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The Bifunctional Role of Hexokinase in Metabolism and Glucose Signaling

The effects of manipulating nutrient supply on plant growth and development have long been known. However, it is only recently that these effects have been understood to consist of more than simply relieving nutrient-limited growth or modifying the allosteric regulation of biochemical pathways. It is now well documented that several nutrients and metabolites act as signaling molecules in multiple pathways that coordinately regulate patterns of gene expression (Coruzzi and Bush, 2001). Our understanding of sugar signaling, particularly glucose signaling, is the most advanced. Jen Sheen and colleagues have been at the forefront of these advances, and earlier this year, Moore et al. (2003) published the results of an insightful series of experiments in *Arabidopsis* that clarified the role of hexokinase in glucose signaling. Using biochemical and genetic tools, the authors showed that hexokinase is required for glucose signaling and separated its enzymatic activity from its signaling function, thus confirming the hypothesis that it is a glucose sensor. Naturally, an important advance such as this raises a number of new and intriguing questions concerning the sensing mechanism and the transduction pathway at the physiological, biochemical, and genomic levels.

Nutrient and metabolite signaling research has a long history in plant biology (Coruzzi and Bush, 2001). Early data came from observations of changes in plant growth and development caused by the application of nitrate-containing fertilizers. This practice results in increased growth as well as changes in amino acid and protein composition, carbon metabolism, phytohormone levels, allocation, and phenology (Stitt, 1999). The nutrient regulation of enzyme activity was first demonstrated >50 years ago. Nitrate treatment was shown to regulate nitrate reductase activity and ni-

trate transport, and in the early 1960s, a link between the rate of photosynthesis and photoassimilate partitioning was demonstrated (Coruzzi and Bush, 2001). Since the time these observations were made, research into carbon and nitrogen signaling has been pursued vigorously, with a significant component of recent work focusing on the sugar signals involved in plant growth and development.

Sugars have been implicated in a wide variety of signaling processes in higher plants (Jang and Sheen, 1994; Koch, 1996; Rolland et al., 2002a). Metabolite regulatory phenomena are not only the result of the allosteric regulation of enzyme activity, but key metabolites also are signaling molecules that function in complex control pathways that coordinately regulate patterns of gene expression. This has been demonstrated by the regulation of gene expression using hexose and sucrose analogs as well as nonmetabolizable and partially metabolizable hexoses. Metabolism, growth, development, and abiotic and biotic stress responses all are regulated, at least in part, by sugars (Koch, 1996; Rolland et al., 2002a). Thus far, responses to sugars are best understood at the transcriptional level, although some data indicate effects on mRNA stability (Rolland et al., 2002a).

An ever-growing body of literature suggests a number of different carbon-based regulatory pathways are active in higher plants. Even before CO₂ is fixed during photosynthesis, its concentration is monitored dynamically by guard cells as one component of the intricate signaling pathways that regulate stomatal opening (Hedrich et al., 2001, and references therein), and its abundance can even affect stomatal density in developing leaves (Lake et al., 2001). Independent signaling pathways for sucrose (Chiou and Bush, 1998; Rook

et al., 1998; Vaughan et al., 2002), glucose (for a recent, comprehensive review, see Rolland et al., 2002a), trehalose-6-phosphate (Eastmond and Graham, 2003), and possibly fructose (Pego and Smeekens, 2000; German et al., 2003) are indicated. In the case of glucose, it appears that more than one signaling pathway may be operational (Jang and Sheen, 1997; Xiao et al., 2000).

The role of glucose as a signaling molecule in unicellular organisms has been studied extensively. In *Escherichia coli*, glucose supply is monitored by Mlc, a repressor of the glucose phosphotransferase (uptake) system (for a recent review, see Plumbridge, 2002). Multiple glucose-sensing mechanisms are present in yeast. These include the nontransporting glucose carrier homologs Snf3 and Rgt2, hexokinases, and cAMP (for a comprehensive review, see Rolland et al., 2002b). Studies in mammalian systems are relatively recent. In insulin-secreting pancreatic islet β -cells, glucose signaling appears to be a function of the amount of ATP generated by catabolism. Because flux through glucokinase is the rate-limiting step in glucose catabolism in these cells, this enzyme is considered the primary glucose sensor (for recent reviews, see Rolland et al., 2001; Schuit et al., 2001).

