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RESEARCH ARTICLE

Hexokinase as a Sugar Sensor in Higher Plants

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The mechanisms by which higher plants recognize and respond to sugars are largely unknown. Here, we present evidence that the first enzyme in the hexose assimilation pathway, hexokinase (HXK), acts as a sensor for plant sugar responses. Transgenic Arabidopsis plants expressing antisense hexokinase (*AtHXK*) genes are sugar hyposensitive, whereas plants overexpressing AtHXK are sugar hypersensitive. The transgenic plants exhibited a wide spectrum of altered sugar responses in seedling development and in gene activation and repression. Furthermore, overexpressing the yeast sugar sensor YHXK2 caused a dominant negative effect by elevating HXK catalytic activity but reducing sugar sensitivity in transgenic plants. The result suggests that HXK is a dual-function enzyme with a distinct regulatory function not interchangeable between plants and yeast.

INTRODUCTION

Sugars are not only important energy sources and structural components; they are also central regulatory molecules controlling physiology, metabolism, cell cycle, development, and gene expression in prokaryotes and eukaryotes. In higher plants, sugars affect growth and development throughout the life cycle, from germination to flowering to senescence (Steeves and Sussex, 1989; Brusslan and Tobin, 1992; Graham et al., 1992; Bernier et al., 1993; Sheen, 1994; Thomas and Rodriguez, 1994; Dangl et al., 1995). Recently, it has become apparent that sugars are physiological signals repressing or activating plant genes involved in many essential processes, including photosynthesis, glyoxylate metabolism, respiration, starch and sucrose synthesis and degradation, nitrogen metabolism, pathogen defense, wounding response, cell cycle regulation, pigmentation, and senescence (Chen et al., 1994; Knight and Gray, 1994; Lam et al., 1994; Sheen, 1994; Herbers et al., 1995; Mita et al., 1995; Reynolds and Smith, 1995).

Studies with a variety of plant species have also shown that sugar homeostasis appears to be tightly regulated. Elevated sugar levels cause stunted growth, reduced photosynthesis, enhanced anthocyanin accumulation, and curled, chlorotic, and necrotic leaves (Casper et al., 1986; von Schaewen et al., 1990; Dickinson et al., 1991; Sonnewald et al., 1991, 1995; Tsukaya et al., 1991; Huber and Hanson, 1992). In addition, environmental factors, such as elevated CO_2 (Stitt, 1991; Stitt et al., 1991; van Oosten et al., 1994; Nie et al., 1995), and intrinsic genetic variations, such as different invertase levels (Goldschmidt and Huber, 1992), have been proposed to affect photosynthetic capacity through sugar regulation. Although sugar signal transduction pathways are well characterized in prokaryotes (Saier et al., 1990, 1995) and unicellular eukaryotes (Entian and Barnett, 1992; Gancedo, 1992; Johnston and Carlson, 1992; Trumbly, 1992; Ronne, 1995; Thevelein and Hohmann, 1995), relatively little is known about the molecular and biochemical mechanisms underlying sugar responses in multicellular eukaryotes, especially in sugar-producing higher plants.

Using a single-cell system and the sugar-repressible promoters of genes involved in photosynthesis, we have initiated the dissection of the sugar signal transduction pathway in higher plants (Jang and Sheen, 1994). We have shown that plant cells can sense and respond to several hexose signals that are perceived intracellularly. The phosphorylation of hexoses by hexokinase (HXK) appears to be critical for signaling because only the hexoses and glucose analogs that can be phosphorylated by HXK are effective. Although in mammals extensive glucose metabolism is essential for signaling (Efrat et al., 1994; Grupe et al., 1995; Newgard and McGarry, 1995), sugar metabolism beyond the phosphorylation of glucose in plants is not required to elicit sugar responses. This conclusion is based on the findings that 2-deoxyglucose (2-dGlc) and mannose, which are not metabolized through glycolysis, are both potent elicitors of sugar responses. In addition, when delivered directly into maize leaf cells by electroporation, glucose, but not glucose 6-phosphate or other downstream metabolites in the glycolytic pathway, triggers the repression. It has been proposed that

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HXK is the sugar sensor mediating the repression of genes involved in photosynthesis (Jang and Sheen, 1994). This notion is supported by the observation that a specific HXK inhibitor, mannoheptulose, can block glucose repression in plant cells (Jang and Sheen, 1994). Recently, HXK has also been proposed to be the sugar sensor in the repression of genes involved in the glyoxylate cycle in plants (Graham et al., 1994).

Although HXK is commonly known as the glycolytic enzyme that catalyzes the ATP-dependent conversion of hexoses to hexose 6-phosphates, it has been implicated as a glucose sensor in lower and higher eukaryotes, including yeast (Entian, 1980; Entian and Frölich, 1984; Entian et al., 1985; Rose et al., 1991) and mammals (German, 1993; Matschinsky et al., 1993; Mueckler, 1993; Grupe et al., 1995). Our previous studies (Jang and Sheen, 1994) suggested that sugar sensors might be evolutionarily conserved in all eukaryotes. Here, we report the identification of two Arabidopsis HXK (*AtHXK*) genes by genetic complementation of a yeast *hxk1 hxk2* double mutant (Ma and Botstein, 1986). Genes encoding HXK in plants show significant sequence similarity to the genes encoding HXK in yeast and the mammalian glucokinase (GLK or HXKIV) genes but are structurally distinct from the mammalian HXKI, HXKII, and HXKIII genes. We demonstrate that HXK is a sensor mediating diverse sugar responses by manipulating its expression in transgenic plants. These plants exhibit predicted sugar hyposensitivity or hypersensitivity. Interestingly, although yeast and higher plants share similar HXK catalytic activity, their HXKs are not interchangeable for the regulatory function that controls sugar signal transduction.

RESULTS

Molecular Characterization of the Arabidopsis HXK-Encoding Genes

To elucidate the role of HXK in sugar responses in whole plants, we used Arabidopsis as a model system. We first identified the Arabidopsis HXK genes from an expression library (Minet et al., 1992) by functional complementation of the yeast *Saccharomyces cerevisiae hxk1 hxk2* double mutant (DBY2219) that completely lacks HXK activity. Two cDNAs, *AtHXK1* and *AtHXK2* (2.0 and 1.9 kb, respectively), were able to complement the yeast mutant. The complements were able to grow on a selection plate containing fructose as the sole carbon source. Several clones of each *AtHXK* cDNA were isolated and reintroduced into DBY2219 to confirm the result of the complementation analysis. Mutated yeast cells transformed with either the *AtHXK1* or *AtHXK2* cDNA restored growth on the selection medium. The vector pFL61 (Minet et al., 1992) alone was not able to complement the mutant (Figure 1).

DNA sequence analyses of *AtHXK1* and *AtHXK2* revealed open reading frames coding for 496 and 502 amino acids,

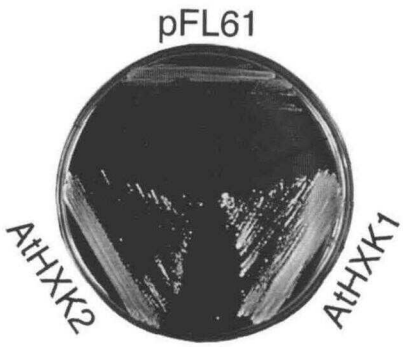

Figure 1. Complementation of the Yeast *hxk1 hxk2* Double Mutant.

