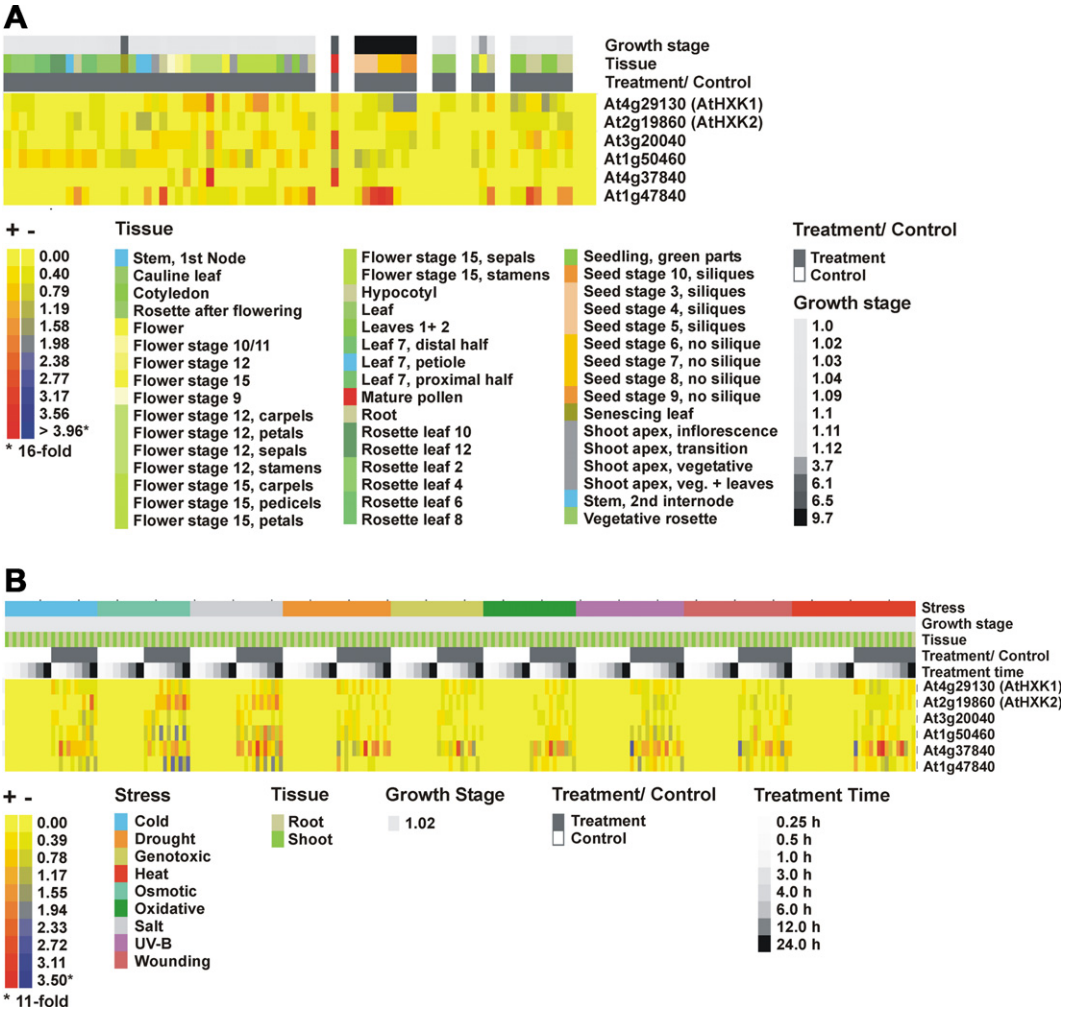

Fig. 2. Expression profiles of the six hexokinase (HK) genes of *Arabidopsis*, in the different plant parts (A) and in response to abiotic stresses (B), using the Expression Browser on Affymetrix ATH1 array data from the AtGenExpress Consortium Tissue and Stress series, respectively (http://bbc.botany.utoronto.ca/). The HK sequences were named after their AGI numbers: *At4g29130* (*AtHXK1*), *At2g19860* (*AtHXK2*), *At3g20040*, *At1g50460*, *At4g37840* and *At1g47840*. Each gene of the AtGenExpress Tissue series was considered as a 'treatment' and compared to a median value ('control') calculated across all wild-type samples in the data set used by Schmid et al. (2005). As for the AtGenExpress Stress series, stress treatments were stored as separate projects, each with a corresponding wild-type for control. The color scale indicates $\log_2$-transformed levels of expression above (red) or below (blue), the control (yellow) (Toufighi et al., 2005). Values are means of three independent expression estimates from triplicate Affymetrix ATH1 arrays (Schmid et al., 2005). Growth stages are depicted in Supplementary Table 2 according to Boyes et al. (2001). Seed stages are described further by Schmid et al. (2005). The reader is referred to the above-cited website for details on procedures for each microarray experiment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

degradation takes place at the transition between these two phases (Baud and Graham, 2006). Cell division during heart to torpedo stages in the *Arabidopsis* seed has been correlated to a high hexose/Suc ratio, which decreased during subsequent cell elongation and lipid storage (Baud et al., 2002). Consequently, the aforementioned statement that a high hexose/Suc ratio favors cell division during sink initiation (Weber et al., 1997), may apply to the *Arabidopsis* seed (Baud et al., 2002). Furthermore, expression of *At1g47840* peaked at these stages (Fig. 2A), suggesting that the At1g47840 protein product may support cell division in some way. However, total HK activity has been shown to rise three to four days later, during the maturation phase (Focks and Benning, 1998; Baud and Graham, 2006). Uncorrelated levels of *At1g47840* transcript and HK activity suggest that some regulation may take place at the post-transcriptional and/or posttranslational levels, and that the catalytic activity of At1g47840 may not be involved in its function. It may then be proposed that At1g47840 supports cell division as a hexose sensor, a function discussed later.