In higher plants, like mammals, hexose-signaling studies are relatively recent. Before the initial observations of the sugar regulation of gene expression in the late 1980s, sugar effects on photosynthesis, growth, and development were presumed to be the result of metabolic fluctuations. In pioneering work with a transient gene expression system, Sheen (1990) showed that glucose, sucrose, or acetate applied to maize protoplasts led to the repression of seven photosynthetic genes. A clear demonstration of the carbohydrate regulation of photosynthetic gene expression in

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whole plants was provided by Krapp et al. (1993). These authors showed that carbohydrate accumulation in the mesophyll results in a concomitant downregulation of photosynthetic gene expression in plants fed glucose through the transpiration stream. Subsequent studies by Graham et al. (1994) using cucumber cell suspension cultures, and by Jang and Sheen (1994) using the maize protoplast transient expression system, led to the hypothesis that the sugar signal was perceived by hexokinase. These authors used a variety of sugars, glucose analogs, and metabolic intermediates to demonstrate that glucose affects the expression of genes that encode enzymes in both photosynthesis (Jang and Sheen, 1994) and the glyoxylate cycle (Graham et al., 1994). Both groups demonstrated that the provision of relatively low concentrations of sugars that are substrates of hexokinase resulted in decreased levels of gene expression. Mannoheptulose, a competitive inhibitor of hexokinase, blocked the effect of these sugars. Glucose analogs that are transported across the plasma membrane but are not phosphorylated by hexokinase, nontransported analogs, and sugar phosphates did not alter gene expression significantly. The provision of excess inorganic phosphate or ATP did not block the observed response, indicating that their depletion during metabolism did not constitute the signal for decreased gene expression. Jang et al. (1997) then constructed transgenic plants that expressed either sense or antisense constructs of the *Arabidopsis* genes *HEXOKINASE1* (*HXK1*) and *HXK2*, which encode different hexokinase isoforms. Using both seedling development parameters and analysis of gene expression as markers, plants that overexpressed *HXK* genes exhibited glucose-hypersensitive characteristics, whereas antisense plants were hyposensitive (Jang et al., 1997). Together, these data supported the hypothesis that hexokinase is the putative sensor for hexose signaling.

The work of Moore et al. (2003) represents a significant advance in our understanding of the role that hexokinase plays

in glucose signaling and perception. The authors present data describing the effect of two independent knockout mutations for the HXK1 isoform (*glucose insensitive2-1* [*gin2-1*] and *gin2-2*) on plant growth and development. The *gin2-1* nonsense mutant showed broad growth defects during both reproductive and vegetative stages of development. These defects became more pronounced as light intensity increased. Importantly, glucose phosphorylation capacity was reduced by only ~50%, as a result of the activity of other endogenous enzymes. Moreover, the decrease in enzyme capacity did not reduce glucose-6-phosphate or fructose-6-phosphate content. Thus, Moore et al. concluded that glucose-6-phosphate metabolism is uncoupled from HXK1-dependent signaling.

Because the experiments described above separated HXK1-mediated glucose metabolism from glucose signaling at the physiological level, Moore et al. (2003) attempted to uncouple the phosphorylation activity of hexokinase from its sensing/signaling activity by generating point mutations in the catalytic domains of the protein. The engineered proteins exhibited no phosphorylation activity, yet in a protoplast-dependent glucose repression assay, the catalytically inactive HXK1 still exhibited glucose signaling activity. To investigate this observation in planta, they transferred these constructs into *gin2-1* plants and scored them for glucose-sensitive changes in growth and development. Significantly, glucose-dependent changes in development and gene expression were restored to the levels seen in wild-type plants, confirming HXK1's pivotal role in glucose signal transduction. In an additional series of growth experiments, the authors provided evidence for interactions between the HXK1 glucose signaling pathway and plant responses to auxin and cytokinin.

The data presented by Moore et al. (2003) clearly demonstrate that glucose signaling requires the presence of hexokinase in the plant. Their results also show that glucose signaling is not the result of the accumulation or depletion of downstream metabolic products or of changes

in the ATP:ADP ratio, as have been hypothesized previously (Jang and Sheen, 1997; Halford et al., 1999). Together, these data provide compelling evidence that hexokinase-mediated glucose signaling is not dependent on its catalytic activity per se. Thus, hexokinase plays two functionally distinct roles in the plant.

How does this model for the bifunctional nature of hexokinase fit with what is known about the characteristics and roles of hexokinases across the phyla? Bacterial hexokinases have a relatively low molecular mass (32 to 37 kD) and are almost universally specific for a single hexose—glucose, fructose, or mannose (reviewed by Cárdenas et al., 1998)—and do not appear to be involved in sugar sensing. In yeast, two hexokinase isoforms (PI and PII) are present, as well as a glucokinase. These two hexokinases have both metabolic and putative glucose sensory roles (for review, see Rolland et al., 2002b). Vertebrates have four isoforms of hexokinase, three of which are 100 kD, and the fourth of which is 50 kD (hexokinase type IV or D, also called glucokinase [Cárdenas et al., 1998]), similar in size to hexokinases in higher plants and yeast. The three 100-kD hexokinases are encoded by genes that appear to have a single 50-kD ancestor that was duplicated and fused and then underwent further duplication in the genome (Cárdenas et al., 1998). This is significant in that the type-I and -III isoforms have a catalytic function that resides solely in the C-terminal half, whereas the N-terminal half has a noncatalytic (allosteric regulatory) role (Wilson, 2003). Interestingly, *Arabidopsis* also has a putative 97-kD isoform. In β -cells, it appears that the 50-kD type-IV is involved in glucose sensing via regulation of the ATP:ADP ratio (reviewed by Schuit et al., 2001). The yeast, animal (50-kD isoform), and *Arabidopsis* hexokinases show 30 to 33% amino acid sequence identity. It will be interesting to learn if all of them exhibit both enzymatic and signaling activity that could be attributable to a common, bifunctional ancestor.

