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Molecules of Interest

Putting plant hexokinases in their proper place

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ABSTRACT

Hexokinases (HXKs), catalysts of the first essential step in glucose metabolism, have emerged as important enzymes that mediate sugar sensing in many organisms, including plants. The presence of several types of plant HXK isozymes, located in different intracellular locations, has been suggested. However, recent studies have indicated that most plants have only two types of HXKs, a plastidic stromal isozyme and membrane-associated isozymes located mainly adjacent to the mitochondria, but also in the nucleus. The membrane-associated isozymes are involved in sugar sensing and regulate gene expression. The central role of HXKs in plant development and the increasing interest in their role necessitate the correction of inaccuracies that have spread concerning the substrate specificity and intracellular localization of HXK isozymes, as these inaccuracies are affecting the hypothesized roles presented for these isozymes and shaping future research in this active field.

1. Introduction

Hexoses such as glucose and fructose must be phosphorylated to glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) for further metabolism. While fructose can be phosphorylated by either hexokinase (HXK) or fructokinase (FRK), glucose can be phosphorylated only by HXK, as no glucose-specific glucokinase has been found so far in plants (Dai et al., 2002). Furthermore, the affinity of HXK to glucose is two orders of magnitude higher than its affinity to fructose and two orders of magnitude lower than the affinity of FRK to fructose. It is, therefore, likely that HXK phosphorylates mainly glucose *in planta* (Granot, 2007).

G6P is used in several major metabolic pathways: (1) it fuels downstream glycolysis and respiration; (2) it is a key metabolite in sucrose biosynthesis; (3) it is the starting substrate for the oxidative pentose phosphate pathway (OPPP); (4) it may be used for starch biosynthesis; and (5) it is the substrate of trehalose (a glucose–glucose disaccharide) synthesis. Accordingly, HXK, which is necessary for glucose phosphorylation in plants, is destined to be a key player in sugar metabolism.

Theoretical considerations may predict temporal and spatial expression pattern of HXKs in various plants tissues. As photosynthesis is being carried out in source (photosynthetic) tissues, there is presumably no need for HXK, as there is seemingly no production of free glucose (Fig. 1). Atmospheric carbon is incorporated via the Calvin cycle in the chloroplast to yield triose-phosphate (triose-P). Two molecules of triose-P are combined, in the chloroplast or in the cytoplasm, to form fructose 1,6-biphosphate (F1,6BP). F1,6BP is dephosphorylated to form F6P, which may undergo isomerization to yield G6P. Hence, G6P is formed in photosynthetic tissues during the day without any need for HXK. G6P may be converted into glucose 1-phosphate (G1P) and the latter attached to UDP or ADP to form UDP-glucose (UDP-G) or ADP-glucose (ADP-G), respectively. ADP-glucose is used for starch synthesis in the chloroplast, to store extra sugar and to create a pool of reserve carbohydrates. In the cytoplasm, UDP-G and F6P are combined to form sucrose 6-phosphate (Suc-P), which is dephosphorylated to produce sucrose. This sucrose can then be stored in vacuoles or exported out of the photosynthetic (source) tissues to non-photosynthetic (sink) tissues, where it serves as initial substrate for all organic metabolic pathways. Neither of the above described pathways involves free hexoses, glucose and fructose. Hence, apparently, there is no need for HXK in photosynthetic tissues during the day.

In most plants, the starch stored during the day is used during the following night (Zeeman et al., 2007). Starch degradation may yield G1P, glucose or maltose (a glucose–glucose disaccharide), but only glucose and maltose are exported (via specific carriers) to the cytoplasm (Zeeman et al., 2007). The breakdown of maltose in the cytoplasm also yields glucose monomers, and both the plastidic and the cytoplasmic glucose must be phosphorylated by HXK before they can be further metabolized. Accordingly, HXKs might be expected to be found during the dark period in both the chloroplasts and the cytoplasm of photosynthetic cells, to phosphorylate glucose to G6P, which is required for sucrose formation, respiration and other metabolic pathways.