The Arabidopsis HXK cDNAs *AtHXK1* and *AtHXK2* provided HXK catalytic activity and supported the growth of the yeast *hxk1 hxk2* double mutant on the fructose plate. pFL61 is the vector control.

respectively (Figure 2). *AtHXK1* and *AtHXK2* share 82% nucleotide identity, and AtHXK1 and AtHXK2 share 85% amino acid identity. Results of data base searches and sequence comparisons showed that AtHXK proteins share significant sequence identity with human (Nishi et al., 1992) and rat (Magnuson et al., 1989) GLK (34 to 35%) and yeast (Stachelek et al., 1986; Prior et al., 1993) HXKs (36 to 38%) (Figure 2). Conserved ATP and sugar binding domains (Bork et al., 1993) were found in the deduced protein sequences of both *AtHXK* genes (Figure 2). A part of the *AtHXK1* gene showed 100% identity to a previously reported Arabidopsis HXK cDNA (Dai et al., 1995) that encodes a predicted polypeptide that is 61 amino acids shorter than AtHXK1.

In mammals, four HXK-encoding genes have been isolated. HXKI, HXKII, and HXKIII all consist of a duplicated, highly homologous polypeptide sequence (Kogure et al., 1993). Each half of the mammalian HXK polypeptide is similar to that of the 50-kD GLK polypeptide and the yeast HXK proteins involved in glucose sensing and signaling. Although HXK activities have been detected in various plant tissues (Miernyk and Dennis, 1983; Doehlert, 1989; Schnarrenberger, 1990; Renz et al., 1993), the sequence and arrangement of putative activity domains of the higher plant HXK proteins were not clear before this study and a recent report (Dai et al., 1995) were undertaken. We show here that the overall sequence of the two Arabidopsis HXK proteins are similar to those of the mammalian GLK and yeast HXK proteins but distinct from those of the mammalian HXK proteins.

Both AtHXK1 and AtHXK2 were selected based on their ability to confer fructose utilization in the yeast *hxk1 hxk2* double mutant. Nevertheless, their amino acid sequence identities to a plant fructokinase (Smith et al., 1993) are quite low (<20%). Both AtHXK1 and AtHXK2 are also capable of phosphorylating glucose (data not shown), which implies that they are unlikely to be either fructokinase or GLK.

We determined the chromosomal positions of *AtHXK1* and *AtHXK2* by segregation analysis of their restriction

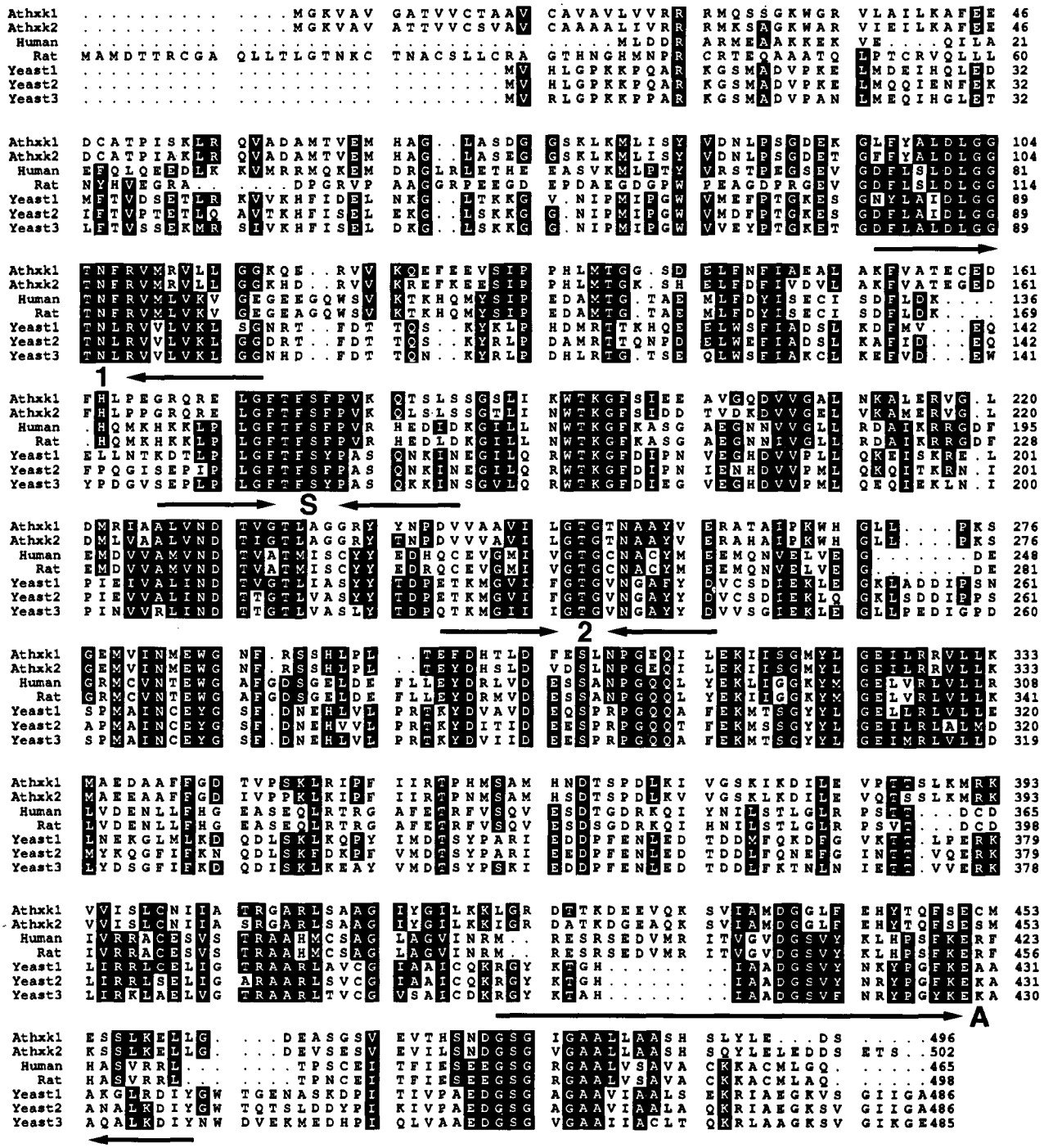

Figure 2. The HXK Sequence Is Conserved in Eukaryotes.

A comparison of predicted amino acid sequences of Arabidopsis AtHXK1 and AtHXK2, human GLK (Nishi et al., 1992), rat GLK (Magnuson et al., 1989), *S. cerevisiae* HXK1 (Yeast1) and HXK2 (Yeast2) (Stachelek et al., 1986), and *Kluyveromyces lactis* RAG5 (Yeast3) (Prior et al., 1993) is shown. The ATP binding site (Bork et al., 1993) includes underlined regions 1, 2, and A, indicating the conserved phosphate 1 and 2 and adenosine interaction regions, respectively. The conserved sugar binding domain (Bork et al., 1993) is indicated in the underlined S region. Identical and similar residues are boxed and highlighted. Dots were introduced to optimize alignment. GenBank accession numbers for AtHXK1 and AtHXK2 are U28214 and U28215, respectively.

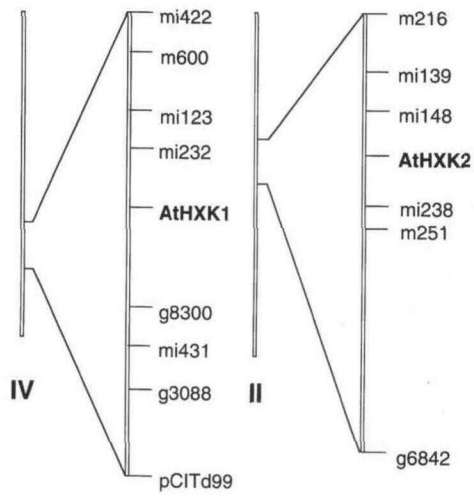

Figure 3. Map Positions of *AtHXK1* and *AtHXK2*.

AtHXK1 is located on chromosome 4, and *AtHXK2* is located on chromosome 2.

fragment length polymorphisms in recombinant inbred lines (Nam et al., 1989; Hauge et al., 1993; Lister and Dean, 1993). *AtHXK1* is located on chromosome 4 (Schmidt et al., 1995) and is flanked by mi232 and g8300. *AtHXK2* is located on chromosome 2 (Zachgo et al., 1996) and is flanked by mi148 and mi238 (Figure 3).

