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The role of hexokinase in plant sugar signal transduction and growth and development

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Abstract

Previous studies have revealed a central role of *Arabidopsis thaliana* hexokinases (AtHXK1 and AtHXK2) in the glucose repression of photosynthetic genes and early seedling development. However, it remains unclear whether HXK can modulate the expression of diverse sugar-regulated genes. On the basis of the results of analyses of gene expression in *HXK* transgenic plants, we suggest that three distinct glucose signal transduction pathways exist in plants. The first is an AtHXK1-dependent pathway in which gene expression is correlated with the AtHXK1-mediated signaling function. The second is a glycolysis-dependent pathway that is influenced by the catalytic activity of both AtHXK1 and the heterologous yeast Hxk2. The last is an AtHXK1-independent pathway in which gene expression is independent of AtHXK1. Further investigation of *HXK* transgenic *Arabidopsis* discloses a role of HXK in glucose-dependent growth and senescence. In the absence of exogenous glucose, plant growth is limited to the seedling stage with restricted true leaf development even after a 3-week culture on MS medium. In the presence of glucose, however, over-expressing *Arabidopsis* or yeast *HXK* in plants results in the repression of growth and true leaf development, and early senescence, while under-expressing *AtHXK1* delays the senescence process. These studies reveal multiple glucose signal transduction pathways that control diverse genes and processes that are intimately linked to developmental stages and environmental conditions.

Introduction

Sugar effects on plant growth and development have often been attributed to sugar metabolism. However, recent evidence has shown that sugars can act as regulatory signals that control the expression of diverse genes involved in many processes in the plant life cycle (Koch, 1996; Jang and Sheen, 1997; Smeekens and Rook, 1997; Smeekens, 1998; Lalonde *et al.*, 1999; Roitsch, 1999; Sheen *et al.*, 1999). Plant hexokinase (HXK) has been shown to be involved in sugar sensing and signaling, and is proposed to be a dual-function enzyme with both catalytic and regulatory functions (Jang and Sheen, 1994; Jang *et al.*, 1997; Perata *et al.*, 1997; Umemura *et al.*, 1998; Pego *et al.*, 1999). Although additional evidence is needed to further elucidate how HXK functions as a sugar sensor, it has been shown that HXK-dependent signaling functions can be uncoupled with HXK-dependent metabolism (Jang *et al.*, 1997; Moore and Sheen, 1999). Transgenic *Arabidopsis* plants over- or under-expressing AtHXK show hypersensitivity and hyposensitivity to glucose, respectively. Furthermore, *Arabidopsis* plants over-expressing yeast *Hxk2* (*YHXK2*) were less sensitive to glucose repression of seedling development despite a several-fold increase of HXK catalytic activity (Jang *et al.*, 1997). More recently, it has been shown that low levels of mannose can block germination of *Arabidopsis* without affecting internal levels of ATP or inorganic phosphate. This germination repression can be overcome by a hexokinase competitive inhibitor, mannoheptulose, that might block the normal HXK conformational change induced by glucose and hence

inhibits both catalytic and signaling functions of HXK (Pego *et al.*, 1999).

Analogous mechanisms have also been found in yeast, in that hexokinase PI (Hxk1) and PII (Hxk2) are required for triggering glucose repression, and the *Hxk2* gene plays a major role in glucose repression (Entian, 1980; Rose *et al.*, 1991). It was shown that glucose 6-phosphate, the product of glucose phosphorylation catalyzed by Hxk, is not the trigger for glucose repression in yeast. For example, both glucokinase (Glk1) and Hxk1 can provide yeast cells with high levels of sugar-phosphorylating activities, but Glk1 cannot substitute Hxk2, and Hxk1 can only partially substitute Hxk2 in establishing glucose repression (Rose *et al.*, 1991). Recently, several novel mutant alleles of yeast *Hxk2* have been isolated whose ability to cause glucose repression is uncoupled with Hxk catalytic activity (Hohmann *et al.*, 1999). In several of those reported *hxk2* mutants, Hxk activity is about 100-fold lower than that in the wild type, but mutants can still initiate and maintain similar levels of glucose repression on a marker gene. In summary, these studies indicate that yeast Hxk2 has a distinct regulatory function in addition to its catalytic activity in metabolism.

Although a HXK-dependent sugar signal transduction pathway has been established, HXK-independent sugar signaling pathways also exist in plants (Jang and Sheen, 1997; Smeekens, 1998; Lalonde *et al.*, 1999; Roitsch, 1999; Sheen *et al.*, 1999). For example, while glucose analogue 6-deoxyglucose can be transported across the plasma membrane but cannot be phosphorylated by HXK, it activates the expression of genes encoding cell-wall invertase (*CIN*), sucrose synthase, and phenylalanine amonia-lyase1 (*PAL1*) (Godt *et al.*, 1995; Roitsch *et al.*, 1995; Ehness *et al.*, 1997). Likewise, 3-*O*-methylglucose is not a substrate of HXK but can also activate a patatin class I promoter in transgenic *Arabidopsis* (Martin *et al.*, 1997). These findings suggest that other sugar signaling pathways may exist in addition to the HXK-dependent sugar signaling pathway. However, it has been pointed out that the use of different plant systems has generated inconsistent or contradictory results (Roitsch, 1999), and more evidence is needed to support an HXK-independent signal transduction pathway. In addition, it remains unclear how sugars regulate the expression of diverse genes involved in pathogen defense, hormone biosynthesis and signaling, and carbon and nitrogen metabolism (Sheen *et al.*, 1999).

