

Overexpression of Arabidopsis Hexokinase in Tomato Plants Inhibits Growth, Reduces Photosynthesis, and Induces Rapid Senescence

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Sugars are key regulatory molecules that affect diverse processes in higher plants. Hexokinase is the first enzyme in hexose metabolism and may be a sugar sensor that mediates sugar regulation. We present evidence that hexokinase is involved in sensing endogenous levels of sugars in photosynthetic tissues and that it participates in the regulation of senescence, photosynthesis, and growth in seedlings as well as in mature plants. Transgenic tomato plants overexpressing the Arabidopsis hexokinase-encoding gene *AtHKK1* were produced. Independent transgenic plants carrying single copies of *AtHKK1* were characterized by growth inhibition, the degree of which was found to correlate directly to the expression and activity of *AtHKK1*. Reciprocal grafting experiments suggested that the inhibitory effect occurred when *AtHKK1* was expressed in photosynthetic tissues. Accordingly, plants with increased *AtHKK1* activity had reduced chlorophyll content in their leaves, reduced photosynthesis rates, and reduced photochemical quantum efficiency of photosystem II reaction centers compared with plants without increased *AtHKK1* activity. In addition, the transgenic plants underwent rapid senescence, suggesting that hexokinase is also involved in senescence regulation. Fruit weight, starch content in young fruits, and total soluble solids in mature fruits were also reduced in the transgenic plants. The results indicate that endogenous hexokinase activity is not rate limiting for growth; rather, they support the role of hexokinase as a regulatory enzyme in photosynthetic tissues, in which it regulates photosynthesis, growth, and senescence.

INTRODUCTION

Sugars are central compounds in nature that serve as essential metabolic nutrients and structural components for most organisms. They are also major regulatory molecules that control gene expression, metabolism, physiology, cell cycle, and development in prokaryotes and eukaryotes (Newgard and McGarry, 1995; Ronne, 1995; Saier et al., 1995). In plants, it has been shown that sugars regulate the expression of a broad spectrum of genes involved in many essential processes (e.g., Sheen, 1990; Thomas and Rodriguez, 1994; Stitt and Sonnewald, 1995; Graham, 1996; Koch, 1996; Smeekens, 1998). Furthermore, sugars affect developmental and metabolic processes throughout the life cycle of the plant. These processes include germination,

growth, flowering, senescence, sugar metabolism, and photosynthesis (von Schaewen et al., 1990; Dickinson et al., 1991; Stitt et al., 1991; Goldschmidt and Huber, 1992; Huber and Hanson, 1992; Bernier et al., 1993; Ding et al., 1993; Yang et al., 1993; Thomas and Rodriguez, 1994; Dangl et al., 1995; Kovtun and Daie, 1995; Jang and Sheen, 1997; Perata et al., 1997; Prata et al., 1997; Smeekens and Rook, 1997; Wingler et al., 1998).

Photosynthesis is regulated by sugar levels, and this regulation overrides that of light, tissue type, and developmental stage (Sheen, 1990; von Schaewen et al., 1990; Krapp et al., 1993). Increased concentrations of externally supplemented sugar or internal modification of sugar content inhibits photosynthesis and causes stunted growth of various plants (von Schaewen et al., 1990; Dickinson et al., 1991; Sonnewald et al., 1991, 1993; Goldschmidt and Huber, 1992; Jones et al., 1996; Jang et al., 1997). In addition, many species exhibit a progressive decline in the rate of photosynthesis after full leaf expansion, and it has been

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suggested that photosynthesis is feedback regulated by the accumulation of carbohydrates in source leaves (Hensel et al., 1993; Jiang et al., 1993; Wingler et al., 1998). Senescence is also thought to be regulated by sugars. It has been hypothesized that leaf senescence is initiated when the photosynthetic rate drops below a certain threshold. At this threshold, the leaf no longer contributes fixed carbon to the rest of the plant (Ding et al., 1993; Hensel et al., 1993; King et al., 1995; Gan and Amasino, 1997; Wingler et al., 1998).

The mechanisms by which higher plants recognize and respond to sugars are largely unknown. Recently, it was demonstrated that hexokinase, which catalyzes sugar phosphorylation, the first step of hexose metabolism, is involved in glucose sensing (Jang and Sheen, 1994; Jang et al., 1997; Perata et al., 1997; Prata et al., 1997). Sugar-repressible, photosynthetic, and metabolic genes have been used to show that phosphorylation of sugars by hexokinase is critical for sugar signaling. For example, only sugars that can be phosphorylated by hexokinase repressed expression of photosynthetic genes, such as chlorophyll *a/b* binding protein (*CAB1*) (Jang and Sheen, 1994). Furthermore, steps in sugar metabolism beyond the phosphorylation of glucose are not required to elicit sugar-mediated regulation of such photosynthetic genes (Jang and Sheen, 1994).