Genomic DNA gel blot analysis using either *AtHXK1* or *AtHXK2* as a probe revealed at least two *AtHXK* genes corresponding to the two isolated *AtHXK* cDNAs that were hybridized at the same level of stringency (Figure 4). For example, in the lane with DNA that was digested with BglII, *AtHXK1* hybridized strongly with a band near 8.4 kb and weakly to a band near 4.3 kb. However, the intensity of the two bands was the reverse when *AtHXK2* was used as a probe. RNA gel blot analyses using a gene-specific probe showed that both *AtHXK1* and *AtHXK2* detected RNA bands of ~2 kb with similar expression patterns. However, *AtHXK1* was expressed at higher levels in rosette leaves and stems. The transcripts of both *AtHXK1* and *AtHXK2* were more abundant in siliques, flowers, and roots but less abundant in leaves and stems (Figure 5).

AtHXK as a Sugar Sensor in Plants

To test the hypothesis that HXK acts as a sugar sensor in intact plants, we established a plant model in which sense and antisense *AtHXK* genes were introduced into Arabidopsis to alter AtHXK levels. This reverse genetic approach has been shown to be a powerful tool for the functional analysis of diverse plant gene products (Rodermel et al., 1988; van der Krol et al., 1988; Mol et al., 1990; Sonnewald et al., 1991; Weigel and Nilsson, 1995). Wild-type (ecotype Bensheim) Arabidopsis plants were transformed with binary vectors carrying fusions of the cauliflower mosaic virus 35S RNA promoter and *AtHXK1* or *AtHXK2* in the sense or antisense orientation. We used a constitutive promoter because it should bypass the endogenous transcriptional regulation of the *AtHXK* genes.

Hundreds of transgenic Arabidopsis plants were generated by using a modified Agrobacterium-mediated root transformation method (Czako et al., 1992). Transgenic lines of the T_3 generation homozygous for the transgenes were selected for further analyses. The presence of the transgene was determined by neomycin phosphotransferase expression, which confers kanamycin resistance, and T-DNA integration was determined by DNA gel blot analyses (data not shown).

We first examined the sugar sensitivity of transgenic plants, based on bioassays in which striking seedling phenotypes could be easily scored when grown on plates containing 6% glucose or 0.8 mM 2-dGlc. For example, greening and expansion of cotyledons, initiation of true leaves, and elongation of the hypocotyl and root were suppressed in control (wild-type) Arabidopsis seedlings grown on 6% glucose plates under constant light (Figure 6A, right). These inhibitory effects caused by glucose were observed in six different Arabidopsis ecotypes, including Bensheim, C24, Columbia, Landsberg *erecta*, RLD, and Wassilewskija (data not shown). In addition, the greening of cotyledons was inhibited at a low

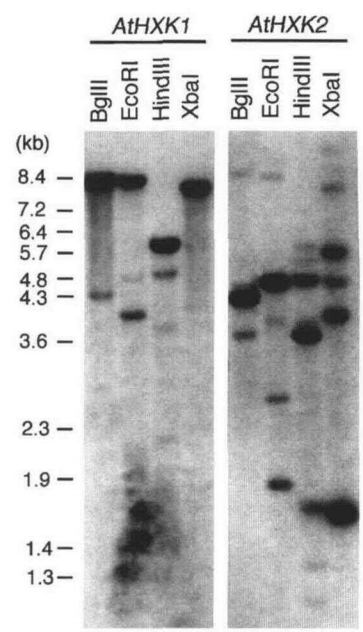

Figure 4. AtHXK Is Encoded by a Multigene Family.

Arabidopsis (Landsberg *erecta*) genomic DNA was digested with BglII, EcoRI, HindIII, or XbaI. A full-length *AtHXK1* or *AtHXK2* cDNA was used as a probe. Numbers at left are molecular length markers in kilobases.

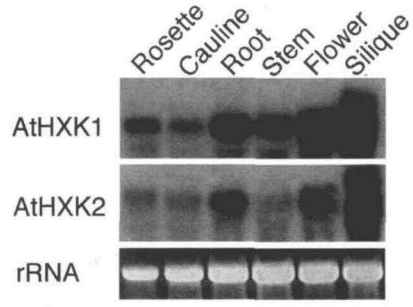

Figure 5. *AtHXK* Expression Patterns.

Total RNA was extracted from rosette leaves (Rosette), cauline leaves (Cauline), stems, flowers, and siliques of greenhouse-grown Arabidopsis plants. Total root RNA was derived from plants grown in liquid culture. The RNA gel blot was hybridized with the full-length cDNA probe of either *AtHXK1* or *AtHXK2*. rRNA was used as the control to ensure equal loading of the lanes. Staining was with ethidium bromide.

concentration of 2-dGlc in the control plants (Figure 6B, right). This phenotype is consistent with the finding that 2-dGlc is a potent sugar signal. It can trigger global repression of genes encoding photosynthetic proteins (Jang and Sheen, 1994).

Compared with the control plants, the plants carrying the 35S:*AtHXK1* sense transgene (*sense-AtHXK1*) showed hypersensitivity to 6% glucose, as indicated by extremely stunted cotyledons, hypocotyls, and roots (Figure 6A, left). In contrast, the plants carrying the 35S:*AtHXK1* antisense transgene (*anti-AtHXK1*) greened, expanded, and elongated normally on 6% glucose plates (Figure 6A, middle), suggesting that they are relatively hyposensitive to glucose. Figure 6C illustrates that glucose hypersensitivity and hyposensitivity are displayed homogeneously in the transgenic plant populations. As in the glucose assay, the *sense-AtHXK1* plants were hypersensitive to 2-dGlc, as shown by the severe inhibition of cotyledon greening (Figures 6B, left, and 6D, left). The *anti-AtHXK1* plants were 2-dGlc hyposensitive and appeared green (Figures 6B, middle, and 6D, right). Similar phenotypes were observed in multiple independent transgenic lines generated with either sense or antisense *AtHXK1* or *AtHXK2* (Table 1). The sugar hyposensitivity in the transgenic lines carrying the 35S:*AtHXK1* sense transgene presumably resulted from cosuppression (Napoli et al., 1990; Matzke and Matzke, 1993; Flavell, 1994). A few transgenic lines showed phenotypes similar to those of the wild-type plants. These phenotypes are most likely due to variations in transgene expression.

To rule out the possibility that the difference in sugar response between the transgenic and the control plants is due to an osmotic effect, we used mannitol and 3-*O*-methylglucose in control experiments. No apparent difference was observed between the control and the transgenic plants when they were plated on 6% mannitol (Figure 6E) or 6% 3-*O*-methylglucose, a glucose analog that is not phosphorylated by HXK (Figure 6F).

AtHXK Mediates the Effects of Sugar on Plant Growth and Development

Sugars have long been known to affect plant growth and development (Steeves and Sussex, 1989; Kovtun and Daie, 1995). However, the effects of sugar are often attributed to the consequences of sugar metabolism or osmotic stress rather than to specific sugar sensing and signaling. The availability of transgenic plants with altered sugar responses offers a unique opportunity to examine sugar-dependent phenomena systematically. Using wild-type and transgenic seedlings, we first compared the effects of sugar on two responses, hypocotyl elongation and cotyledon greening and expansion. Three independent transgenic lines from each type of the transgene were examined. One of three transgenic lines, including 9A14-2 (*sense-AtHXK1*), 225B3-4 (*sense-AtHXK2*), A9Af2-6 (*anti-AtHXK1*), and A225Bf8-5 (*anti-AtHXK2*), was used for detailed quantitative analyses. These transgenic lines were also used in subsequent experiments for the gene expression analysis.