In addition to germination and seedling development, sugars have a broad influence on other processes such as internode elongation, root formation, and mature leaf development (von Schaewen *et al.*, 1990; Dickinson *et al.*, 1991; Jiang *et al.*, 1993; Weber *et al.*, 1998), embryogenesis and organ differentiation (Tang *et al.*, 1999), as well as leaf senescence (Ding *et al.*, 1993; Wingler *et al.*, 1998). However, it has been shown that over-expression of *AtHXK1* can promote senescence in transgenic tomato but not in *Arabidopsis* under normal growth conditions (Dai *et al.*, 1999). The differential response was presumably due to different plant species with different sugar sensitivity (Dai *et al.*, 1999). Alternatively, it may be possible that the manifestation of sugar responses is dependent on other signaling pathways triggered by hormones, light, and environmental stimuli that crosstalk with the sugar signaling pathways (Zhou *et al.*, 1998; Németh *et al.*, 1998; Sheen *et al.*, 1999). It remains unclear whether HXK plays a role in plant senescence under different developmental and environmental conditions.

To further define complex sugar responses in plants, we exploited various transgenic lines with altered levels of HXK and examined the expression of glucose-regulated genes involved in diverse functions and processes. Results of the gene expression analyses show that three distinct glucose signal transduction pathways are mediating the expression of genes regulated by sugars in plants. Furthermore, glucose, mediated through AtHXK1, can modulate plant growth and senescence and enhance the expression of a gene (*SAG21*) associated with senescence initiation (Weaver *et al.*, 1998).

Materials and methods

Plant materials and growth conditions

Seeds of *35S-AtHXK1*, *35S-antiAtHXK1*, *35S-YHXK2* (Jang *et al.*, 1997), and the wild-type *Arabidopsis thaliana* (ecotype Bensheim) were germinated and grown in the dark for 6 days on MS plates supplemented with 6% glucose. The etiolated seedlings were illuminated for 4 h before tissues were collected for RNA extraction. These 6-day old seedlings were used in RNA blot analysis to determine the expression of *CAB1*, *PC*, and *rbcS*. A liquid culture system (Cheng *et al.*, 1992) was employed in the experiments designed to determine the expression of other genes in *Arabidopsis*. Ten seeds of each genotype were germinated and grown for 14 to 15 days in a 250 ml

Erlenmeyer flask with 50 ml 1/2 MS medium supplemented with 2% sucrose. The cultures were grown at 25 °C, under constant white light (90 μmol m^{-2} s^{-1}) and constant agitation at 130 rpm. Seedlings were then transferred to the dark for 2–3 days in a glucose-free medium to deplete endogenous sugars. Plants were finally treated with 6% glucose for 4 h in the light before they were collected for RNA extraction (Jang *et al.*, 1997).

RNA blot hybridization

Total RNA isolation and RNA gel blot analyses were performed as described (Jang *et al.*, 1997). Probes of *CAB1*, *rbcS*, and *UBQ* were as described previously (Jang *et al.*, 1997). Probes of *PC* (Zhou *et al.*, 1998), *CHS* (Kubasek *et al.*, 1998), and *PAL1* and *PAL3* (Wanner *et al.*, 1995) were as described. *PLD* probe was an EST clone (accession number R30508) which shows sequence identity to *PLDα* (Dyer *et al.*, 1995). The other probes were obtained by PCR using a cDNA library (Minet *et al.*, 1992) and the following primers: *AS1* (accession number L29083), forward, 5′-CGGAATTCGTACTGGTAGTCATT; reverse, 5′-GCTCTAGAAGTGACGTGCAGTGA; *CIN1* (accession number X74514), forward, 5′-GCTCTAGAATGACCAAAGAAGTT; reverse, 5′-CGGAATTCCGTACCAATCGTGTA; *ERA1* (accession number U44849), forward, 5′-CGGGATCCTTAAGGCAGCTGGGTCCG; reverse, 5′-CGGAATTCAGATTCGAGGAGCCACCC; *PR1* (accession number M90508), forward 5′-CACAAGATTATCTAAG; reverse, 5′-GATCATAGTTGCAAC; *PR5* (accession number M90510), forward, 5′-CACTCTAGTAGGCGAT; reverse, 5′-CGTAGTTAGCTCCGGTA. The PCR products were cloned into pBluescript II SK.

Senescence analysis

The *HXK* transgenic and the wild-type plants were grown in 1/2 or 1/10 MS medium with or without 3% glucose for 21 days under constant white light (90 μmol m^{-2} s^{-1}), 25 °C, and constant agitation at 130 rpm. Ten seeds of each genotype were cultured in a 250 ml Erlemeyer flask with 50 ml medium. At day 21, duplicates of samples were photographed, collected for the quantitation of chlorophyll contents, petiole lengths, and dry weight, or used for RNA blot analysis. Chlorophyll was extracted from the fresh leaves by 100% methanol for 24 h. The chlorophyll contents (chl a + chl b, mg per gram fresh weight) were determined by measuring the absorption at OD_{665} and OD_{650}, respectively. *SAG21* probe was obtained via PCR from a cDNA library (Minet *et al.*, 1992) and by standard cloning procedures: *SAG21* (accession number AF053065), forward, 5′-CGGGATCCATGGCTTTAAAACATATGCA; reverse, 5′-CCATCGATCGCAGCTGCCTTGATTCT. RNA gel blot analysis was conducted as described (Jang *et al.*, 1997).