It was observed elsewhere that a peak of HK activity coincided with starch degradation and storage lipid deposition during seed development in *Arabidopsis* (Baud and Graham, 2006). This may suggest an additional role for HK in directing carbon flow from Suc or transitory starch-derived Glc to lipid synthesis. In support of that view, incorporation of Glc into seed lipids was compromised in the *Arabidopsis* mutant *wrinkled1* that lacked HK activity (Focks and Benning, 1998). Furthermore, the onset of the SuSy path of Suc cleavage during the

maturation phase coincided with a shift from ATP-dependent activities to pyrophosphate (PP_i)-consuming activities (Baud and Graham, 2006). This switch to more energy-efficient reactions is viewed as an adaptive response to the low O_2 tensions that limit ATP production in developing oilseeds (Rolletschek et al., 2005; Baud and Graham, 2006). Nonetheless, HK activity displayed the same temporal pattern as SuSy activity rather than decreasing like the other ATP-dependent enzyme activities measured (Baud and Graham, 2006). Thus, the data may indicate an absolute requirement for HK in storage lipid deposition. Only the *AtHXK2* gene was slightly induced during the rise in total HK activity, the other HK genes were unaffected or even repressed, like *AtHXK1* (Fig. 2A; Baud and Graham, 2006). Again, the results strongly suggest posttranscriptional and/or posttranslational regulation of HK activity during seed development. Moreover, the question as to which HK isoform(s) may contribute G6P for seed lipid synthesis remains unresolved. The *Wrinkled1* gene has been found to code for a transcription factor, WRI1, which regulates expression of some glycolytic genes (Cernac and Benning, 2004). WRI1 is likely to impact on HK activity since the latter was abolished in the *wrinkled1* mutant (Focks and Benning, 1998). However, mRNA levels of the six HK genes were similar in wild-type and *wrinkled1* seeds, suggesting that WRI1 does not modulate HK gene transcription (Ruuska et al., 2002). Collectively, these findings suggest that HK is subject to posttranscriptional and/or posttranslational regulation in the seed, but the regulatory mechanisms involved remain unknown.

In Fig. 2A, *At3g20040*, *AtHXK1* and *At4g37840* were expressed at highest levels in stamen and pollen grain whereas *At1g50460* was strongly repressed. This suggests specific roles for At3g20040, AtHXK1 and At4g37840 gene products in male reproductive tissues. In particular, pollen development relies on Suc import from the apoplast and its hydrolysis by cwINV (Goetz et al., 2001). It may, therefore, be proposed that meiosis, or starch or lipid reserve synthesis during subsequent pollen maturation (Clément et al., 1994), relies on utilization by HK of hexoses arising from Suc hydrolysis. By contrast, all six ESTs were expressed at rather basal levels in other plant parts (Fig. 2A). Similar observations have been made recently with rice (*Oryza sativa*) plants, which expressed one pollen-specific and eight ubiquitous HK genes (Cho et al., 2006). In tomato, also, *LeHXK2* and *LeHXK3* were found at low levels in flower and in root and apex, respectively, whereas *LeHXK1* and *LeHXK4* were ubiquitously and highly expressed (Kandel-Kfir et al., 2006). However, gene expression constitutes only one of several levels at which regulation can occur, as exemplified by HK gene and activity data from the developing seed. Unfortunately, HK regulation at the protein and activity levels and the mechanisms involved, have been largely ignored in plants. Above all, the link has often been missing between HK genes and their protein products and corresponding activities for the vast majority of plant organs investigated. It is thus conceivable that HK activity in some plant organs may result from a single or limited number of isoforms. In support of that view, a stromal HK (GenBank AY260967) was shown to be the main Glc-phosphorylating enzyme in the moss *Physcomitrella patens*, accounting for 80% of total activity in protonemal tissue (Olsson et al., 2003). A similar organ/tissue preponderance has been documented in other eukaryotic organisms. In rat (*Rattus norvegicus*), HK I is expressed at high levels in brain, whereas HK II predominates in muscle, and HK IV in hepatocytes and pancreatic β-islets. It was noticed that their kinetic properties made these HK isoforms particularly suited to their presumed roles in their respective locations (Cárdenas et al., 1998). In plant HK research, the transcriptomic data need to be complemented with activity profiles in order to determine which HK isoforms contribute to total HK activity. Examination of their kinetic properties may then help to elucidate their individual roles.