Arabidopsis seedlings were first grown on plates containing 2 to 6% glucose for 6 days in the dark. Because hypocotyls elongate extensively in the dark (Chory, 1993; Deng, 1994; Kendrick and Kronenberg, 1994), the inhibitory effect caused by glucose could be quantified easily. These dark-grown seedlings were then exposed to light for 12 hr to determine the effect of glucose on cotyledon expansion and greening triggered by light (Chory, 1993; Deng, 1994; Kendrick and Kronenberg, 1994).

In the control plants, hypocotyl length was inversely proportional to glucose concentration (Figures 7A and 8A). Under similar growth conditions, the *sense-AtHXK1* plants were hypersensitive to glucose, as determined by the severe reduction of hypocotyl length. Figures 7B and 8A show that the length of the hypocotyl of *sense-AtHXK1* plants was reduced 90% when grown on 6% glucose plates compared with those grown on 2% glucose plates. In contrast, the reduction of hypocotyl elongation in *anti-AtHXK1* plants was only near 50% when plants were grown on 6% glucose plates compared with those grown on 2% glucose plates (Figures 7C and 8A). As shown by the quantitative analysis (Figure 8A), although glucose $<2\%$ promotes seedling growth in the presence of other nutrients, hypocotyl inhibition by glucose $>2\%$ appears to reflect a specific sugar response mediated through AtHXK.

In addition to the repression of hypocotyl elongation, glucose (4 to 6%) also suppressed light-inducible cotyledon development in the control plants (Figures 7A and 8B). In the *sense-AtHXK1* plants, suppression was greater, as indicated by the etiolated and small cotyledons of seedlings treated with 4 to 6% glucose (Figures 7B and 8B). In contrast, glucose (2 to 6%) did not inhibit the development of

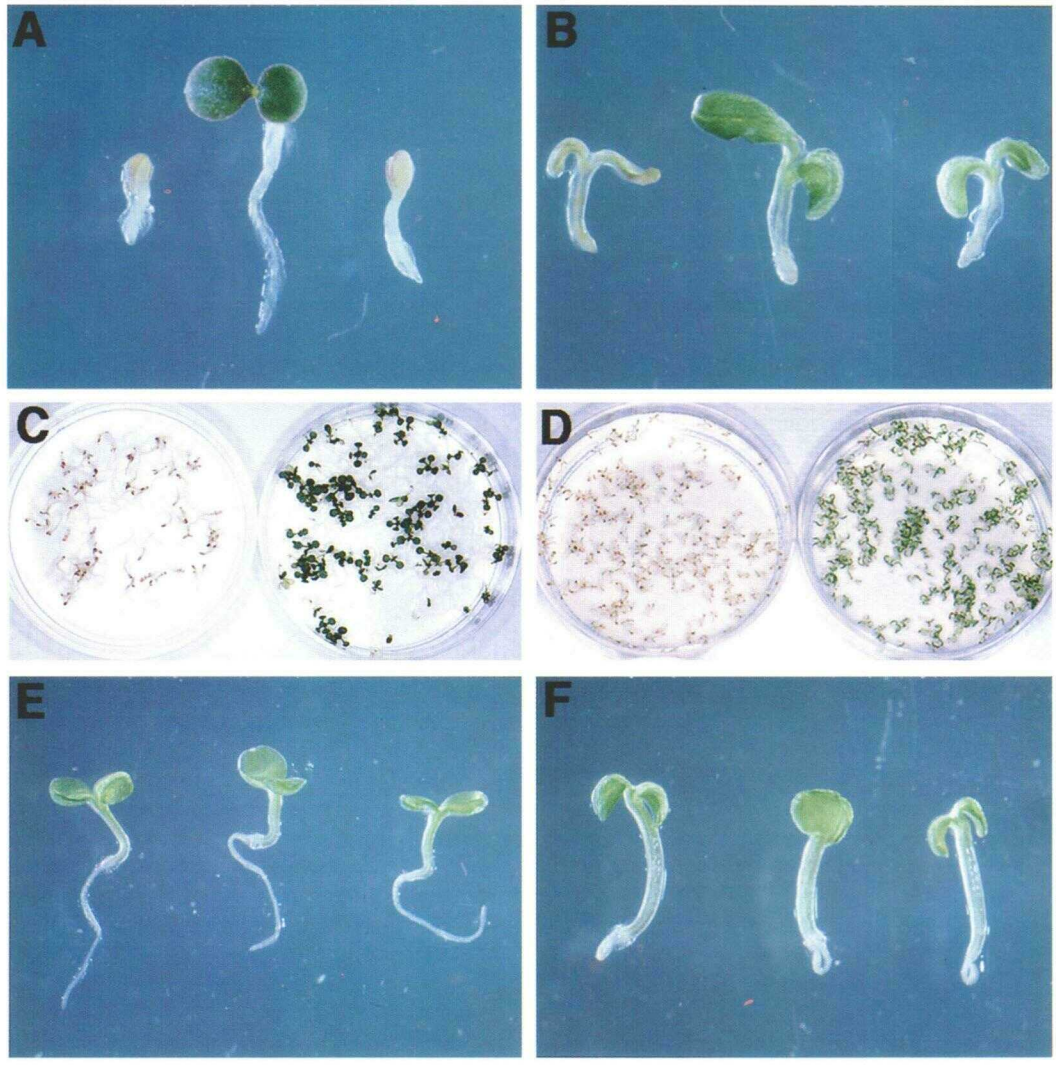

Figure 6. HXK Is a Sugar Sensor in Plants.

Arabidopsis seedlings were grown under constant light for 7 days.

(A) and **(B)** Transgenic plants overexpressing sense (left) and antisense (middle) *AtHXK1* were grown on half-strength Murashige and Skoog plates containing 6% glucose or 0.8 mM 2-dGlc, respectively. Wild-type control plants (right) are shown for comparison.

(C) and **(D)** A T_3 homozygous population of the *sense-AtHXK1* (left) and *anti-AtHXK1* (right) plants were grown on 6% glucose or 0.8 mM 2-dGlc plates, respectively.

(E) and **(F)** *Sense-AtHXK1* (left), *anti-AtHXK1* (middle), and control (right) plants were grown on half-strength Murashige and Skoog plates containing 6% mannitol or 6% 3-*O*-methylglucose, respectively.

the *anti-AtHXK1* plants, as determined by the expanded and green cotyledons (Figures 7C and 8B). The observed difference in the greening of the cotyledons was consistent with the measurements of chlorophyll content in the control and the transgenic plants (Figure 8B). Consistent with the results of the previous sugar-sensing assay, altered sugar responses during hypocotyl and cotyledon development were also observed in *sense-AtHXK2* and *anti-AtHXK2* plants (data not shown). Although the appearance of the *sense-AtHXK* and the *anti-AtHXK* plants was similar to that of the wild-type plants in the greenhouse, flowering time was altered in the transgenic plants (data not shown), implying that other developmental programs were also affected.

AtHXK Mediates Sugar-Dependent Gene Repression and Activation

Numerous genes have recently been shown to be regulated by sugar signals in higher plants. To determine whether

Table 1. Sugar Sensitivity in T_3 Homozygous Transgenic Plants[a]

		Glucose (6%)			2-dGlc (0.8 mM)		
Transgenic Plants	Total Lines	Hypo	Hyper	NA	Hypo	Hyper	NA
Sense-AtHXK1	13	0	11	2	0	13	0
Sense-AtHXK2	13	2[b]	8	3	1[b]	12	0
Anti-AtHXK1	14	9	3	2	13	0	1
Anti-AtHXK2	14	10	0	4	11	1	2

[a] Phenotypes were determined based on light-grown 7-day-old seedlings on half-strength Murashige and Skoog plates containing either 6% glucose or 0.8 mM 2-dGlc. *Sense-AtHXK* plants are transgenic plants carrying the 35S:*AtHXK* sense construct, and *anti-AtHXK* plants are transgenic plants carrying the 35S:*AtHXK* antisense construct. Sugar hyposensitive (Hypo) and hypersensitive (Hyper) phenotypes were scored. Transgenic lines with no altered phenotypes are designated as NA.