Results

The expression of photosynthetic genes is mediated by AtHXK1-dependent pathway

It has been shown previously that glucose repression of seedling development in *Arabidopsis* is controlled by an AtHXK1-mediated glucose signaling mechanism that is likely uncoupled with glucose metabolism (Jang *et al.*, 1997). This notion is supported, in principle, by the result of a five-fold increase of hexose phosphorylation activity but no inhibition of early seedling development in plants ectopically expressing yeast *Hxk2* (*YHXK2*). This was in contrast to the plants over-expressing *AtHXK1* in which both an elevated hexose phosphorylation activity and a strong inhibition of germination and early seedling development were observed. The results indicated that AtHXK1 has a specific signaling function not replaceable by *YHXK2* (Jang *et al.*, 1997).

To determine the role of AtHXK1 in glucose-regulated photosynthetic gene expression, we examined the effect of exogenous glucose on the expression of *CAB1* (chlorophyll *a/b*-binding protein), *PC* (plastocyanin), and *rbcS* (ribulose-1,5-bisphosphate carboxylase small subunit) genes in the *HXK* transgenic and the wild-type plants. The expression levels of *CAB1*, *PC*, and *rbcS* were very low in the wild-type plants as photosynthetic genes are repressed by glucose which overrides light activation (Sheen, 1990). The expression of these genes was further reduced in *35S-AtHXK1* plants, indicating that they are hypersensitive to glucose (Figure 1A). In contrast, no repression was observed in *35S-antiAtHXK1* plants (Figure 1A) since their expression levels were similar to those in the derepression condition (0–2% glucose, data not shown). Interestingly, the glucose repression of these photosynthetic genes was also diminished in two independent lines of *35S-YHXK2* plants (Figure 1A), although it was shown that their HXK activities were similar to those in *35S-AtHXK1* plants

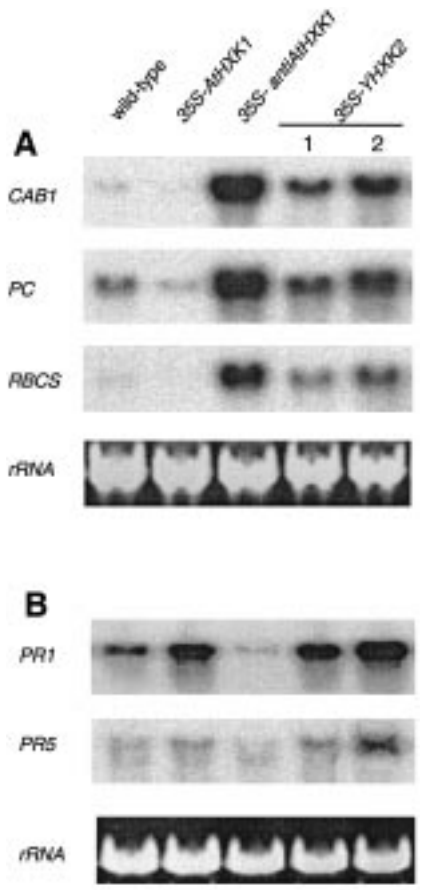

Figure 1. Effects of altered HXK levels on the expression of genes associated with photosynthesis and defense response. Four homozygous transgenic lines with altered HXK levels and the wild type (Bensheim ecotype) were used for the RNA blot analyses using ^{32}P-labeled probes. *35S-AtHXK1*, *AtHXK1* over-expressing line; *35S-antiAtHXK1*, *AtHXK1* under-expressing line; *35S-YHXK2*, yeast *HXK2* mis-expressing line. Total RNA (5 μg) was loaded in each lane using rRNA bands as loading controls. A. Photosynthetic gene expression mediated by the AtHXK1 signaling function. RNA samples were extracted from seedlings grown on MS plates with 6% glucose in the dark for 6 days and illuminated for 4 h. The analyzed genes encode: CAB1, chlorophyll *a*/*b*-binding protein; PC, plastocyanin; rbcS, ribulose-bisphosphate carboxylase small subunit. B. Expression of defense-responsive genes mediated by the HXK catalytic function. RNA samples were extracted from plants grown in liquid culture for 18 days under constant white light and treated with 6% glucose for 4 h after a glucose depletion step (see Materials and methods for growth conditions). PR, pathogenesis-related proteins.

(Jang *et al.*, 1997). The derepression of photosynthetic genes shown here correlates with the derepression of early seedling development in the *35S-YHXK2* that we reported earlier (Jang *et al.*, 1997). Together these data indicate that the regulation of *CAB1*, *PC*, and *rbcS* expression is mediated through an AtHXK1-dependent pathway. Presumably, the metabolism downstream of HXK does not have an effect on the expression of these photosynthetic genes. Furthermore, the yeast Hxk2 could not substitute AtHXK1 signaling function in the repression of photosynthetic gene expression.