Further evidence for the involvement of hexokinase in the repression of photosynthetic genes was recently presented by Jang et al. (1997). Using transgenic Arabidopsis seedlings expressing sense and antisense Arabidopsis hexokinase (*AtHXK1*), they analyzed the role of hexokinase in whole plants. Transgenic seedlings overexpressing *AtHXK1* were reported to be hypersensitive to increasing concentrations of exogenous glucose (2 to 6%), with reduced hypocotyl elongation, low expression of photosynthetic genes, and repressed greening of the cotyledons. Transgenic seedlings in which the expression of hexokinase was reduced by using an antisense approach showed a markedly reduced ability to sense the presence of exogenous glucose, and they displayed accelerated hypocotyl elongation and enhanced expression of photosynthetic genes. In a separate experiment, in which a heterologous hexokinase from yeast was introduced and overexpressed in Arabidopsis plants, no enhanced sensitivity to glucose was observed. Therefore, it was suggested that Arabidopsis hexokinase is a dual-function enzyme that plays a role in sugar sensing and signaling in addition to its role in phosphorylating hexoses (Jang et al., 1997).

In this study, we analyzed under natural growth conditions the role of hexokinase in transgenic tomato plants that exhibited varying levels of hexokinase activity. We were thereby able to assess the role of hexokinase in the response to endogenous sugar levels. The effects of increased hexokinase activity were observed for seedlings as well as for mature plants that were grown using standard horticultural procedures. We demonstrate that hexokinase regulates photosynthesis, growth, and senescence in plants overexpressing *AtHXK1* in photosynthetic tissues.

RESULTS

Analysis of *AtHXK1*

To analyze the role of hexokinase in plant development, we previously isolated an Arabidopsis hexokinase cDNA (Dai et al., 1995) by complementation of a yeast triple mutant (*hvk1 hvk2 glk1*) that lacks glucose and fructose phosphorylating ability and therefore is unable to grow either on glucose or on fructose (Fraenkel, 1982). The Arabidopsis hexokinase cDNA complemented the inability of the mutant yeast cells to grow on glucose and fructose. The hexokinase cDNA that we isolated is identical to the *AtHXK1* clone isolated by Jang et al. (1997). They determined that *AtHXK1* encodes hexokinase on the basis of analogous results from complementation assays and on its similarity to other known hexokinases.

We analyzed the enzymatic properties of *AtHXK1* in protein extracts prepared from the mutant yeast cells expressing *AtHXK1*. The enzyme showed K_m values of 44 μ M for glucose and 17 mM for fructose (Figures 1A and 1B), which are values similar to those of hexokinases from plant sources (Renz and Stitt, 1993); therefore, we characterized the enzyme as hexokinase (EC 2.7.1.1) that has a preferential affinity for glucose.

Generation of Transgenic Tomato Lines with Differing Levels of *AtHXK1* Expression and Activity

To analyze the effects of modified hexokinase activity on fruits and vegetative parts of the plant, we introduced *AtHXK1* under the control of the cauliflower mosaic virus 35S promoter into tomato by means of Agrobacterium-mediated transformation. We obtained 64 independent R_0 regenerants and analyzed them by using polymerase chain reaction and DNA and RNA gel blot analyses to determine the presence, genomic integration, copy number, and expression level of *AtHXK1*. Three independent transgenic plants, HK4, HK37, and HK38, each with a single copy of *AtHXK1* and with differing expression levels of the gene, were chosen. Plants homozygous for *AtHXK1* were obtained by self-pollinating each transgenic plant, and the expression of *AtHXK1* in the homozygous progeny was analyzed.

HK4 and HK38 plants had high expression of *AtHXK1*, whereas HK37 plants had a moderate expression level (Figure 2A). Expression in leaf, stem, root, and fruit tissues was similar within each line. To test whether the differing expression levels of *AtHXK1* among the three lines could be correlated with activities of hexokinase, we measured phosphorylation activity with glucose and fructose in protein extracts prepared from leaves of the transgenic plants and compared it with phosphorylation activity of control plants. As shown in Figures 2B and 2C, hexokinase activities with glucose and fructose directly correlated with the *AtHXK1* expression level.