[b] Sugar hyposensitivity presumably results from cosuppression.

AtHXK is involved in sugar-dependent gene expression, we compared the expression levels of the sugar-repressible (Sheen, 1994) chlorophyll *a/b* binding protein (*CAB1*) and ribulose-1,5-bisphosphate carboxylase small subunit (*RBCS*) genes and one sugar-inducible gene, nitrate reductase (*NR1*) (Cheng et al., 1992), in the control, *sense-AtHXK*, and *anti-AtHXK* plants. We first examined 6-day-old seedlings grown on 6% glucose. Transcription of the *CAB1* and *RBCS* genes was low in the control plants but nearly abolished in both *sense-AtHXK1* and *sense-AtHXK2* plants exhibiting sugar hypersensitivity (Figure 9A). In contrast, both genes were expressed at high levels in both *anti-AtHXK* plants, indicating sugar hyposensitivity of gene expression. Consistent with the idea that the *sense-AtHXK* plants are hypersensitive to sugars, *NR1* was highly activated in both *sense-AtHXK1* and *sense-AtHXK2* plants but in neither the *anti-AtHXK* nor the control plants (Figure 9A). The data suggest that AtHXK is important for both sugar-repressible and sugar-inducible gene expression in higher plants.

To show that AtHXK regulates gene expression under physiological conditions, we examined *CAB1* and *RBCS* expression in 18-day-old green plants in the absence of exogenous sugars. Results showed that the transcript levels of both genes were nearly fivefold (quantitative data not shown) lower in the *sense-AtHXK* plants than in the *anti-AtHXK* or the control plants (Figure 9B). This differential expression is most likely due to the repression of light inducibility by endogenous sugars mediated through overexpressed AtHXK, because both genes showed uniformly low expression

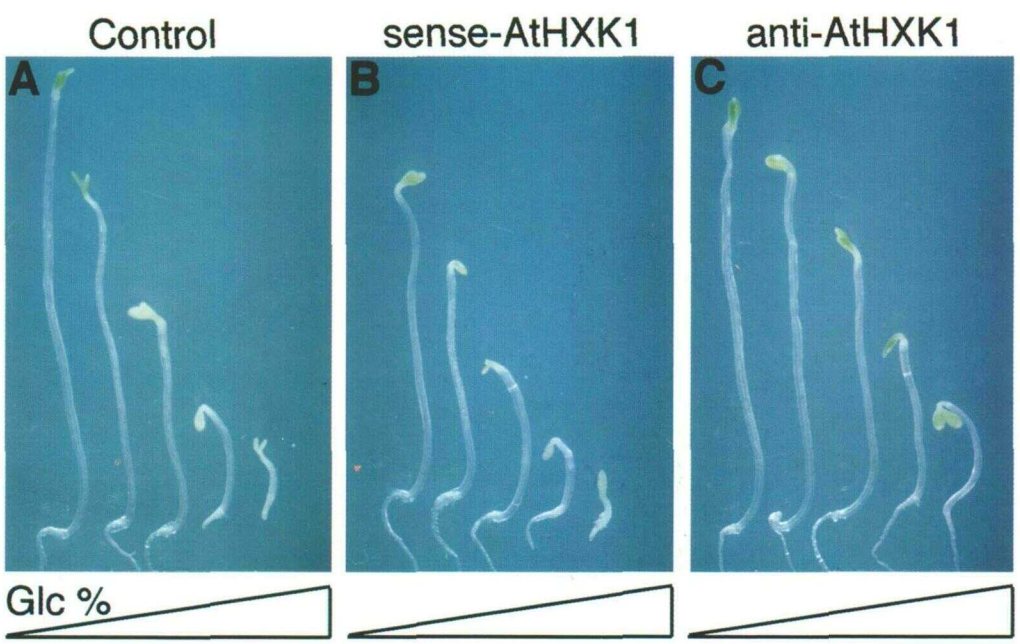

Figure 7. AtHXK Mediates Sugar Effects on Seedling Growth and Development.

Arabidopsis seedlings were grown on various concentrations (2, 3, 4, 5, and 6%) of glucose (Glc %) in the dark for 6 days and then illuminated for 12 hr.

(A) Wild-type (Control) plants.

(B) Transgenic plants carrying the 35S:*AtHXK1* sense construct (sense-AtHXK1).

(C) Transgenic plants carrying the 35S:*AtHXK1* antisense construct (anti-AtHXK1).

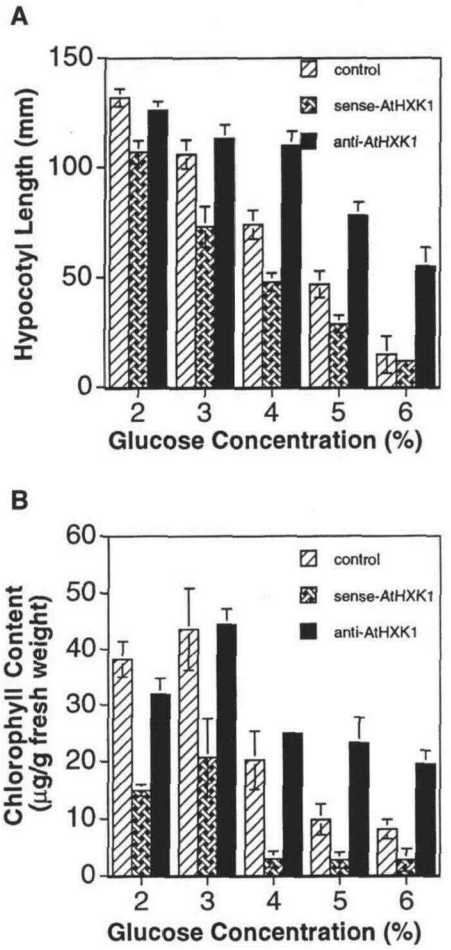

Figure 8. AtHXK Mediates Sugar Effects on Hypocotyl Elongation and Greening of Seedlings.

(A) Quantitation of hypocotyl length.
(B) Quantitation of chlorophyll content.
Seedlings partly shown in Figure 7 were used. Error bars represent standard deviations from 20 duplicated measurements.

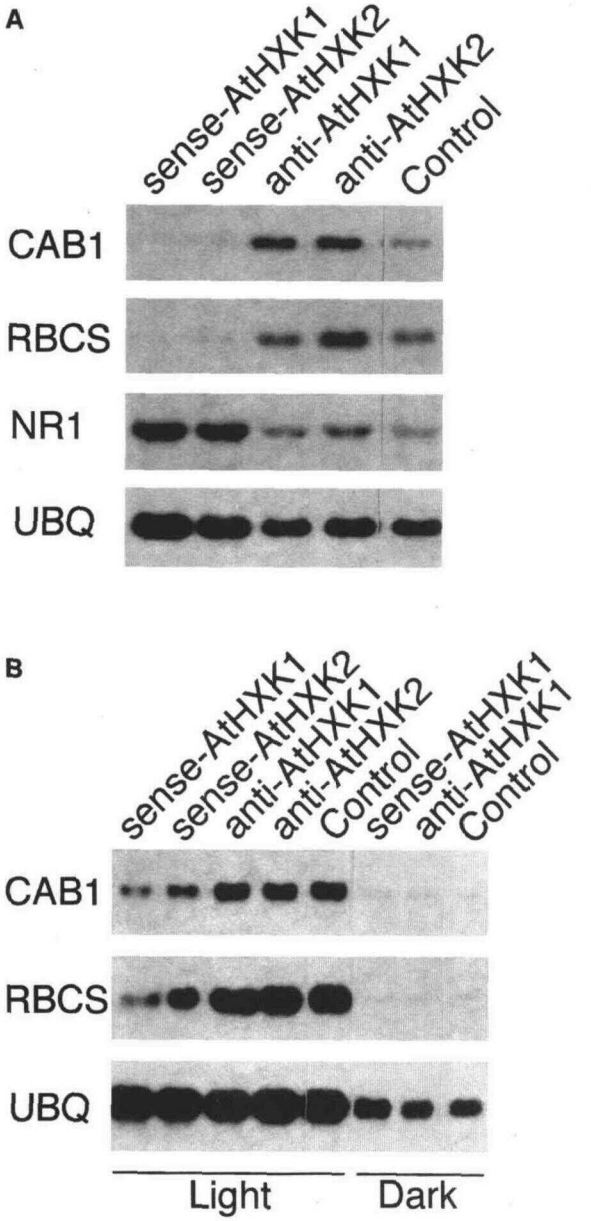

Figure 9. AtHXK Mediates Gene Repression and Activation.