The expression of PR1 *and* PR5 *is dependent on glycolysis*

Tobacco transgenic plants over-expressing a yeast invertase in the vacuole or cell wall showed concomitant accumulation of sugars and pathogenesis-related (PR) transcripts (*PAR-1*, *PR-1b*, *SAR8.2*, and *PR-Q*), as well as enhanced resistance to potato virus Y (Herbers *et al.*, 1996a). In tobacco leaf disks, *PAR1* and *PR-Q* transcripts can also be induced by glucose, fructose and sucrose (Herbers *et al.*, 1996b). It was proposed that the increased *PR* gene expression and decreased *CAB* gene expression are mediated by hexose signaling through a secretory pathway involving extracellular sugar recognition (Herbers *et al.*, 1996a; LaLonde *et al.*, 1999; Roitsch, 1999). To test whether HXK can modulate the effect of sugars on the expression of genes involved in defense, we analyzed the expression of two *Arabidopsis PR* genes (*PR1* and *PR5*) in the wild-type and various types of *HXK* transgenic plants. Because the expression of *PR* was below detectable level at the 6-day old seedling stage (data not shown), we adopted a liquid culture system in which the repression of *rbcS* gene and the activation of nitrate reductase gene (*NR*) induced by sugars were well established (Cheng *et al.*, 1992). Our results showed that glucose induction of *PR1* and *PR5* expression was higher in *35S-AtHXK1* plants than in the wild-type plants (Figure 1B). Loss of *PR* gene induction in *35S-antiAtHXK1* plants indicated the requirement of AtHXK1. However, unlike the regulation of *CAB1*, *PC* and *rbcS*, the induction of *PR1* and *PR5* was exaggerated to the same extent in two independent *35S-YHXK2* lines as in *35S-AtHXK1* (Figure 1B). These results suggest that *PR1* and *PR5* induction by glucose may depend on HXK catalytic activity but not the signalling function of AtHXK1. In this case, the signal(s) may not be glucose itself but rather an

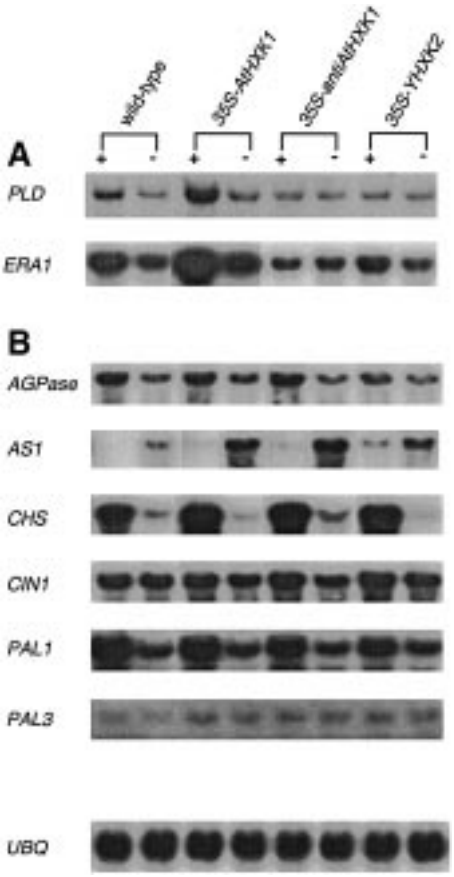

Figure 2. Expression of diverse glucose-regulated genes mediated by AtHXK1-dependent or AtHXK1-independent signaling pathways. Three homozygous transgenic lines with altered HXK levels and the wild type (Bensheim ecotype) were used for the RNA blot analyses with ^{32}P-labeled probes. *35S-AtHXK1*, *AtHXK1* over-expressing line; *35S-antiAtHXK1*, *AtHXK1* under-expressing line; *35S-YHXK2*, yeast *HXK2* mis-expressing line. RNA samples were extracted from plants grown in liquid culture for 16 days under constant white light then treated with (+) or without (−) 6% glucose for 4 h after a glucose depletion step (see Materials and methods for growth conditions). Total RNA (5 μg) was loaded in each lane. The expression of ubiquitin (*UBQ*) was used as a control which was not regulated by glucose. The analyzed genes encode: AGPase, ADP-glucose pyrophosphorylase; AS1, asparagine synthetase; CHS, chalcone synthase; CIN1, cell-wall invertase; ERA1, enhanced response to ABA; PAL1, phenylalanine amonia-lyase1; PAL3, phenylalanine amonia-lyase3; PLD, phospholipase D; UBQ, ubiquitin. A. Gene expression mediated by AtHXK1. B. Gene expression not mediated by AtHXK1.

unknown metabolite(s) downstream in the glycolytic pathway.