Unlike in photosynthetic (source) tissues, in which HXK might be needed *a priori*, mainly during the dark period, in sink (non-photosynthetic) tissues, HXK might be required during both dark and

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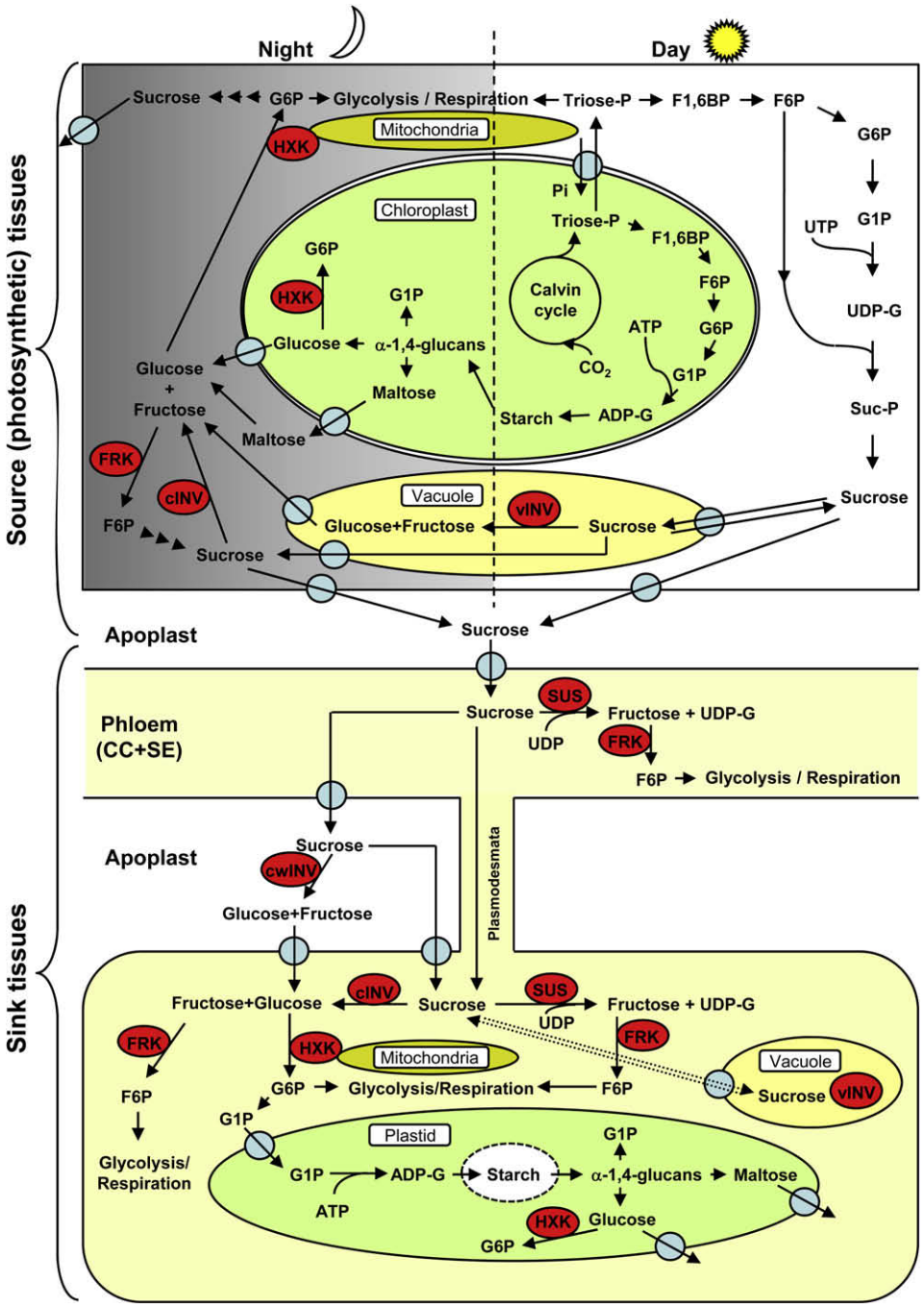