(A) AtHXK mediates gene repression and activation in 6-day-old seedlings grown on 6% glucose plates. The repression of *CAB1* and *RBCS* and the activation of *NR1* were examined in the transgenic plants carrying the 35S:*AtHXK1* sense construct (sense-AtHXK1), transgenic plants carrying the 35S:*AtHXK2* sense construct (sense-AtHXK2), transgenic plants carrying the 35S:*AtHXK1* antisense construct (anti-AtHXK1), transgenic plants carrying the 35S:*AtHXK2* antisense construct (anti-AtHXK2), and wild-type (Control) plants by RNA gel blot analysis. Ubiquitin (UBQ) expression was used as a control.
(B) AtHXK mediates gene repression in 18-day-old green plants without exogenous glucose treatment. The plants were first dark adapted and then treated with (Light) or without (Dark) light.

in the transgenic and the control plants grown in the dark (Figure 9B; data not shown for *AtHXK2*).

Altered AtHXK Expression in Transgenic Plants

To confirm that the observed sugar hypersensitivity or hyposensitivity in transgenic plants correlates with transgene expression, we conducted RNA gel blot and immunoblot analyses. RNA gel blot analyses were performed by using gene- and strand-specific sense 1 and sense 2 and antisense 1 and antisense 2 probes. In 6-day-old seedlings, the transcript levels of *AtHXK1* (sense 1 RNA) in the *sense-AtHXK1* plants and *AtHXK2* (sense 2 RNA) in the *sense-*

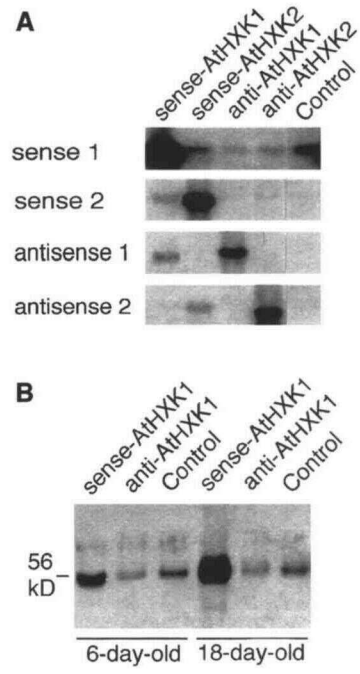

Figure 10. Transgene Expression.

(A) RNA gel blot analysis of the transgene expression. RNA samples were derived from 6-day-old seedlings grown on 6% glucose plates. Gene- and strand-specific sense 1 and sense 2 and antisense 1 and antisense 2 probes were used to detect *sense-AtHXK1*, *sense-AtHXK2*, *anti-AtHXK1*, and *anti-AtHXK2* transcripts, respectively. The blot that hybridized with the sense 1 probe was exposed longer. Wild-type plants were used as the control.

(B) Immunoblot analysis of AtHXK1 expression. Transgenic plants carrying the 35S:*AtHXK1* sense construct (sense-AtHXK1) showed elevated, and transgenic plants carrying the 35S:*AtHXK1* antisense construct (anti-AtHXK1) showed reduced expression of AtHXK1. Proteins were extracted from 6- or 18-day-old plants. Wild-type plants were used as the control. A molecular mass marker in kilodaltons (kD) is shown at left.

AtHXK2 plants were >20-fold higher than those in the control plants (Figure 10A). The antisense RNA of *AtHXK1* (antisense 1 RNA) and *AtHXK2* (antisense 2 RNA) were also highly expressed in the respective transgenic plants carrying antisense *AtHXK* genes (Figure 10A). The strand-specific antisense 1 and antisense 2 probes generated by polymerase chain reaction inevitably carried small amounts of the sense 1 and sense 2 probes and revealed weak signals corresponding to sense *AtHXK* transcripts (Figure 10A). In contrast, the endogenous *AtHXK1* transcript in the *anti-AtHXK1* and the *anti-AtHXK2* plants was reduced to <20% of the control level (Figure 10A). Although the sense 2 blot showed that the levels of the endogenous *AtHXK2* transcript were not reduced in *anti-AtHXK1* and *anti-AtHXK2* plants under this specific growth condition, significant reduction of the endogenous *AtHXK2* transcript was observed in 18-day-old green plants (data not shown). The results indicate that either the *anti-AtHXK1* or the *anti-AtHXK2* RNA is capable of reducing the endogenous RNA levels of both *AtHXK1* and *AtHXK2*, presumably because of the high level of sequence identity (Figure 2). Because the level of the endogenous *AtHXK2* transcript is extremely low in 6-day-old plants, the effect of the *anti-AtHXK2* construct might be reflected by the reduction of *AtHXK1* RNA in *anti-AtHXK2* plants (Figure 10A, sense 1 blot).

Immunoblot analyses were also performed with 6-day-old young seedlings and 18-day-old green plants. The results showed that AtHXK1 expression was five- to 10-fold higher in the *sense-AtHXK1* plants than in the control plants. In the *anti-AtHXK1* plants, the level of AtHXK1 was significantly lower than that in the control plants, although not completely abolished (Figure 10B).

Sugar Signaling Is Uncoupled from Sugar Metabolism in Plants

Although the effects of sugar in plants could be the result of unbalanced sugar metabolism or signaling from metabolic intermediates (Stitt et al., 1995), our previous studies of sugar sensing in a single-cell system in which sugars and metabolites can be delivered and monitored do not support this view (Jang and Sheen, 1994). To determine whether sugar metabolism is required for AtHXK-mediated sugar signal transduction in intact plants, we sought to overexpress a heterologous HXK that would provide sufficient catalytic activity for sugar metabolism but no regulatory function that would initiate the sugar signal transduction pathway in the transgenic plants. Yeast HXK2 (YHXK2) has been shown to be the sensor mediating glucose repression and has been proposed to have catalytic and regulatory functions (Entian et al., 1985). We reasoned that heterologous YHXK2 would be a good candidate for uncoupling sugar signaling from sugar metabolism in higher plants.