The demonstration by Moore et al. (2003) of the two roles of hexokinase in

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glucose metabolism and sensing raises a number of intriguing questions about the bifunctional nature of the hexokinase protein itself, the roles of other *HXK* gene family members, and the nature of glucose sensing and signaling at the level of cell physiology. To separate the metabolic and signaling functions of HXK1, the authors generated two catalytically inactive HXK1 mutants by single amino acid changes in two separate catalytic domains (G104D and S177A). Significantly, neither of these mutations appears to be involved in glucose binding. Models for the reaction mechanism of yeast hexokinase hypothesize that glucose binds before ATP and then ADP is released, followed by glucose-6-phosphate (DelaFuente and Sols, 1970; Kuser et al., 2000). Based on the corresponding amino acid positions of the yeast hexokinase PII crystal structure, the S177A mutation appears to occupy a pivotal domain involved in the conformational change that binds the glucose molecule, simultaneously opening the nucleotide binding site. This domain also borders the mouth of the conserved hydrophobic channel that is hypothesized to be the release tunnel for the proton generated as a result of product formation (Kuser et al., 2000). Kuser et al. (2000) hypothesize that proton release is essential to decrease local pH, thus allowing the negative charges of the two adjacent phosphate groups to drive each other apart and release the glucose-6-phosphate from hexokinase, followed by ADP. By contrast, the G104D mutation is in a corresponding region of yeast hexokinase PII associated with binding phosphate 1 of ATP. The positions of these two mutations indicate that the glucose binding domain may be intact, and because both proteins retain their glucose-dependent signaling function, they still can bind glucose (Moore et al., 2003).

Of course, we cannot exclude the possibility that glucose binds at a secondary site. This raises two questions about the properties of glucose binding to the engineered enzymes. First, where is glucose binding, and second, what are the kinetics of binding and release? Two obvious sce-

narios for binding are that glucose is bound and not released, or that glucose binds and releases with kinetics comparable to those of the wild-type protein but without being phosphorylated because of the inability of hexokinase to bind ATP. Both of these scenarios support plausible mechanisms for glucose sensing in HXK1 wherein substrate binding causes a change in conformation that initiates a signaling cascade. However, a related question that remains to be answered is whether hexokinase senses free glucose in a linear, concentration-dependent manner or whether it is a flux sensor (Koch, 1996) that senses either upward and/or downward deviations from a fixed or dynamic set point.

Rolland et al. (2002a) proposed several possible mechanisms involved in glucose signaling. These include protein kinases and phosphatases that may or may not be conserved elements of yeast and/or mammalian sugar signaling pathways and the involvement of 14-3-3 proteins and Ca^{2+} as a second messenger. However, other proteins may be involved. For example, a signaling protein that transduces the glucose signal directly to the nucleus could be in dynamic equilibrium between bound and unbound states with HXK1. It is easy to imagine a change in that equilibrium as a function of glucose binding to the enzyme. In mammalian systems, hexokinase type III has been localized to the nucleus associated with the nuclear envelope porin (Wilson, 2003), whereas yeast hexokinase PII has been localized to nuclear DNA-protein *cis*-regulatory structures (reviewed by Rolland et al., 2002b). Although the precise role of hexokinase in these examples is not yet understood, it is clear that there may be multiple solutions to this signaling question.

The data presented by Moore et al. (2003) refer specifically to HXK1. This raises the obvious question about the contributions of HXK2 and the four additional hexokinase-like enzymes to glucose or hexose sensing. Both HXK1 and HXK2 have similar substrate activity profiles and appear to operate in similar concentration

ranges (Dai et al., 1999; Gonzali et al., 2002). Unfortunately, nothing is known about the biochemical properties of the four remaining hexokinase-like isoforms, although unpublished data appear to indicate that HXK-like genes also possess sugar sensing and signaling functions (B. Moore and J. Sheen, unpublished data, cited in Rolland et al., 2002a). Likewise, very little is known about the intracellular localization of the various isoforms. Without more detailed information on substrate specificities and cellular and subcellular localization, it is impossible to determine whether these isoforms are functionally redundant or whether there is complete separation or partial overlap.

Moore et al. have provided compelling evidence that HXK1 is a glucose sensor and that glucose sensing interacts with additional signaling networks that control plant growth and development. With this critical advance comes a new series of challenging questions. From a reductionist approach: what are the other players in the glucose signal transduction pathway, what are their targets, and how do they interact? From the physiologist's perspective: how are the complex networks of sugar, hormone, and nutrient signaling integrated across the plant as a multicellular organism? It seems we still have a few challenges on our doorstep.

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