3.1.4. Some HK isoforms may have stress-specific roles in plants

As for responses to abiotic stresses, ESTs *At1g47840*, *At1g50460*, *AtHXK2* and *At4g37840* clearly exhibited responsive expression patterns (Fig. 2B). The *At4g37840* gene was the most responsive in all stress treatments: genotoxic stress by bleomycin and mytomycin, cold, drought, heat, salt, UV-B, wounding, osmotic and oxidative stress. Its expression peaked within the first 3 h of treatment, sometimes followed by repression compared to the control. Most pronounced effects on expression of *At4g37840* were observed under heat, drought, salt and UV-B stress, with generally greater amplitude in the root than in the shoot (Fig. 2B). In response to cold, osmotic and salt stress, *At1g47840* and *At1g50460* were consistently repressed in the root and in the shoot, respectively, and as early as 1 h of treatment. On the contrary, *AtHXK2* was clearly induced in shoot and root within 3 h of osmotic and salt stress (Fig. 2B). Accordingly, *AtHXK2* was found to be induced 2–3-fold under cold, osmotic and salt stress in published microarray experiments (Kreps et al., 2002). In addition, significant induction of *AtHXK2* by chilling was observed in wild-types but not in chilling-lethal mutants, suggesting a role for AtHXK2 in acclimation to suboptimal temperatures (Provart et al., 2003). Hypoxia is another stress that significantly induced *AtHXK2*, together with *At1g47840* (Liu et al., 2005; Loreti et al., 2005). On the contrary, *At4g37840* was dramatically repressed (Loreti et al., 2005). It is worth noting that HK helped sustain high glycolytic activity in maize root tips during hypoxic acclimation, thereby improving their survival under low O_2 tensions (Bouny and Saglio, 1996). Taken together, these data suggest that the HK isoforms encoded by the *At1g47840*, *At1g50460*, *AtHXK2* and *At4g37840* genes may be implicated in specific stress responses. Unfortunately, HK is not often mentioned in proteome profiling studies. This may not contradict the data cited above, but rather point to HK as a low abundance protein not eas-

ily detectable in complex protein mixtures. It should then be made clear that variations in activity of a protein that escapes detection in large profiling studies, may still impact dramatically on metabolism.

3.2. HK distribution at the subcellular level

3.2.1. HKs can be soluble in the cytosol or associated with organelles

It has already been mentioned that HK isoforms could be distinguished on the basis of their subcellular localizations. Several studies have shown that a given tissue could contain HK isoforms localized to different cell compartments. In maize roots, active HK isoforms have been found in cytosolic, Golgi and mitochondrial fractions (da-Silva et al., 2001). Cytosolic, mitochondrial and plastidic HK isoforms have been characterized in developing castor oil seeds (Miernyk and Dennis, 1983). Soluble and mitochondrial HKs have also been partially purified from pea and spinach leaves (Baldus et al., 1981; Schnarrenberger, 1990). In pea roots, plastid-associated HK accounted for 16% of total HK activity (Borchert et al., 1993), suggesting the presence of other HK isoforms in other cell compartments. The possibility of cross-contaminations between purified fractions cannot be dismissed for these studies. Nevertheless, several HKs identified by their coding gene, have been localized *in situ* and could be distinguished from HK activities in other cell compartments. Two HK isoforms, At1g50460 and AtHXK2 (At2g19860), have been identified on the outer mitochondrial membrane of *Arabidopsis* suspension cells (Giegé et al., 2003). AtHXK1 (At4g29130) transfected in maize protoplasts has been found in nuclear fractions (Yanagisawa et al., 2003), thus suggesting different subcellular locations for the different HK isoforms of *Arabidopsis*. In spinach leaves, the SoHxK1 isoform anchored in the chloroplastic outer envelope membrane was distinct from soluble HK activity, based on immunodetection evidence (Wiese et al., 1999). An HK has been localized in the plastid stroma in various tissues of the moss *Physcomitrella patens*, tobacco, tomato and rice. Other HK isoforms were identified, or their activity measured, in other cell compartments in these cases