We first investigated whether the putative regulatory functions of YHXK2 and the Arabidopsis AtHXK are interchangeable by examining the effect of AtHXK overexpression on glucose repression in the yeast *hxk1 hxk2* double mutant (DBY2219). The assay was based on growth inhibition (glucose repression) mediated by YHXK2 in wild-type yeast cells on a 2-dGlc/raffinose plate. The yeast *hxk1 hxk2* double mutant could grow on the 2-dGlc/raffinose plate due to the lack of YHXK2 and the derepression of the invertase gene (Figure 11A). However, growth was inhibited when these mutant yeast cells were transformed with YHXK2 to restore glucose repression (Ma and Botstein, 1986; Ma et al., 1989). Although both AtHXK1 and AtHXK2 can complement the HXK catalytic activity in the yeast *hxk1 hxk2* double mutant (Figure 1), neither of them can complement the regulatory function of YHXK2 in glucose repression, as indicated by the growth of the transformed cells with either *AtHXK1* or

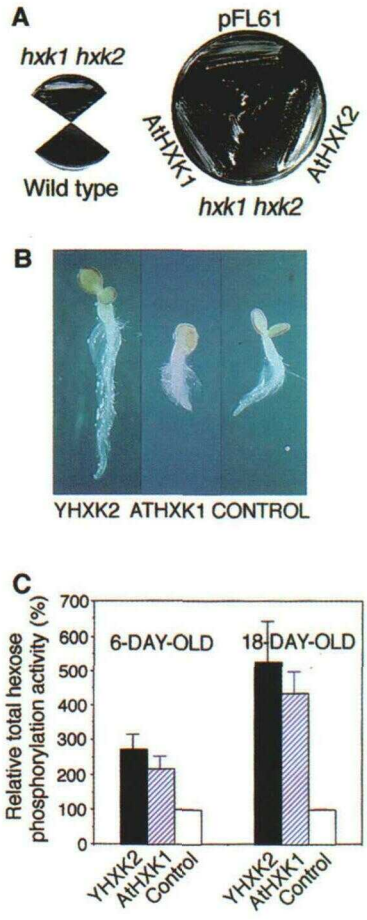

Figure 11. Sugar Signaling Is Uncoupled from Sugar Metabolism.

(A) AtHXK cannot restore glucose repression in the yeast *hxk1 hxk2* double mutant. The growth of the wild-type strain but not the double mutant *hxk1 hxk2* is inhibited on the 2-dGlc/raffinose plate (left). Overexpression of either AtHXK1 or AtHXK2 in the double mutant *hxk1 hxk2* did not restore glucose repression, as shown by their growth on the 2-dGlc/raffinose plate (right) that is similar to the double mutant transformed with vector pFL61.

(B) Dominant negative effect of YHXK2 in transgenic Arabidopsis. Transgenic plants carrying the 35S:*YHXK2* sense construct (*YHXK2* plants), transgenic plants carrying the 35S:*AtHXK1* sense construct (*AtHXK1* plants), and wild-type control plants were grown on 6% glucose plates for 7 days in constant light.

(C) Relative total hexose phosphorylation activities in 6- or 18-day-old *YHXK2*, *sense-AtHXK1*, and control plants. Error bars represent standard deviations.

AtHXK2 (Figure 11A). This result suggests that glucose repression is uncoupled from carbon flux in yeast.

To determine the effect of YHXK2 overexpression in plants, we introduced a cauliflower mosaic virus 35S–*YHXK2* construct into Arabidopsis. The transgenic plants overexpressing YHXK2 (*YHXK2* plants) displayed sugar hyposensitivity instead of sugar hypersensitivity in several assays. For instance, hypocotyl elongation, root growth, and greening of cotyledons were inhibited in the *sense-AtHXK1* or the control but not in the *YHXK2* plants grown on 6% glucose plates (Figure 11B). RNA gel blot analysis revealed that *CAB1* and *RBCS* were not repressed in the *YHXK2* plants grown with 6% exogenous glucose (data not shown). To ensure that *YHXK2* provides hexose phosphorylation activity in plants, we conducted a crude enzymatic assay using both 6- and 18-day-old transgenic plants. Figure 11C shows that total hexose phosphorylation activity in the *YHXK2* plants was 2.5-fold higher than that in the control plants and similar to or higher than that in the *sense-AtHXK1* plants. However, the *YHXK2* plants are sugar hyposensitive rather than hypersensitive (Figure 11B). This dominant negative effect of *YHXK2* in the transgenic plants presumably results from the overexpression of YHXK2, which competes with AtHXK for sugars but is not capable of transmitting the signal. We ruled out the possibility of gene silencing (Napoli et al., 1990; Matzke and Matzke, 1993; Flavell, 1994), based on the detection of transcript levels of *AtHXK1* and *AtHXK2* in *YHXK2* plants that were comparable to the levels in the control plants (data not shown).

DISCUSSION

Here, we report that HXK mediates sugar sensing in higher plants, based on our analyses of transgenic Arabidopsis plants with gain or loss of AtHXK function as well as on the fact that heterologous YHXK2 shows a dominant negative effect. The transgenic plants overexpressing AtHXK show sugar hypersensitivity and the antisense plants show sugar hyposensitivity in a variety of assays. It is apparent that the sugar phosphorylation enzyme HXK, acting as a sugar sensor, is evolutionarily conserved in bacteria, yeast, mammals, and higher plants. However, the dominant negative effect of *YHXK2* in transgenic plants and the apparent lack of signaling by AtHXK in yeast suggest that distinct regulatory domains for protein–protein interactions and diverse downstream signaling molecules might be employed in each system.

Sugars Profoundly Affect Plant Growth and Development

Sugars have long been shown to affect plant growth and development (Steeves and Sussex, 1989; von Schaewen et al., 1990; Sonnewald et al., 1991). However, it was not clear whether sugars act as specific regulatory signals in plant development or merely disturb cellular metabolism and cause osmotic stress. Based on our analyses of three types of transgenic Arabidopsis plants, we demonstrate here that the effects of sugar on seedling development are the conse-

quence of specific sugar sensing and signaling mediated through HXK.

In Arabidopsis seedlings, hypocotyl elongation is enhanced by darkness, auxin, brassinosteroids, and gibberellic acid but reduced by light, cytokinin, and ethylene. We report here that sugar negatively regulates hypocotyl elongation. It raises the possibility that sugars can interact with or regulate other internal and external signals to control hypocotyl elongation. Recent studies have shown that genes essential for the biosynthesis of ethylene are activated by sugars (Sonnewald et al., 1995), whereas genes involved in the biosynthesis of brassinolides are repressed by sugars (Szekeres et al., 1996). In addition, auxin (DeWald et al., 1994) and cytokinin (L. Zhou and J. Sheen, unpublished data) are able to block a subset of sugar responses. The analysis of sugar responses in the existing Arabidopsis mutants with altered hormone levels or responses might provide new insights into the cross-talk between sugar and hormone signal transduction pathways in higher plants.

Sugars as Regulatory Signals Controlling Gene Activation and Repression

Studies of the effects of sugar on gene expression in *Escherichia coli* and yeast have focused primarily on glucose repression (Saier et al., 1990, 1995; Gancedo, 1992; Trumbly, 1992; Ronne, 1995). However, it is apparent that both sugar repression and activation of gene expression are equally important in higher eukaryotes such as mammals (Granner and Pilkis, 1990) and plants (Sheen, 1994; Thomas and Rodriguez, 1994). By analyzing *CAB*, *RBCS*, and *NR* gene expression in transgenic Arabidopsis plants with altered AtHXK expression levels, we have demonstrated here that sugar-mediated gene activation and repression are controlled by the same sugar sensors, AtHXK1 and AtHXK2 (Figures 9A and 9B). Currently, the *cis*-acting elements and *trans*-acting factors involved in sugar regulation are not well characterized. Because sugar-mediated gene expression is affected by many diverse signal transduction pathways, distinct regulatory DNA elements and transcription factors are likely to be employed (Liu et al., 1990; Mass et al., 1990; Sheen, 1990, 1994; Kim et al., 1994; Sadka et al., 1994).