Fig. 1. Schematic presentation of sugar metabolism in source tissues during the day and night and sugar translocation and metabolism in sink tissues. Triose-phosphate (Triose-P), the product of CO_2 fixation in the Calvin cycle, is exported to the cytoplasm. Consecutive cytoplasmic enzymatic steps lead to the formation of G6P independent of HXK. Further metabolism of G6P yields sucrose, which either remains in the cytosol, or is temporarily stored in the vacuole or exported to the apoplast. Within the chloroplast, triose-P is used for the formation of starch during the day. During the dark period, starch is degraded to maltose, glucose and glucose-1-phophate (G1P). Maltose cleavage also releases glucose and vacuolar and cytosolic sucrose might be cleaved by cytosolic (cINV) and vacuolar (vINV) invertases to produce glucose and fructose. While fructose can be phosphorylated by FRK, glucose must be phosphorylated by HXK. Sucrose transported to the apoplast, during the day or night, enters the phloem complex via sucrose transporters. Within the phloem complex, sucrose can be cleaved by sucrose synthase to support phloem metabolism or transported to sink tissues. Sucrose unloading in sink tissues may occur symplasmically via plasmodesmata, or through the apoplast via sucrose transporters. Alternatively, sucrose might be cleaved by apoplastic (cell wall) invertase (cwINV) to produce glucose and fructose that would enter sink cells via specific hexose transporters. The enzymatic steps of sugar metabolism in sink tissues are basically similar to those found in source tissues. Triose-P – triose-phosphate; F1,6BP – fructose 1,6-biphosphate; F6P – fructose 6-phosphate; G6P – glucose 6-phosphate; G1P – glucose 1-phosphate; ATP – adenosine triphosphate; UTP – uridine triphosphate; ADP-G – adenosine diphosphate glucose; UDP-G – uridine diphosphate glucose; Suc-P – sucrose-phosphate; FRK – fructokinase; HXK – hexokinase; cINV – cytosolic invertase; cwINV – cell wall invertase; vINV – vacuolar invertase; SUS – sucrose synthase; CC – companion cells; SE – sieve elements. Blue circles represent transporters.

light periods. Sucrose, a glucose–fructose disaccharide, is the main photoassimilate transported from source to sink tissues in many plants. Some plant families, such as Cucurbitaceae, also translocate raffinose-family oligosaccharides (RFOs), which are galactosyl derivatives of sucrose containing one or more galactose moieties (Keller and Pharr, 1996). However, the metabolism of the RFOs in sink tissues starts with the removal of the galactose moieties, followed by cleavage of the sucrose (Carmi et al., 2003; Gao and

Schaffer, 1999). Hence, cleavage of sucrose is ubiquitous in sink tissues.

Sucrose can be cleaved by cytoplasmic sucrose synthase (SUS) in the presence of UDP to yield UDP-G and fructose, by cytoplasmic or vacuolar invertases (cINV or vINV respectively) to yield intracellular glucose and fructose or by apoplastic – cell wall – invertase (cwINV) to yield extracellular glucose and fructose (Koch, 2004; Roitsch and Gonzalez, 2004). The extracellular glucose and fructose enter the cell via hexose-specific transporters (Sherson et al., 2003). Sink tissues also store starch, whose degradation releases plastidic and cytoplasmic glucose, and glucose may also enter plastids via a plastidic glucose transporter (Butowt et al., 2003). Hence, glucose may exist in the cytoplasm, vacuoles and plastids of sink tissues, awaiting phosphorylation by HXK. Based on the biochemical pathways described above, we would expect to find plant HXK in different intracellular locations and diverse temporal and spatial expression of these enzymes.

2. Identification of HXKs and their substrates

Plant HXKs were first identified in the early 1950s in sink and source tissues of various plants; wheat (*Triticum* spp.) germ, potato (*Solanum tuberosum*) tubers, spinach (*Spinacea oleracea*) leaves, and pea (*Pisum sativum*), mung bean (*Phaseolus aureus*) and oat (*Avena sativa*) seeds (Millerd et al., 1951; Saltman, 1953). The HXKs of these various species were found to be capable of phosphorylating glucose, fructose, mannose and glucosamine, but could not phosphorylate galactose (Saltman, 1953), similar to fungal, mammalian and protozoan HXKs (Cardenas et al., 1984; Fekete et al., 2004; Kroschewski et al., 2000; Otieno et al., 1975; Panneman et al., 1998; Rui and Hahn, 2007; Xu et al., 1995).