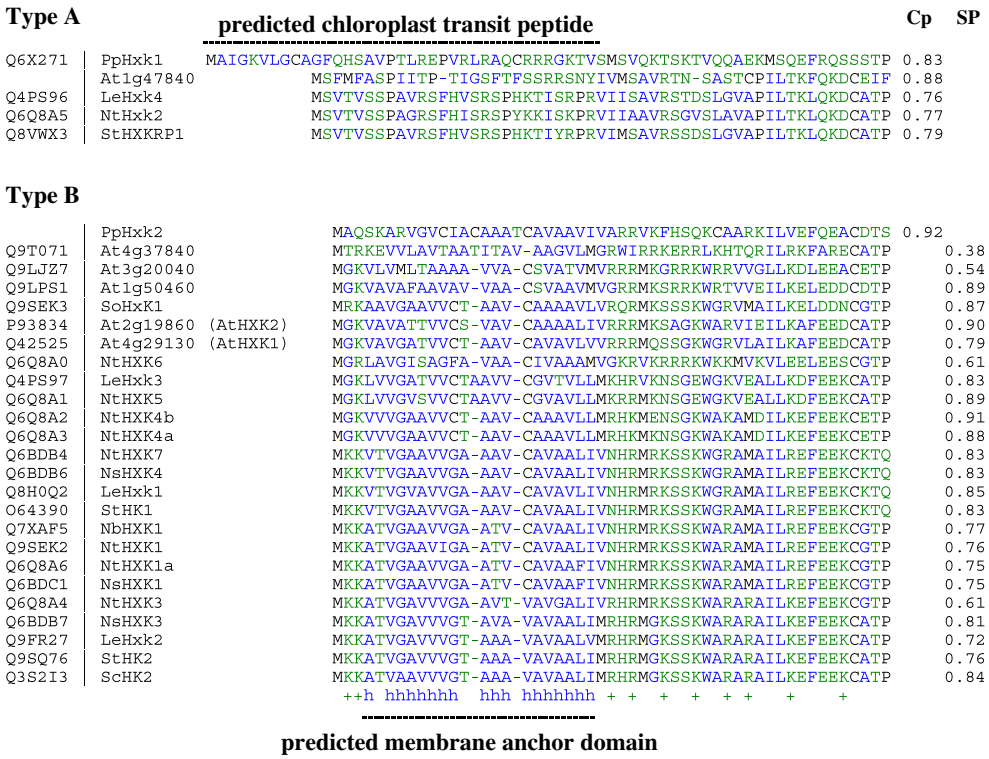

Fig. 3. Multiple alignment of N-terminal sequences of hexokinases from five plant species, and their predicted targeting to the chloroplast (Cp) or to the secretory pathway (SP), using the TargetP 1.1 program. Type A is hexokinases (HKs) with predicted chloroplast transit peptides, using the ChloroP 1.1 program. Type B is HKs with N-terminal membrane anchors, using the SignalP 1.1 program. All programs are available at the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/) (Emanuelsson et al., 2000). Hydrophobic (h) amino acids are represented in blue, polar, positive or basic (+) amino acids are in green, others are in black. UniProt entries of HKs are indicated on the left, followed by their AGI numbers or trivial names, some of which were taken from the following references: AtHXK1 and AtHXK2 (Jang et al., 1997), LeHxk1 to LeHxk4 (Kandel-Kfir et al., 2006), NtHxk2 (Giese et al., 2005), PpHxk1 and PpHxk2 (Olsson et al., 2003), ScHK2 (Claeyssen et al., 2006), SoHxK1 (Wiese et al., 1999), StHK1 and StHK2 (Veramendi et al., 1999; Veramendi et al., 2002). TargetP scores and predictions are shown on the right. Abbreviations indicate the species: At, *Arabidopsis thaliana*; Le, *Lycopersicon esculentum* (*Solanum lycopersicum*); Nb, *Nicotiana benthamiana*; Ns, *Nicotiana sylvestris*; Nt, *Nicotiana tabacum*; Pp, *Physcomitrella patens*; Sc, *Solanum chacoense*; So, *Spinacia oleracea*; St, *Solanum tuberosum*. The N-terminal sequence of PpHxk2 was taken from Olsson et al. (2003), that of StHK1 from Menu et al. (2001). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Olsson et al., 2003; Giese et al., 2005; Kandel-Kfir et al., 2006; Damari-Weissler et al., 2006; Cho et al., 2006).

3.2.2. Importance of the N-terminus in HK targeting

The N-terminus of several plant HKs has been implicated in their targeting to specific cell compartments (Wiese et al., 1999; Olsson et al., 2003; Giese et al., 2005; Claeyssen et al., 2006). This has led us to examine the N-terminal sequences of HKs from *Arabidopsis*, several *Solanaceae*, spinach and the moss *Physcomitrella* (Fig. 3). In each species, one HK shared sequence homology with the stromal HK from *Physcomitrella*, PpHxk1, thus constituting the type A group according to Olsson et al. (2003). These HKs were predicted to contain a transit peptide and to be targeted to the chloroplast, using the ChloroP 1.1 and TargetP 1.1 programs available at the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/) (Emanuelsson et al., 2000). The stromal localization of PpHxk1, NtHxk2 and LeHxk4 has been confirmed experimentally (Olsson et al., 2003; Giegé et al., 2003; Kandel-Kfir et al., 2006; Damari-Weissler et al., 2006). Using the SignalP 1.1 program from the same website, the N-termini of type B were predicted to contain a signal peptide absent from animal and yeast HKs (Fig. 3) (Olsson et al., 2003). Most were predicted to be targeted to the secretory pathway except PpHxk2, which differed significantly in sequence and was predicted to be targeted to the chloroplast (Fig. 3). Some type B HKs have been shown to be anchored in, or associated with, membranes (Wiese et al., 1999; Giegé et al., 2003; Kandel-Kfir et al., 2006; Damari-Weissler et al., 2006). Some contain a prokaryotic attachment site in their N-terminus that may play a role in interactions with membranes (Giegé et al., 2003). Also, type B HKs typically display electropositive surfaces near their membrane anchor domain (Fig. 3) that may enhance their association with negatively charged membrane components (Kandel-Kfir et al., 2006). Lastly, SoHxK1 was inserted into the outer chloroplast membrane via its N-terminal membrane anchor, without need for proteinaceous receptors or ATP (Wiese et al., 1999). Despite these clues, however, the mechanisms of insertion/binding to membranes are still unclear.

Interestingly, different subcellular localizations have been reported for type B HKs, sometimes for a same HK isoform. We recently characterized a putative plasma membrane-anchored HK (Fig. 1) from *Solanum chacoense*, ScHK2 (Claeyssen et al., 2006). In tomato, LeHxk1, LeHxk2 and LeHxk3 have been associated with mitochondrial membranes (Kandel-Kfir et al., 2006; Damari-Weissler et al., 2006). In *Arabidopsis*, At1g50460, AtHXK1 (At4g29130) and AtHXK2 (At2g19860) have been associated with the mitochondrion (Giegé et al., 2003; Rolland et al., 2006). However, AtHXK1 has also been found in the nucleus (Yanagisawa et al., 2003). As for spinach, SoHxK1 was localized not only in the outer chloroplast membrane but also in mitochondrial fractions, as shown by HK activity assay and immunodetection (Wiese et al., 1999). It is not clear whether or not SoHxK1 was present in a peak of HK activity that co-fractionated with that of a Golgi marker after isopycnic sucrose gradient centrifugation (Wiese et al., 1999). If so, this would suggest a localization of SoHxK1 in endomembranes. It is thus possible that plant HKs are subject to dual targeting and/or translocation events. Dual targeting of proteins to mitochondria and chloroplasts has been documented in several instances in plants (Peeters and Small, 2001; Cleary et al., 2002; Duchêne et al., 2005). Also, the protein disulfide isomerase RB60 was targeted to the chloroplast and the endoplasmic reticulum in the green alga *Chlamydomonas reinhardtii* (Levitan et al., 2005). Several dual-targeted proteins have been found to contain two targeting signals in their N-terminus, either in tandem or overlapping (Chabregas et al., 2001; Chew et al., 2003; Levitan et al., 2005). In a similar manner, the N-terminus of type B HKs could have dual-targeting properties not recognized by prediction programs like TargetP 1.1. However, this hypothesis needs to be tested experimentally.