AtHXK1 mediates glucose regulation of diverse plant genes

To further understand the molecular basis of AtHXK1-mediated glucose signal transduction pathways in plants, we analyzed the expression of several functionally distinct sugar-regulated genes in the wild type and three types of *HXK* transgenic lines. To distinguish a direct glucose effect on transcription from those indirect or long-term sugar effects, the same liquid culture system was used for an effective and transient glucose induction (Cheng *et al.*, 1992). The results showed that the induction of *PLD* (phospholipase D, involved in leaf senescence) (Fan *et al.*, 1997) and *ERA* (enhanced response to ABA, involved in ABA signal transduction and meristem cell cycle control) (Culter *et al.*, 1996; Ziegelhoffer *et al.*, 1999) was also dependent on AtHXK1 (Figure 2A). While the activation of *PLD* expression was enhanced by glucose in the wild type and further enhanced in *35S-AtHXK1* plants, it was diminished in both *35S-antiAtHXK1* and *35S-YHXK2* lines (Figure 2A). Similar to the expression of photosynthetic genes (Figure 1A), glucose-regulated *PLD* expression was dependent on the signalling function of AtHXK1. Whereas the expression of *ERA1* was also up-regulated by glucose in the wild type and further enhanced in *35S-AtHXK1* plants (Figure 2A), there was little change in *35S-antiAtHXK1* and only a slight activation in *35S-YHXK2* plants (Figure 2A). Thus, the exaggerated expression of *ERA1* in *35S-AtHXK1* plants was dependent on AtHXK1 level but not HXK catalytic activity *per se*. Reduction of AtHXK1 in *35S-antiAtHXK1* plants abolished glucose induction. Thus the expression of *ERA1* modulated by AtHXK1 was also dependent on the signaling function of AtHXK1.

AtHXK1-independent glucose signaling pathway

In addition to the genes involved in photosynthesis and defense, sugars also regulate the expression of a broad spectrum of genes important for various processes in plants (Koch, 1996). The three types of *HXK* transgenic *Arabidopsis* provide a unique opportunity to gain more insight into the complexity of sugar regulation and to define molecular markers for the dissection of different sugar signaling pathways. We investigated the role of HXK in the modulation of the expression of a set of sugar-regulated genes involved in starch biosynthesis (*AGPase*, ADP-glucose pyrophosphorylase) (Villand *et al.*, 1993), sucrose metabolism (*CIN1*, cell-wall invertase) (Schwebel-Dugue *et al.*, 1994), nitrogen metabolism (*AS1*, asparagine synthetase) (Lam

et al., 1994), stress and pathogen responses as well as secondary metabolism (*CHS*, chalcone synthase; *PAL1* and *PAL3*, phenylalanine ammonia-lyase) (Wanner *et al.*, 1993; Kubasek *et al.*, 1998)).

The results showed that *AGPase*, *CHS*, and *PAL1* were up-regulated, whereas *AS1* was repressed by glucose (Figure 2B). These results are consistent with previous studies conducted in diverse plant species and under different growth conditions (Tsukaya *et al.*, 1991; Krapp and Stitt, 1995; Ehness *et al.*, 1997). Interestingly, the effect of glucose on the expression of these genes was independent of the 3 types of transgenic plants (Figure 2B). The expression of *CIN1* and *PAL3* was not regulated by glucose under this specific condition. As a control, we showed that expression of the ubiquitin gene (*UBQ*) was affected neither by glucose nor by HXK levels (Figure 2B).

AtHXK1 mediates the effect of glucose on plant growth and senescence

Sugars have been implicated in the control of plant growth and senescence. For instance, over- or under-expression of invertases in transgenic tobacco, tomato, or carrot plants could alter sucrose metabolism, shoot production, leaf morphology, and senescence (von Schaewen *et al.*, 1990; Dickinson *et al.*, 1991; Sonnewald *et al.*, 1991; Herbers *et al.*, 1996a; Tang *et al.*, 1999). However, it is not clear whether HXK plays a role in growth and development beyond the seedling stage. To address this question, we examined the sugar effects on mature plant development and senescence in HXK transgenic plants using physiological and molecular approaches.

We showed here that plant growth and development were affected by exogenous glucose in the liquid culture condition (Figure 3A and 3B). In the absence of exogenous glucose, it was observed that the growth of the wild-type and the three types of *HXK* transgenic lines was severely inhibited, and seedlings exhibited a morphology with restricted true leaf development even after a 21-day culture period (Figure 3A). No obvious difference was observed in the chlorophyll contents (Figure 4) or dry weight (data not shown) among the wild-type and transgenic plants in the absence of exogenous sugar. In contrast, extensive plant growth and true leaf development were observed in the presence of 3% glucose (Figure 3B). On the average, 3–4 pairs of expanded true leaves were produced after 21 days. The stature of the plants was much larger in the presence of glucose than that in the absence of glucose (Figure 3A and 3B). However, increased levels of AtHXK1 in the transgenic plants did not yield higher rates of shoot and root growth. Leaves were much smaller and yellower and roots turned brown in *35S-AtHXK1* as compared to those of the wild-type plants. The petioles of the first and second true leaves of *35S-AtHXK1* were significantly ($\alpha = 0.01$) shorter than those of the wild-type plants (Table 1). Dry weight of *35S-AtHXK1* was significantly ($\alpha = 0.01$) less than that of the wild-type plants (Table 1). In contrast, leaves of *35S-antiAtHXK1* plants were darker green