In another study, Turner et al. (1977) characterized two HXK fractions from pea seeds that were able to phosphorylate glucose, mannose and fructose, but found no HXK activity involving galactose as a substrate. However, in a further characterization of the second fraction, Turner and Copeland (1981) reported that this fraction had a limited ability to phosphorylate galactose and 2-deoxy-D-glucose (the latter is a non-metabolic substrate). In another report, three distinct fractions containing HXK activity were separated from castor bean (*Ricinus communis*) seeds, and two of these fractions had some low activity with galactose (Miernyk and Dennis, 1983). These two reports (Miernyk and Dennis, 1983; Turner and Copeland, 1981) are the only experimental studies suggesting that HXK may also phosphorylate galactose. Based on these reports, a role for HXKs in the metabolism of galactose released from RFOs (raffinose-family oligosaccharides) has been recently postulated (Claeyssen et al., 2006; Claeyssen and Rivoal, 2007).

However, the presumed product of galactose phosphorylation by HXK, galactose-6P, is not a substrate of enzymes commonly believed to be involved in galactose metabolism in vascular plants, and there is no known enzyme that could directly interconvert galactose-6P and galactose-1P (Keller and Pharr, 1996). Plants have galactokinases (GalK) that specifically catalyze the phosphorylation of galactose at the C-1 position to form galactose-1P (Dey, 1980; Feingold and Avigad, 1980). It is, therefore, unlikely that plant HXKs phosphorylate galactose and compete with GalK for its substrate, as suggested (Claeyssen et al., 2006; Claeyssen and Rivoal, 2007). The low levels of galactose phosphorylation activity reported by Turner and Copeland (1981) and Miernyk and Dennis (1983) were observed in fractions prepared from seeds that had high levels of galactose and high galactokinase activity (Crawshaw and Reid, 1984; Dey, 1983) and these findings could be the result of the presence of a small amount of real galactokinase in these fractions. Hence, it is most likely that HXKs do not play a role in the metabolism of galactose, neither in sink nor in source tissues.

3. Intracellular localization of HXKs

The intracellular localization of HXK isozymes is currently the subject of intense examination. The first report concerning the localization of plant HXK came from the pioneering study of Millerd et al. (1951) in which cytoplasmic particles of mung bean were isolated and HXK activity was found to be associated with those particles identified as mitochondria. Saltman (1953) found later that HXK is distributed between the soluble cell fraction and the insoluble fraction associated with the mitochondria, but the distribution of the soluble and insoluble fractions is dependent upon the sample preparation method. Thus, for example, when potato tubers were ground according to the procedure found to be optimal for the preparation of intact plant mitochondria, all observed HXK activity was associated with the insoluble mitochondria particles. Similarly, in the case of wheat germ, the proportion of soluble to particle-bound HXK was found to depend on how the tissue was treated prior to homogenization (Saltman, 1953). The results of these studies in potato and wheat suggest that the majority of HXK activity may be associated *in vivo* with the mitochondria. Furthermore, the biochemical characteristics of the soluble and the insoluble enzymes were similar and Saltman (1953) suggested that "it is only inability to prepare the enzyme in a sufficiently delicate fashion that results in its solubilization".