3.2.3. Are some HKs translocated between cell compartments?

Another possible cause for such diverse locations of type B HKs may be their translocation between different membrane systems. In mammals, translocation of some HK isoforms may have functional significance, as well as their binding to actin microfilaments (Pedley et al., 1993; Murata et al., 1997). In *Arabidopsis*, one may ask whether AtHXK1 is translocated between the mitochondrion and the nucleus, and how this may relate to its function in Glc signaling (Rolland et al., 2006). If AtHXK1 is translocated, its binding to unknown molecules may be required as no nuclear targeting motif has been found in its gene sequence (ScanProsite tool, http://ca.expasy.org/). A good candidate may then be the actin cytoskeleton, which has been involved in the function of AtHXK1 in Glc signaling (http://www.arabidopsis.org/servlets/TairObject?type=publication&id=501716579). Accordingly, actin bundles have been proposed to function in protein import from the cytoplasm into the plant nucleus (Collings et al., 2000). Furthermore, AtHXK1 is found in a high-molecular mass protein complex in the nucleus (Fig. 1) (Rolland et al., 2006), which may also be involved in regulation and/or translocation of AtHXK1. Therefore, study of interactions of AtHXK1 with the actin cytoskeleton and the nuclear protein complex may help determine whether translocation is part of its mode of action.

On the question of translocation of type B HKs, their possible association with stromules (stroma-filled tubular extensions of the plastid envelope) also deserves some consideration. In roots, plastids are abundantly interconnected by stromules (Kwok and Hanson, 2004). They have also been found to connect plastids with the nucleus or the plasma membrane in non-green tissues of *Arabidopsis* and tobacco (Kwok and Hanson, 2004). Stromules have been proposed to facilitate coordination of plastid activi-

ties by allowing the transport of stromal molecules between plastids (Köhler et al., 1997). Notably, the stromal HK LeHxk4 has been localized in stromules (Kandel-Kfir et al., 2006). It would be interesting to examine whether this bridge between organelles may also allow for trafficking of their membrane components.

Lastly, the exchange of membrane material between plant organelles may be more widespread a phenomenon than usually thought. Some domains of the ER have been observed in close contact with the mitochondrion, the plasma membrane and the plastid (Staehelin, 1997; Benning et al., 2006). There has been evidence to show or strongly suggest an exchange of membrane lipids at such apposition sites between the ER and these organelles (Staehelin, 1997; Benning et al., 2006). Furthermore, some unicellular algae contain complex plastids with four membranes, the outermost being contiguous with the ER (Nassoury and Morse, 2005). Some preproteins targeted to these plastids are inserted into the ER membrane before being translocated to the plastid via vesicles of the secretory pathway (Nassoury and Morse, 2005). Therefore, it is not inconceivable that membrane-associated HKs may be exchanged between organelles according to similar mechanisms, or between organelles that are connected via stromules. Much remains to be discovered about the mechanisms underlying HK targeting to specific cell compartments or their possible translocation in plant cells. Their elucidation, together with detailed localization of all HK isoforms of a cell, will certainly help unravel their individual roles and modes of action.

4. Functions of plant HKs

4.1. Catalytic function in hexose metabolism

4.1.1. Evidence for a crucial role in regulation of primary metabolism

HK is best known as a glycolytic enzyme, therefore its catalytic function will be discussed first. The central role of glycolysis in plant metabolism has been an incentive for elucidating its regulation. However, its compartmentalization and structure as a network of alternative reactions (Plaxton, 1996) make it difficult to assess the contribution of individual enzymes, such as HK, on overall glycolytic flux. In animals, HK has been shown to exert a high level of control over glycolytic flux. Thus, values of flux control coefficients between 0.7 and 1 have been measured in mammalian erythrocytes, liver, heart, insulinoma and muscle cells (Rapoport et al., 1974; Torres et al., 1988; Kashiwaya et al., 1994; Wang and Iynedjian, 1997; Puigjaner et al., 1997; Jannaschk et al., 1999). In plants, however, the control coefficient of HK over glycolytic flux has not been assessed. Nonetheless, indirect evidence may suggest a crucial role for HK in regulation of primary metabolism. HK overexpression in tomato plants impaired growth and photosynthesis, and induced rapid senescence in photosynthetic tissues (Dai et al., 1999). It also resulted in reduced size of tomato fruit and seed, possibly due to an excessively low ATP/ADP ratio for starch synthesis (Menu et al., 2004). One of HK products, G6P, is a potent activator of PEP carboxylase (PEPC) (Plaxton, 1996). It may then be proposed that HK activity could modulate flux through PEPC, an enzyme involved in the replenishment of the tricarboxylic acid cycle intermediates. By the same token, PEPC activity raises the P_i/PEP ratio, which activates ATP-dependent phosphofructokinase (PFK) and raises overall glycolytic flux (Plaxton, 1996). Another possible regulatory mechanism is the implication of HK in Suc and Glc/Glc-P cycles that may increase energy demand, thus contributing to elevated glycolytic rate (Fernie et al., 2002a; Urbanczyk-Wochniak et al., 2003; Alonso et al., 2005). Interestingly, G6P and, to a lesser extent, M6P and F6P, inhibit the activity of a SNF1-related protein kinase 1 (SnRK1) in spinach (Toroser et al., 2000). SnRK1s are thought to function broadly in carbon partitioning and hexose signaling through regulation of enzyme activity and gene expression (Rolland et al., 2006). In particular, two spinach SnRK1s have been found to phosphorylate SPS and nitrate reductase (NR) (Sugden et al., 1999). Since SPS participates in Suc cycling (Nguyen-Quoc and Foyer, 2001), inhibition of SnRK1 by G6P may result in enhanced Suc cycling activity. It follows that G6P accumulation may facilitate its own catabolism through the glycolytic pathway by acting indirectly on both PEPC and SPS activities. G6P may also alleviate SnRK1-mediated inhibition of NR activity, thereby promoting nitrogen assimilation. Taken together, these results suggest that HK-derived G6P may help sustain glycolytic rate and activate both NR and PEPC, thereby coordinating carbon and nitrogen supplies for amino acid synthesis.