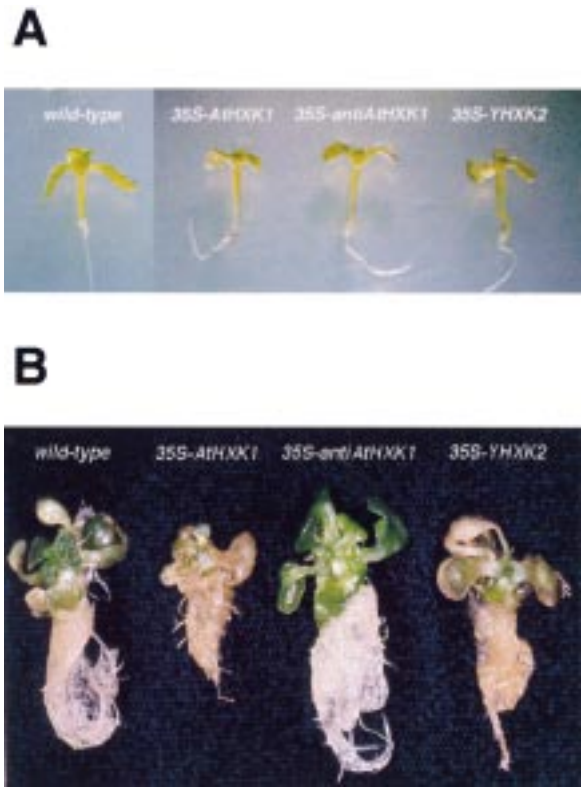

Figure 3. Glucose effects on growth and senescence in *HXK* transgenic plants. Three homozygous transgenic lines with altered HXK levels and the wild type (Bensheim ecotype) were used in the experiment. *35S-AtHXK1*, *AtHXK1* over-expressing line; *35S-antiAtHXK1*, *AtHXK1* under-expressing line; and *35S-YHXK2*, yeast *HXK2* mis-expressing line. A. Limited plant growth in the medium without glucose. The first pair of true leaves were only partially expanded, and no morphological difference between different HXK transgenic lines and the wild-type was seen when plants were grown under constant white light for 21 days in liquid MS medium without glucose. B. Glucose-dependent leaf growth and senescence mediated by HXK. Plants developed 3–4 pairs of true leaves after they were cultured under constant white light for 21 days in a liquid 1/2 MS medium with 3% glucose. In contrast to the wild type and *35S-antiAtHXK1*, decreased leaf size and early senescence were observed in *35S-AtHXK1* and *35S-YHXK2*. Photographs of A and B are shown in the same magnification.

Table 1. Petiole lengths of the first and the second true leaves and dry weight in wild-type and HXK transgenic lines.

	Wild type	*35S-AtHXK1*	*35S-antiAtHXK1*	*35S-YHXK2*
1st true leaf	4.82	2.84	5.99	3.20
2nd true leaf	4.86	2.87	6.09	3.22
Dry weight	0.2938	0.1436	0.3112	0.2375

Plants as shown in Figure 3 were grown for 3 weeks in the presence of 3% glucose in the liquid culture system. Average petiole lengths (mm) of the first and the second true leaves and total dry weight (g) of 10 plants were measured. Values of petiole lengths represent the means of 15 independent measurements and values of dry weight represent the means of 3 independent measurements.

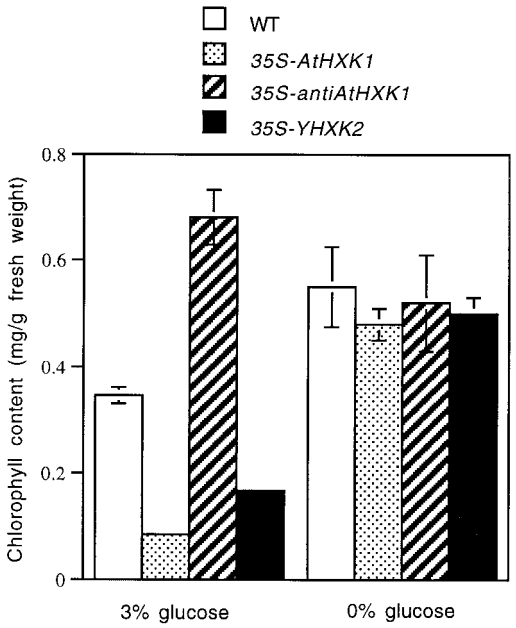

Figure 4. Effects of altered HXK levels on chlorophyll contents. Three homozygous transgenic lines with altered HXK levels and the wild type (Bensheim ecotype) were used in the experiment. *35S-AtHXK1, AtHXK1* over-expressing line; *35S-antiAtHXK1, AtHXK1* under-expressing line; and *35S-YHXK2,* yeast *HXK2* mis-expressing line. Plants were cultured in liquid MS medium without or with 3% glucose under constant white light for 21 days. Chlorophyll contents (chl a + chl b) were determined as described in Materials and methods. Data represent the mean and standard error of three experiments.

and the roots did not show any senescence phenotype as indicated by the white color (Figure 3). When compared with the wild type, the petioles of the first and second true leaves of *35S-antiAtHXK1* plants were significantly ($\alpha = 0.01$) longer which was consistent with a greater dry weight of the whole plant (Table 1). Over-expressing *YHXK2* resulted in the repression of shoot growth and promotion of senescence when compared to the wild-type, but to a lesser extent than that observed in *35S-AtHXK1*. In summary, these results indicate that the effect of glucose on plant growth and development is mediated by AtHXK1.