Similar results were reported in a study in tobacco (*Nicotiana* spp.), in which more than 80% of the total HXK activity of leaf tissue was found to be associated with the mitochondria-containing, particulate fraction and the ratio of the particulate: soluble HXK fraction was influenced by the choice of extraction medium (Sindelarova and Sindelar, 1988). Studies of HXK in pea stems and leaves have shown that practically all of the HXK activity in these tissues is associated with the outer membrane of the mitochondria; these studies reported no evidence of HXK activity in the cytosol (Cosio and Bustamante, 1984; Dry et al., 1983; Tanner et al., 1983). However, additional studies have identified plant HXKs in the soluble fractions of spinach and pea leaves (Baldus et al., 1981; Schnarrenberger, 1990), developing castor bean seeds (Miernyk and Dennis, 1983), soybean (*Glycine max*) nodules (Copeland and Morell, 1985) and maize (*Zea mays*) roots (Galina et al., 1995). Following the publication of these studies, the theory that plant HXKs are not only associated with the mitochondria, but also exist in the cytosol, became widely accepted (Claeyssen and Rivoal, 2007).

With respect to plastids, Stitt et al. (1978) reported that HXK was absent from the stroma of pea chloroplasts, and suggested that HXK might be associated with the external surface of the chloroplast envelope. However, Miernyk and Dennis (1983) did identify an HXK isozyme in the plastidic stroma of developing castor bean seeds.

The major breakthrough concerning the intracellular localization of plant HXKs was achieved with the cloning of HXK genes, starting in the mid-Nineties (Dai et al., 1995; Jang et al., 1997). Soon after, additional HXK genes were isolated and it became evident that various plant species might have up to 10 HXK genes. For example, Arabidopsis (*Arabidopsis thaliana*) has three HXK genes (Karve et al., 2008), four HXK genes have been characterized in tomato (*Solanum lycopersicon*; Granot, 2007) and 10 HXK genes have been characterized in rice (*Oryza sativa*; Cho et al., 2006a). With the sequence of HXK genes on hand, it became possible to look for signal peptides that may indicate the intracellular localization of specific HXK isozymes and to verify their localization using tagged proteins.

Based on their N-terminal amino acid sequences, plant HXK genes have been classified into two major groups, type A and type B (Olsson et al., 2003). Type A HXKs each have a chloroplast transit

peptide of about 30 amino acids, whereas type B HXKs share a common N-terminal hydrophobic membrane anchor domain of about 24 amino acids and are probably associated with membranes. Studies with GFP fusion proteins have localized type A HXK isozymes of moss (*Physcomitrella patens*), tobacco, tomato, rice and Arabidopsis within plastid stroma (Cho et al., 2006a; Giese et al., 2005; Kandel-Kfir et al., 2006; Karve et al., 2008; Olsson et al., 2003). The tomato plastidic HXK (LeHXK4) has also been observed within stromules, which are stroma-filled tubular extensions of the plastid envelope that connect plastids and allow the transport of proteins between plastids (Kandel-Kfir et al., 2006; Kohler et al., 1997).

The intracellular locations of type B HXK isozymes have been determined by several means. Proteomic analysis of mitochondrial proteins has located Arabidopsis AtHXK1 and AtHXK2 on the outside of the mitochondrial membrane (Giege et al., 2003; Heazlewood et al., 2004). The use of GFP fusion proteins has shown that Arabidopsis AtHXK1 and AtHXK2, tomato LeHXK1,2&3, and *Nicotiana benthamiana* NbHXK1 are associated with mitochondria (Balasubramanian et al., 2007; Damari-Weissler et al., 2006; Damari-Weissler et al., 2007; Kandel-Kfir et al., 2006; Karve et al., 2008; Kim et al., 2006). Deletion of the putative N-terminal membrane anchor domains of the Arabidopsis AtHXK1, tomato LeHXK1,2&3, and the *N. benthamiana* NbHXK1 proteins resulted in their localization to the cytosol (Balasubramanian et al., 2007; Damari-Weissler et al., 2006; Kim et al., 2006). Lastly, fusion of the Arabidopsis AtHXK1 N-terminal domain to GFP was sufficient to cause mitochondrial association (Balasubramanian et al., 2007), indicating that the N-terminal membrane anchor domain of a type B HXK determines mitochondrial association.