4.1.2. Integration of location and function for HK isoforms

As suggested earlier, the subcellular localizations of HK isoforms may relate to their individual roles. Putatively plasma membrane-anchored ScHK2 from *S. chacoense* has been proposed to facilitate hexose import into sink cells by maintaining a gradient of concentration across the plasma membrane (Claeyssen et al., 2006). ScHK2 activity was highly sensitive to inhibition by ADP (Table 2), suggesting that hexose import may rely on adequate energy charge for further metabolism into glycolysis (Claeyssen et al., 2006). In spinach leaves, outer chloroplast membrane-anchored SoHxK1 has been proposed to energize Glc that exits the chloroplast during transitory starch degradation. SoHxK1 would help maintain a gradient of Glc across the chloroplast envelope, thereby facilitating its export (Wiese et al., 1999; Weber et al., 2000). Plants may also be equipped with a stromal HK that generates G6P following degradation of transitory starch to Glc (Giese et al., 2005). The resulting accumulation of G6P in the stroma of guard cells may contribute to increasing turgor for stomatal opening (Ritte and Raschke, 2003). In

principle, adjusting the relative activities of cytoplasmic and stromal HKs may be a means for the cell to regulate Glc traffic across the plastid envelope (Olsson et al., 2003).

The localization of HK isoforms may be key to their individual roles by allowing privileged access to ATP supply. Thus, the chloroplast envelope-anchored HK has been claimed to use preferentially chloroplastic ATP in spinach leaf cells (Stitt et al., 1978; Wiese et al., 1999). Similarly, plant HK bound to the mitochondrial membrane has been proposed to gain facilitated access to mitochondrial ATP, while efficiently recycling ADP to sustain oxidative phosphorylation (Dry et al., 1983; Galina and da-Silva, 2000; Yamamoto et al., 2000). This is well established for mammalian cells with high energy needs, e.g. brain or cancer cells, where HK I bound to actively-respiring mitochondria is known to use intramitochondrial rather than cytosolic ATP as substrate (Wilson, 2003). Such substrate specificity of HK I is thought to facilitate coordination between glycolytic and respiratory activities, thus adapting the overall rate of Glc metabolism to energy demand in mammalian cells (Wilson, 2003). HK I binds to the voltage-dependent anion channel (VDAC) and to an adenylate translocator at contact sites between outer and inner mitochondrial membranes, in a G6P-sensitive manner (Wilson, 2003). Consequently, the rate of G6P utilization in mammalian cells may impact on glycolytic and respiratory rates by governing, at least partly, the extent to which HK I is bound to mitochondria (Wilson, 2003). In plants, the presence of an intact glycolytic sequence on the cytosolic face of mitochondria may ensure a direct supply of pyruvate to support mitochondrial respiration (Giegé et al., 2003). Therefore, there may be some coordination between cytoplasmic glycolysis and mitochondrial respiration in plant systems. However, we mentioned previously that HK binding to the mitochondrion was not always sensitive to G6P in plants (Dry et al., 1983; Tanner et al., 1983). The mechanisms involved thus require further investigation as they may differ greatly from those in mammals. Notably, sensitivity of plant HK isoforms to ADP inhibition may be of prime importance for their individual roles. In contrast with the cytosolic isoform, mitochondrial membrane-bound HK has proven highly sensitive to ADP inhibition in maize roots (Galina et al., 1995; Galina and da-Silva, 2000). Blockage of NDP-sugar formation by ADP has led to the proposal that this isoform feeds G6P to energy-demanding pathways only under an adequate ATP/ADP ratio, thus acting as an energy charge sensor in plant cells (Galina and da-Silva, 2000). Under a low ATP/ADP ratio, ATP use may be restricted to cytosolic HK for cell metabolic maintenance as this HK activity is less affected by ADP inhibition (Galina and da-Silva, 2000). Upon return to a sufficiently high ATP/ADP ratio, putative plasma membrane-anchored HK may resume hexose import and commitment to glycolysis (Claeyssen et al., 2006). Mitochondrial HK may also be relieved from ADP inhibition, resuming production of G6P for energy-costly biosyntheses of NDP-sugars and cell wall polysaccharides (Galina and da-Silva, 2000). These considerations also raise intriguing questions about which HK isoforms may contribute to Suc and Glc/Glc-P cycles. The recruitment of ADP-sensitive HK isoforms may lead to self-regulatory substrate cycles as their impact on the ATP/ADP ratio of the cell may feedback regulate these same isoforms. Elucidation of the regulatory mechanisms underlying Suc and Glc/Glc-P cycles may, therefore, reveal additional complexity in HK contribution to these substrate cycles and, possibly, to glycolysis regulation in plants.