Many plant genes show quick response (<3 hr) to either low (<20 mM) (Graham et al., 1994; Jang and Sheen, 1994) or high (>100 mM) (Cheng et al., 1992; Chevalier et al., 1995) sugar concentrations. For instance, the maize *CAB5* gene responds to 1 to 10 mM of glucose in <3 hr (Jang and Sheen, 1994), and de novo protein synthesis is not required (J.-C. Jang and J. Sheen, unpublished data). However, other genes display a slow response (>48 hr) and require high sugar levels (Nakamura et al., 1991; Mason et al., 1992; Sadka et al., 1994; Koch et al., 1995). It is possible that distinct sugar-signaling pathways might be responsible for quick and slow sugar responses. Alternatively, the slow response might be indirectly regulated by sugars that alter cellular physiological and metabolic statuses. The availability of transgenic Arabidopsis plants with altered AtHXK levels should provide useful tools for distinguishing diverse sugar responses in plants. In addition, factors such as carbon and nitrogen balance, hormonal levels, and developmental stages can all influence sugar responses and should be considered when studying sugar regulation of gene expression in plants (Krapp et al., 1993; DeWald et al., 1994; Kovtun and Daie, 1995; L. Zhou and J.-C. Jang, unpublished data).

Sugar Signaling Mediated through HXK Is Uncoupled from Sugar Metabolism

It has been suggested that the effects of hexoses in plants could result from the effects of metabolites downstream of hexose phosphorylation (Stitt et al., 1995). However, our previous studies (Jang and Sheen, 1994) of sugar sensing in a single-cell system indicated the uncoupling of sugar signaling from sugar metabolism. For instance, metabolites downstream of glucose in the glycolytic pathway do not cause the repression of a glucose-sensitive reporter gene. In addition, both 2-dGlc and mannose can be phosphorylated by HXK, but the end products cannot be efficiently metabolized further, triggering strong repression (Jang and Sheen, 1994).

We show here that overexpression of Arabidopsis AtHXK1 and AtHXK2 results in sugar-hypersensitive phenotypes. However, overexpressing yeast YHXK2, despite enhancing catalytic activity, does not provide the regulatory function for sugar signaling in the transgenic plants (Figure 11). On the contrary, glucose responses are diminished in the transgenic plants overexpressing YHXK2. Analogously, AtHXK1 and AtHXK2 restore the catalytic (Figure 1) but not the regulatory (Figure 11A) function in the yeast *hxk1 hxk2* double mutant.

These observations suggest that AtHXK is a bifunctional enzyme with catalytic and regulatory activities. It is possible that specific HXK proteins are required for sugar signaling in each organism, and carbon flux might not be critical for triggering glucose repression. Furthermore, it has been shown that the genes encoding HXK from milk yeast (*Kluyveromyces lactis*) (Prior et al., 1993) and from fission yeast (*Schizosaccharomyces pombe*) (J.-C. Jang and J. Sheen, unpublished data) can complement the catalytic but not the regulatory function of YHXK2 in the *S. cerevisiae hxk1 hxk2* double mutant. These results further support the notion that glucose signaling may be uncoupled from sugar metabolism in yeasts and plants.

It is now evident that HXK acts as a sugar sensor in the signal transduction pathway mediating glucose-regulated gene expression in yeast, mammals, and plants. In yeast and higher plants, glucose repression does not require the glycolytic pathway, except for the first phosphorylation step by HXK. In mammals, glucose-mediated insulin secretion and insulin gene expression require glycolysis (German,

1993; Matschinsky et al., 1993; Mueckler, 1993; Grupe et al., 1995). In addition, insulin secretion has been shown to be enhanced by the overexpression of heterologous yeast YHXK2 (Epstein et al., 1992). However, a distinct glucose-signaling pathway similar to that in plants and yeast has recently been reported to mediate sugar-activated acetyl-CoA carboxylase gene expression in mammalian cells (Brun et al., 1993). It is likely that the structure and the role of HXK in sugar sensing and signaling are evolutionarily conserved in all eukaryotes.

METHODS

Yeast Manipulations and the Glucose Repression Assay

The *Saccharomyces cerevisiae hxk1 hxk2* double mutant (DBY2219) (Ma and Botstein, 1986) was transformed with an *Arabidopsis thaliana* expression library (Minet et al., 1992), as described by Gietz et al. (1992). The glucose repression assay (Ma et al., 1989) was performed using a YP plate (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% Bacto-agar) with 2% raffinose as the carbon source in the presence of 0.02% 2-deoxyglucose (2-dGlc). The glucose analog 2-dGlc mimicks glucose in that it induces strong repression of the invertase gene (*SUC2*) but is itself unable to be used as a carbon source. Wild-type yeast strains with normal glucose repression mechanisms cannot grow under these assay conditions. However, *SUC2* expression is derepressed in the *hxk1 hxk2* double mutant, allowing raffinose to be hydrolyzed to release fructose for its growth on the assay plate. Double-mutant *hxk1 hxk2* was transformed with either *AtHXK1* or *AtHXK2* to determine its function in glucose repression in yeast.

Plant Material

For the RNA gel blot and immunoblot analyses and the hexose phosphorylation assay, both 6-day-old seedlings and 18-day-old plants were used. For 6-day-old seedlings, seeds were germinated on plates containing half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with or without 6% glucose. Seedlings were grown in the dark for 6 days and then exposed to white light (120 μE m^{-2} sec^{-1}) for 4 hr. After being grown in the light for 15 days, 18-day-old green plants were dark adapted for 3 days and illuminated for 4 hr. Growth conditions for the 18-day-old plants were described previously (Cheng et al., 1992).

Molecular Analysis

DNA sequencing, DNA and RNA isolation, and gel blot analyses were performed as described by Ausubel et al. (1987). Amino acid sequence analysis was performed using the PILEUP and PRETTYBOX programs of the Genetics Computer Group (Madison, WI) set to standard parameters. For gene- and strand-specific probe synthesis used in the RNA gel blot analysis, polymerase chain reactions were used as described previously (Greenberg et al., 1994). Sense primers are 5′-ATGGGTAAAGTAGCTGTT-3′ and 5′-ATGGGTAAAGTGGCAGTTGCAA-3′ for *AtHXK1* and *AtHXK2*, respectively. Antisense primers are 5′-TTAAGAGTCTTCAAGGTAGAG-3′ and 5′-TTAACTTGTTTCAGAGTCATCTTC-3′ for *AtHXK1* and *AtHXK2*, respectively. The plasmid (pBluescript KS+; Stratagene, La Jolla, CA) containing either the *AtHXK1* or *AtHXK2* full-length cDNA was used as a template for the polymerase chain reaction. For immunoblot analysis, *AtHXK1* containing the entire open reading frame was subcloned into PET-19b (Novagen, Madison, WI) for overexpression in *Escherichia coli*. AtHXK1 was gel purified and used for the production of rabbit polyclonal antibodies. The antibodies were affinity purified, and immunoblot analyses were performed using the Phototope-Star immunoblot detection kit (New England Biolabs, Beverly, MA).

Plant Transformation

Root tissues of Arabidopsis ecotype Bensheim were used in the plant transformation experiments, as described by Czako et al. (1992).

Analysis of Sugar Sensitivity in Arabidopsis

Transgenic lines of the T_3 generation homozygous for the transgene were used for the analysis of sugar sensitivity. The transgenic and the control (wild-type) lines were germinated and grown in constant white light (100 μE m^{-2} sec^{-1}) for 7 days on half-strength Murashige and Skoog plates containing either 6% glucose or 0.8 mM 2-dGlc. To observe the effect of sugar on growth and development, transgenic and control lines were germinated and grown on half-strength Murashige and Skoog plates containing 2 to 6% glucose. Seedlings were grown in the dark for 6 days and illuminated with white light (100 μE m^{-2} sec^{-1}) for 12 hr. Quantitation of hypocotyl length and chlorophyll content was done by measuring 20 and 200 seedlings, respectively, from each glucose treatment in the control and the transgenic plants. Determination of chlorophyll content was performed as described by Graan and Ort (1984).

Hexose Phosphorylation Assay

The enzymatic assay for the total hexose phosphorylation activity was performed as described by Renz and Stitt (1993).

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