In contrast to all of the type B HXKs examined, it has been suggested by means of subcellular fractionation that the spinach SoHXK1 may be localized on the outer membrane of the chloroplast (Wiese et al., 1999). Accordingly, it has also been suggested that the primary function of SoHXK1 located on the outer membrane of the chloroplast might be to directly phosphorylate glucose leaving the chloroplasts (as a product of hydrolytic starch breakdown), in order to maintain a steep glucose concentration gradient across the plastidic membrane (Wiese et al., 1999). However, the N-terminal membrane anchor domain of SoHXK1 is very similar to those of the mitochondria-associated HXKs (Damari-Weissler et al., 2007). Indeed, the expression of a fusion protein of SoHXK1::GFP in tobacco and Arabidopsis protoplasts showed that SoHXK1::GFP is associated with the mitochondria, similar to the other type B HXK isozymes (Damari-Weissler et al., 2007). A protein containing the first 45 amino acids of SoHXK1 fused to a truncated protein of AtHXK1 was also associated with the mitochondria (Damari-Weissler et al., 2007).

These observations contradict the results obtained through subcellular fractionation and plastid isolation, which suggested that SoHXK1 is attached to the outer membrane of the chloroplast envelope (Wiese et al., 1999). Intensive proteomic analysis of Arabidopsis chloroplast envelope membranes did not reveal any HXK-chloroplast envelope associations (Ferro et al., 2003). Hence, none of the HXK isozymes deduced from the numerous HXK genes cloned so far seem to be located on the outer plastidic envelope, to maintain a steep glucose concentration gradient across the plastidic membrane (Wiese et al., 1999). Maltose is probably the main starch degradation product exported from the chloroplasts (Smith et al., 2005; Zeeman et al., 2007) and, therefore, there might not be a need for any steep glucose gradient between the chloroplast and the cytoplasm.

In a recent report, it was suggested that a new HXK (ScHK2) might be located on the plasma membrane of *Solanum chacoense*, a wild relative of the cultivated potato (Claeyssen et al., 2006). This suggestion was based on analyses of this enzyme's N-terminal amino acid sequence using various programs that predict intracellular localization. These programs predicted the localization of ScHK2 in the secretory pathway and endoplasmic reticulum and, therefore, a role for a plasma membrane HXK has been proposed (Claeyssen et al., 2006; Claeyssen and Rivoal, 2007). However, the N-terminal amino acid sequence of this new HXK is identical to that of the other mitochondria-associated, type B HXKs that were also predicted to be part of the secretory pathway by the same programs (Claeyssen et al., 2006; Damari-Weissler et al., 2006; Kandel-Kfir et al., 2006). Hence, ScHK2 is most likely a mitochondria-associated HXK, similar to the other type B HXKs.

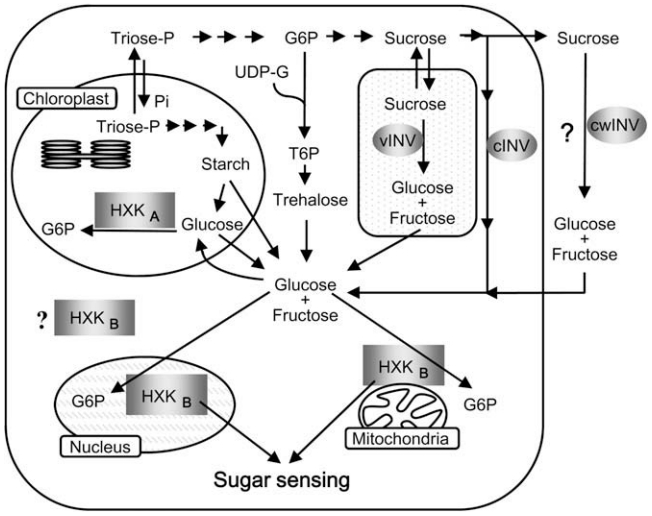

Fig. 2. Schematic presentation of intracellular localization of HXK and its connection to sugar metabolism and sugar sensing in photosynthetic cells. Two types of HXK were identified and characterized in plant cells. Type A HXKs (HXK_A) are located in the stroma of plastids and type B HXKs (HXK_B) are associated with mitochondria. The mitochondria-associated HXKs might also appear in the nucleus and are involved in sugar sensing. The presence of a cytosolic HXK is questioned. Accordingly, glucose might be phosphorylated in the plastidic stroma, adjacent to the mitochondria or within the nucleus. Triose-P – triose-phosphate; G6P – glucose 6-phosphate; T6P – trehalose 6-phosphate; UDP-G – UDP-glucose; cINV – cytosolic invertase; cwINV – cell wall invertase; vINV – vacuolar invertase.