4.1.3. Implications of HK sensitivity to ADP in stress responses

Sensitivity of some HK isoforms to inhibition by ADP may impact greatly on metabolism of plant cells under O_2 deprivation. Seeds, bulky organs such as potato tuber, or roots of flooded plants experience low ATP/ADP ratios as they become hypoxic, i.e. as respiration is limited by low O_2 tensions (Drew, 1997; Geigenberger, 2003). Plant cells under hypoxia are also characterized by a drop of cytoplasmic pH from 7.5 to 6.8, which compromises their survival (Gout et al., 2001). Cytoplasmic acidosis and a low ATP/ADP ratio have been claimed to diminish activities of some HK isoforms in hypoxic tissues (Renz and Stitt, 1993; Galina et al., 1995; Bouny and Saglio, 1996; Claeyssen et al., 2006). Thus, inhibition of putative plasma membrane-anchored HK may impede hexose import from the extracellular space under hypoxic stress (Claeyssen et al., 2006). Also, production of G6P for energy-costly pathways may be shut down due to ADP inhibition of mitochondrial HK in hypoxic maize root tips (Galina and da-Silva, 2000). Such impairments may provide a basis for the identification of HK as a major limiting step of glycolysis in maize root tips deprived of O_2 (Bouny and Saglio, 1996). The maintenance of glycolytic flux in acclimated root tips, a requirement for their survival under hypoxia (Xia et al., 1995), was attributed to induced HK activity and its reduced inhibition due to higher cytoplasmic pH (Bouny and Saglio, 1996). Similarly, the flood tolerance of some species of the *Echinochloa* genus has been correlated to specific HK isoforms with acidic pH optima under hypoxia (Fox et al., 1998). These results suggest a major part for some HK isoforms in survival of plant tissues under low O_2 environments. A good candidate may be cytosolic, ADP-insensitive HK, which has been proposed to transiently generate ATP and Glc from ADP and G6P in hypoxic maize root tips (Galina et al., 1995). However, this process remains to be demonstrated. Nonetheless, these data on HK activities agree with those from HK gene expression patterns in that HK appears to play a primordial role in the response of plant cells to hypoxic stress. The above findings suggest that HK has evolved to multiple isoforms with specific subcellular localizations and kinetic properties that serve their individual roles in plant metabolism, under normal and stress conditions.

4.2. Function in hexose sensing and signaling

4.2.1. HK is a hexose sensor: evidence and mode of action

Beyond its function in hexose metabolism, HK acts as a hexose sensor in plants (Rolland et al., 2006), thus exhibiting a feature common to higher and lower Eukaryotes (Rolland et al., 2001). Sugars serve as nutrients and structural components, but also as signaling molecules that control metabolism and growth and development throughout the plant life cycle (Rolland et al., 2006). At the molecular level, mutant screenings have revealed extensive connections between sugar and hormone signaling pathways (Leon and Sheen, 2003; Rolland et al., 2006). Sugars also activate or repress genes involved in cell cycle regulation, photosynthesis, carbon and nitrogen metabolism, stress responses, germination, vegetative and reproductive development, and senescence (Koch, 1996; Rolland et al., 2002). HK was first implicated as a hexose sensor in mediating repression of photosynthetic and glyoxylate cycle genes by Glc, Fru, Man and Gal (Graham et al., 1994; Jang and Sheen, 1994). Glc analogs and the HK inhibitor mannoheptulose have been used to show that HK mediated gene repression by hexoses, and the arrest of seed germination by Man (Graham et al., 1994; Jang and Sheen, 1994; Pego et al., 1999). Intermediates of downstream Glc metabolism such as P_i or ATP were ineffective in triggering the signal (Jang and Sheen, 1994; Pego et al., 1999). More direct evidence for a role of AtHXK1 and AtHXK2 in Glc sensing was provided with the characterization of transgenic *Arabidopsis* plants with altered HK levels (Jang et al., 1997). Plants overexpressing AtHXK1 or AtHXK2 exhibited Glc hypersensitivity whereas antisense plants were hyposensitive, based on seedling development and marker gene expression. Interestingly, heterologous expression of the yeast Glc sensor HK PII elevated the catalytic activity but reduced Glc sensitivity in the transgenics, suggesting distinct sensing mechanisms in yeast and plants (Jang et al., 1997).