There was a notable early senescence in *35S-AtHXK1* and *35S-YHXK2* plants as reflected by the yellow shoots in the presence of 3% glucose (Figure 3B). This suggested that the overexpression of *AtHXK1* or *YHXK2* caused a decrease in chlorophyll synthesis and/or an increase in chlorophyll degradation as a result of early leaf senescence. In the presence of 3% glucose, chlorophyll contents in *35S-AtHXK1* and *35S-YHXK2* plants was about 25% and 50% of that in the wild type, respectively, whereas chlorophyll contents in *35S-antiAtHXK1* plants was about two-fold higher than that of the wild type (Figure 4). In the absence of glucose, there was no obvious difference in the chlorophyll contents among the wild-type and all 3 types of *HXK* transgenic plants. To determine whether the reduction of chlorophyll contents and the brown color in root systems were associated with the onset of senescence, we analyzed the expression of a senescence-associated marker gene, *SAG21* (Weaver *et al.,* 1998). *SAG21* was highly expressed in *35S-AtHXK1* plants but not detectable in *35S-antiAtHXK1* under the same conditions (Figure 5). Over-expression of *YHXK2* in plants resulted in higher levels of expression of *SAG21* when compared with the wild type. This result indicates that early senescence in *35S-YHXK2* is likely caused by altered metabolism due to elevated catalytic activity of HXK.

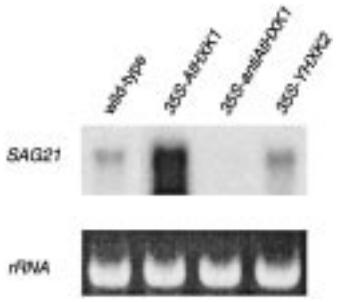

Figure 5. *SAG21* expression in *HXK* transgenic plants. Three homozygous transgenic lines with altered levels of HXK and the wild type (Bensheim ecotype) were used for the RNA blot analyses with ^{32}P-labeled *SAG21* probe, *35S-AtHXK1*, *AtHXK1* over-expressing line; *35S-antiAtHXK1*, *AtHXK1* under-expressing line; and *35S-YHXK2*, yeast *HXK2* mis-expressing line. The expression of a senescence-associated gene (*SAG21*) was positively correlated with the levels of HXK in the transgenic and the wild-type plants. RNA samples were extracted from plants cultured under constant white light for 21 days in liquid 1/2 MS medium with 3% glucose. Total RNA (5 μg) was loaded in each lane with rRNA bands as loading controls.

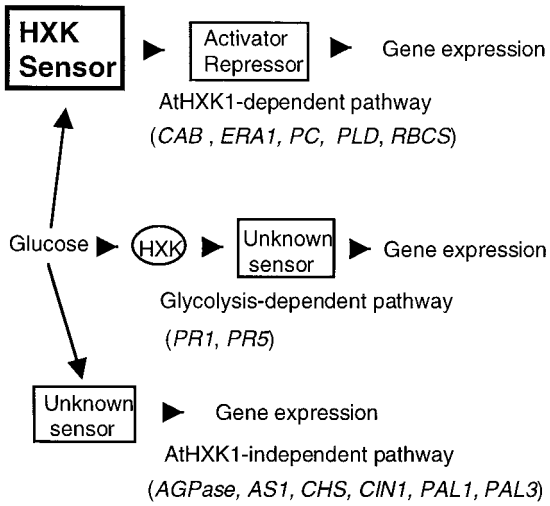

Figure 6. Three glucose signal transduction pathways in plants.

Discussion

Multiple glucose signal transduction pathways exist in plants

While sugar-regulated gene expression has been studied for years, it is not clear what the direct sugar signal is, whether sugar metabolism is important, whether HXK is involved in the sensing process, or whether the signalling and/or catalytic activities of HXK are required. Results of the present study suggest that at least three distinct glucose signal transduction pathways exist in plants. The first is an AtHXK1-dependent pathway, in which gene expression is mediated through the signaling function of AtHXK1 (Figures 1, 2, and 6). Apparently, glucose-phosphorylating activity *per se* is not crucial for this pathway since over-expression of a heterologous *YHXK2* caused dominant negative or little effect on the expression patterns of *CAB1*, *PC*, *PLD*, and *rbcS* despite a several-fold increase in HXK catalytic activity. The simplest interpretation is that YHXK2 can effectively phosphorylate glucose in the cells but fails to provide the signaling function due to other reasons. For example, the conformational change of HXK upon the binding of glucose needs to be specific in order to interact with a second protein that is involved in signal transduction but not metabolism. It is noteworthy that AtHXK1 and AtHXK2 are likely to be membrane proteins (predicted by Protein Secondary Structure Prediction Tool, http://www.tuat.ac.jp/~mitaku/adv-SOSUI), whereas YHXK2 is a soluble protein present in both the cytoplasm and the nucleus (Randez-Gil *et al.*, 1998). Distinct cellular localization has provided a possible rationale for the inability of YHXK2 to replace the signaling function of AtHXK1 in the regulation of *ERA1* expression. Alternatively, it may be possible that metabolism downstream of hexose phosphorylation has also been altered in *YHXK2* plants and the change could, indirectly but ultimately, affect the expression of photosynthetic genes.