The evidence presented so far suggests that different plant species have a single HXK located in the stroma within plastids and several HXK isozymes associated with the mitochondria (Fig. 2). The only exception to this is a single monocot OsHXK7 that lacks the N-terminal membrane anchor domain and was localized in the cytosol (Cho et al., 2006a). However, in dicots, all of the HXKs examined so far have either a plastidic signal peptide (type A) or an N-terminal membrane anchor domain (type B; Claeyssen et al., 2006; Granot, 2007; Olsson et al., 2003).

4. HXK and sugar sensing

HXKs have emerged as important enzymes that mediate glucose sensing in plants, regulating photosynthesis, growth and leaf senescence (Rolland et al., 2006). However, to date, sugar sensing functions of HXKs have been shown only for the mitochondria-associated (type B) HXKs, mainly with the Arabidopsis *AtHXK1* (Balasubramanian et al., 2007; Dai et al., 1999; Jang et al., 1997; Moore et al., 2003). It has been suggested that a small fraction of the mitochondria-associated HXK isozymes are transported to the nucleus, where they regulate the expression of various genes, such as chlorophyll a/b binding protein (*CAB*; Cho et al., 2006b; Yanagisawa et al., 2003). The questions of which membranes the mitochondria-associated HXKs are associated with, whether they dissociate from these membranes and become cytosolic under

natural physiological conditions and how they arrive at the nucleus remain to be explored.

Regulation of photosynthesis by type B HXK implies the presence of free glucose in the cytoplasm of photosynthetic tissues during the day. That is in contrast to the theoretical considerations described above, which questioned the presence of free glucose and the need for HXK in photosynthetically active tissues. The source of the free glucose sensed by the type B HXK in photosynthetic tissues during the day is not known and this glucose could potentially be from starch degradation, cleavage of some of the exported extracellular sucrose followed by glucose import or cleavage of intracellular sucrose or trehalose (Fig. 2). Trehalose is cleaved to glucose monomers by trehalase. Among the various options, trehalose cleavage as a source of glucose sensed by HXK seems plausible as a trehalose pathway has already been suggested to be involved in sugar sensing (Paul, 2007). Trehalose is the product of G6P and UDP-G joined together by trehalose 6-P synthase to yield trehalose 6-P (T6P), which is then dephosphorylated to form trehalose. It has been suggested that T6P has a regulatory role, accelerating starch biosynthesis and reducing starch degradation, perhaps conveying the status of the sucrose level and sugar metabolism in the cytosol to plastids (Kolbe et al., 2005; Lunn et al., 2006; Paul, 2007). High G6P levels followed by increasing levels of T6P may indicate a need to preserve more triose-P in the chloroplast for starch biosynthesis, rather than spending it on glycolysis and sucrose synthesis. Increasing levels of T6P are followed by increasing levels of trehalose, whose cleavage yields free glucose that could then be sensed by HXK, inhibit the expression of photosynthetic genes and repress the rate of photosynthesis.

A sugar sensing role for type B HXK has been shown mainly with respect to source photosynthetic tissues. The underlying assumption is that type B HXKs have sugar sensing roles only within the cells in which they are expressed. However, type B HXKs are expressed not only in photosynthetic, but also in non-photosynthetic tissues (Jang et al., 1997; Kandel-Kfir et al., 2006). Furthermore, even classical source tissues like leaves are heterogeneous, composed of different types of photosynthetic and non-photosynthetic cells, and most studies cannot differentiate between effects on various cell types. Hence, expression analysis of HXK genes in sub-tissues of leaves, stems and other organs, accompanied by tissue-specific expression of HXK genes might shed light on the roles of individual type B and type A HXKs in specific tissues.

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