Compelling evidence that HK indeed fulfills two separate functions in *Arabidopsis* came with the characterization of two *Glc insensitive 2* (*gin2*) mutants carrying a mutation in the *AtHXK1* gene (Moore et al., 2003). One *gin2* mutant displayed broad growth defects that became more pronounced with increased light intensity, i.e. in physiological conditions where Glc signaling was enhanced (Moore et al., 2003). Furthermore, the phosphorylating activity of AtHXK1 was uncoupled from its sensing function through point mutations in its two catalytic domains. The engineered proteins displayed no phosphorylating activity, yet they still mediated Glc responses in gene expression, growth and senescence. Also, these constructs restored growth and gene expression to wild-type levels when expressed in *gin2* plants (Moore et al., 2003). Therefore, it was unambiguously demonstrated that Glc sensing and signaling in plants required AtHXK1 but not hexose-Ps or other downstream metabolic products. Moreover, Glc sensing by AtHXK1 was not dependent on its catalytic activity *per se*, thus proving that HK exhibits two distinct functions in hexose metabolism and sensing in plants (Moore et al., 2003). Lastly, the AtHXK1-dependent Glc signaling was shown to interact with auxin and cytokinin signaling pathways. Therefore, HK is a Glc sensor that integrates environmental, nutritional and hormonal cues in the signaling network that governs growth and development (Moore et al., 2003). The broad spectrum of Glc responses affected in *gin2* mutants, including gene expression, cell proliferation, vegetative and reproductive development, reproduction, and senescence, has confirmed the central role of HK-dependent signaling in plant life (Moore et al., 2003). However, much remains to be discovered about the molecular mechanisms underlying hexose sensing and signaling by HK. First, how HK transmits the signal is unclear. It has been suggested that HK may undergo a change in conformation or oligomeric state upon Glc binding, thereby triggering a signal transduction cascade (Frommer et al., 2003; Moore, 2004). Secondly, it is not known whether the presence of AtHXK1 in both mitochondrial and nuclear fractions is significant to its signaling function. Membrane localization of AtHXK1 has been suggested to be key for its signaling function (Xiao et al., 2000). In maize, inhibition of mitochondrial HK by ADP, mannoheptulose and glucosamine compared to insensitive cytosolic HK, has been taken as evidence for the role of mitochondrial HK in hexose sensing (da-Silva et al., 2001). However, this interesting hypothesis awaits more direct demonstration. Glc has been shown to enhance proteasome-dependent degradation of the transcription factor EIN3 in the nucleus through AtHXK1 signaling (Yanagisawa et al., 2003). This suggests that at least the nuclear localization is important for HK-dependent signaling. Other transcription factors have been implicated in Glc signaling (Rolland et al., 2006), which may constitute additional targets for AtHXK1. In these regards, identification and characterization of the partner molecules that interact with AtHXK1 in the nucleus (Fig. 1) will provide useful clues on its regulatory interactions (Rolland et al., 2006). These investigations may also clarify whether AtHXK1 is translocated from the mitochondrion to the nucleus, or mediates distinct signaling events depending on its subcellular distribution.

4.2.2. Importance of HK in the hexose signaling network

Additional sugar signaling pathways have been unraveled that do not rely on the signaling function of HK. Thus, glycolytic-dependent hexose signaling has been found that involved the catalytic activity of HK and downstream hexose metabolism (Xiao et al., 2000; Lejay et al., 2003). The metabolic role of HK in hexose sensing may help explain the recurrent idea that sugar fluxes into metabolism are more important than their steady-state levels to initiate a response (Koch, 1996; Lalonde et al., 1999; Smeekens, 2000). Furthermore, hexose and Suc responses have been shown to trigger specific signaling pathways that do not involve HK (Chiou and Bush, 1998; Smeekens, 2000;

Xiao et al., 2000; Tiessen et al., 2003). HK-independent Glc sensing and signaling may involve cell-surface receptors as extracellular sensors, such as Regulator of G-protein signaling 1 (RGS1) (Chen and Jones, 2004). Targets and processes downstream of RGS1 await further elucidation (Chen and Jones, 2004). HK-independent signaling of Suc has been shown to involve SnRK1 (Tiessen et al., 2003; Halford et al., 2004), which may interact with T6P at least in some cases (Schluepmann et al., 2004; Kolbe et al., 2005). It should be added that FK and GalK have been proposed as additional intracellular sugar sensors besides HK (Pego and Smeekens, 2000; Rolland et al., 2002; Sherson et al., 2003). In particular, FK has been hypothesized to regulate HK activity, by analogy with the mammalian model (Pego and Smeekens, 2000). These considerations reveal a complex interplay of various sugar sensors and signal transduction pathways, and raise questions about their coordination with HK-dependent signaling. In that perspective, sensitivity of SnRK1 activity to inhibition by G6P or F6P (Toroser et al., 2000) suggests possibilities of cross-talk between glycolysis-dependent and HK-independent signaling pathways. Also, the question is open as to which HK isoforms may be involved in HK- versus glycolysis-dependent signaling, and whether their localization or kinetic properties may play a role in one pathway or the other.

5. Conclusions and perspectives

It is now well established that plant HK fulfills a catalytic function and another in hexose sensing and signaling. The catalytic function of HK occupies a central place in primary metabolism by providing hexose-Ps to glycolysis and other major pathways in several cell compartments (Fig. 1). The level of control of HK on primary metabolism still awaits quantification and hence, determining the flux control coefficient of HK over these pathways will mark a major progress. Of equal importance is the function of HK in hexose sensing and signaling as it integrates environmental and intrinsic cues in the signaling network that controls growth and development (Moore et al., 2003; Rolland et al., 2006). In addition, there may be much left to discover about plant HK if the implication of mammalian HK in apoptosis (Kim and Dang, 2005) proves to be conserved in plants. This may well be the case as mitochondrion-associated NbHXK1 has recently been involved in the control of apoptosis in *Nicotiana benthamiana* cells (Kim et al., 2006). The main future challenge will be to clarify how individual HK isoforms of a plant cell contribute to each function. We are only beginning to understand how the kinetic and regulatory properties of HK isoforms, and their subcellular localizations, may suit them to specific roles. The implication of HK in glycolysis-dependent hexose signaling suggests that a given HK isoform may contribute to hexose metabolism and/or signaling, depending on environmental conditions and developmental stage.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2006.12.001.

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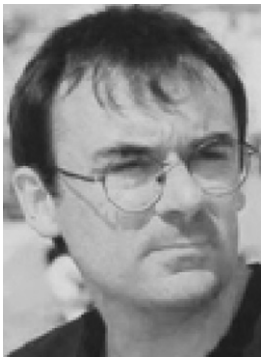

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