The second pathway is dependent on the catalytic activity of HXK. The glucose-activated expression of *PR1* and *PR5* was similar in *35S-AtHXK1* and *35S-YHXK2* plants (Figure 1). Thus, the expression of *PR* genes may depend on the levels of an unknown metabolite downstream of HXK in the glycolytic pathway. This glycolysis-dependent pathway may be similar to the pathway for glucose-induced insulin responses in mammals. It is well established that insulin synthesis and secretion requires glycolysis, and the intermediates of anaerobic glycolysis between fructose 1,6-diphosphate and phospho*enol*pyruvate are essential for β-cell glucose sensing in the pancreatic islets (German, 1993). It remains unclear what direct metabolic signals and sensors are for the regulation of *PR* genes. Although the *PR* gene expression may be linked to salicylate induction (Herbers *et al.*, 1996a), our analysis of the regulation of *PAL1* gene, important for salicylate biosynthesis, suggests that *PAL1* is regulated by a third distinct glucose signaling pathway that is independent of AtHXK1 (Figures 2B and 6).

Our present data indicate that the expression of *AGPase*, *AS1*, *CHS*, and *PAL1* is mediated by an AtHXK1-independent pathway (Figure 6). Components in this AtHXK1-independent signaling pathway are currently unknown. Nevertheless, the existence of two glucose transporter-like proteins, Rgt2 and Snf3, as sugar sensors in yeast has prompted some speculations that similar mechanisms may exist in plants (LaLonde *et al.*, 1999; Roitsch, 1999; Sheen *et al.*, 1999). It is intriguing that neither Rgt2 nor Snf3 appears to be able to transport glucose. Rather, they behave like typical cell surface receptors that can transduce the glucose signal to a HXK-independent signaling pathway, leading to a transcriptional repression of authentic sugar transporters (Johnston, 1999). In *Arabidopsis*, 26 homologous sequences of monosaccharide transporters have been identified (Lalonde *et al.*, 1999). It is possible that sugar transporter-like proteins and other extracellular sugar-binding proteins serve as sugar sensors to perceive and transmit the glucose signal in AtHXK1-independent pathway(s).

Gene regulation influenced by crosstalk between sugar and other signalling pathways

One major complication in understanding sugar responses in plants is the existence of crosstalk between sugar and other signaling pathways. It seems that sugar production and sugar signal transduction pathways are intimately linked to other regulatory pathways to coordinate nutrient distribution and utilization, growth and development (Sheen *et al.*, 1999). For instance, genes involved in both carbon (*AGPase*) and nitrogen (*AS1*) metabolism were regulated by glucose. *ERA1* and *PLD*, involved in ABA signaling and senescence, were regulated by glucose through the AtHXK1-dependent pathway (Figure 6). Interestingly, *WIGGUM (WIG)*, involved in the control of floral and apical meristem size and floral organ number in *Arabidopsis*, has recently been cloned and turned out to be *ERA1* (Ziegelhoffer *et al.*, 1999). Induction of *ERA1* by glucose suggests that AtHXK1-mediated sugar signaling may interact with ABA signaling cascades in turn to modulate meristem cell division. Genetic analysis of the glucose-insensitive mutant *gin1* in *Arabidopsis* has unraveled the crosstalk between glucose and ethylene signaling (Zhou *et al.*, 1998). Furthermore, the analysis of the *Arabidopsis prl1* mutant (pleiotropic regulatory locus) has revealed complex interactions between sugars and many other plant hormones (Németh *et al.*, 1998). In conclusion, our results in the analysis of sugar-regulated gene expression are consistent with previous genetic studies suggesting the existence of crosstalk between sugar and other signaling pathways in plants.

Role of AtHXK1 in glucose-regulated plant growth and development

It was previously shown that high levels of glucose could block expansion and greening of cotyledons and inhibit shoot development through an AtHXK1-dependent mechanism (Jang *et al.*, 1997). Here we further show that glucose exerts two additional levels of regulation in leaf development: energy production for growth, and a signal molecule for the modulation of leaf expansion and senescence. In the absence of glucose, true leaf development was inhibited. The addition of 3% glucose promoted true leaf expansion in an AtHXK1-dependent manner. Overexpression of *AtHXK1* did not enhance but rather inhibited leaf expansion (Figure 3B). The inhibition of true leaf expansion may be linked to AtHXK1-dependent promotion of early leaf senescence manifested by chlorophyll reduction (Figure 4) and the activation of *SAG21* for senescence initiation (Figure 5). One possible underlying molecular mechanisms would be that the glucose activation of *PLD* and *ERA1* through the AtHXK1-dependent pathway mimics ABA- and ethylene-promoted senescence in detached leaves (Fan *et al.*, 1997).

The complexity of sugar sensing and sugar-regulated gene expression has just begun to be unraveled. In the near future, new technology such as DNA microarray will provide a global view of sugar-regulated gene expression at the whole-genome level (Schena *et al.*, 1995; DeRisi *et al.*, 1997). Phenotypic and genetic analyses of sugar signaling mutants and molecular cloning of these mutant loci will further elucidate the signaling network involving sugar, hormone, light, and other environmental stimuli in plants.

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