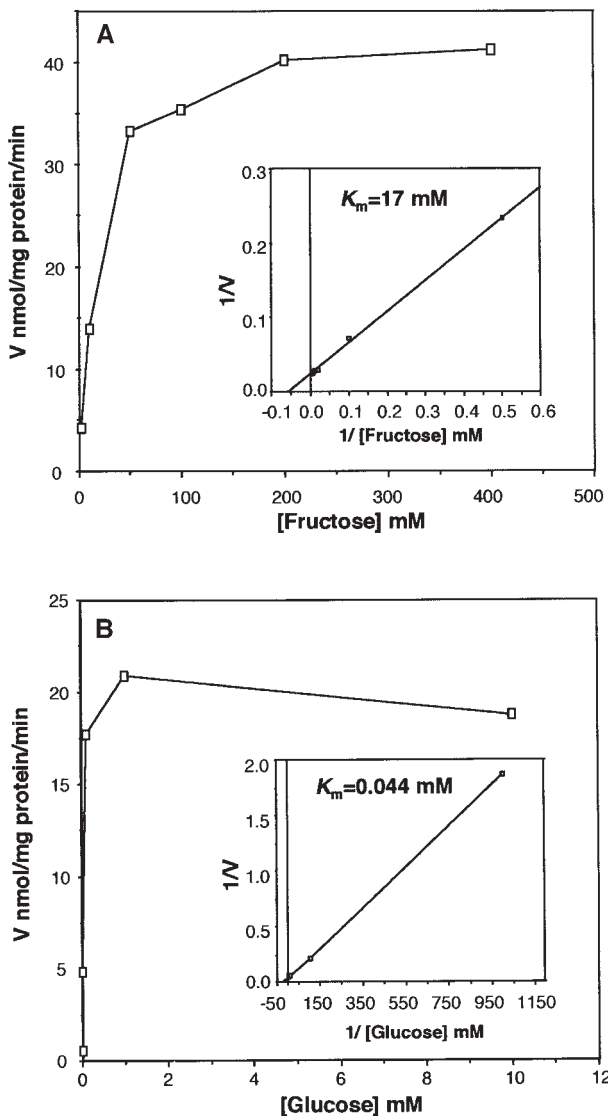


Figure 1. Kinetic Plots of *AtHXK1* When Expressed in Yeast.

(A) Hexose phosphorylation activity of *AtHXK1* with fructose.

(B) Hexose phosphorylation activity of *AtHXK1* with glucose.

Activity was measured using extracts prepared from DFY632 yeast cells transformed with pFL61-*AtHXK1*. Each data point is the mean of three independent experiments. DFY632 cells transformed with pFL61 without *AtHXK1* showed no phosphorylation activity. The insets show a double-reciprocal plot of the data. K_m values were calculated from the intercept on the horizontal axis of the linear portion of the double-reciprocal plot. V , nanomoles of fructose 6-phosphate or glucose 6-phosphate produced per milligram of protein extract per minute.

AtHXK1 Expression and Activity within Heterozygous and Homozygous Plants

To analyze the effect of *AtHXK1* copy number on hexokinase expression and activity, we developed segregating populations of HK38 and HK4 plants. These plants carried one (heterozygous), two (homozygous), or no copies of *AtHXK1*. Hexokinase expression and activity levels in HK38 heterozygous plants were approximately half those in homozygous plants (Figures 3A and 3B). Similar results were obtained with segregating HK4 plants. We concluded that hexokinase activity in the transgenic plants is also determined by gene dosage.

Glucose 6-Phosphate/Glucose and Fructose 6-Phosphate/Fructose Ratios in Transgenic Tomato Plants

Increased activity of hexokinase is expected to increase the level of phosphorylated sugars in the plant and accordingly may change the ratios of glucose 6-phosphate and fructose 6-phosphate to the respective nonphosphorylated sugars. We measured the levels of fructose, glucose, fructose 6-phosphate, and glucose 6-phosphate in young and mature leaves of heterozygous and homozygous HK4 plants. There was an approximately twofold increase in the amount of phosphorylated sugars in mature leaves of heterozygous plants and an approximately fourfold increase in those of homozygous plants when compared with plants without *AtHXK1*. The increase in the phosphorylated sugars was accompanied by a net reduction in the total amount of glucose and fructose. Hence, the fructose 6-phosphate/fructose and glucose 6-phosphate/glucose ratios increased approximately three- to fourfold in heterozygous plants and eightfold in homozygous plants (Figures 4A and 4B). Similar results were obtained with young leaves (data not shown). We concluded that the increased activity of hexokinase affected the amounts and proportions of sugars and phosphorylated sugars in the plant.

Overexpression of *AtHXK1* Inhibits Growth

Transgenic plants overexpressing *AtHXK1* exhibited several unique phenotypes. One of these was growth inhibition, the degree of which could be directly correlated with *AtHXK1* expression and activity (Figure 5A). HK37 plants were stunted compared with wild-type plants, and HK4 and HK38 plants were more stunted, showed reduction of the internodal distances, and had smaller leaves. Accordingly, the growth of homozygous HK4 plants, with two copies of *AtHXK1*, was more inhibited than was growth of heterozygous plants (Figure 5B). Similar results were obtained with heterozygous and homozygous HK38 plants, indicating that growth inhibition was directly correlated with *AtHXK1*

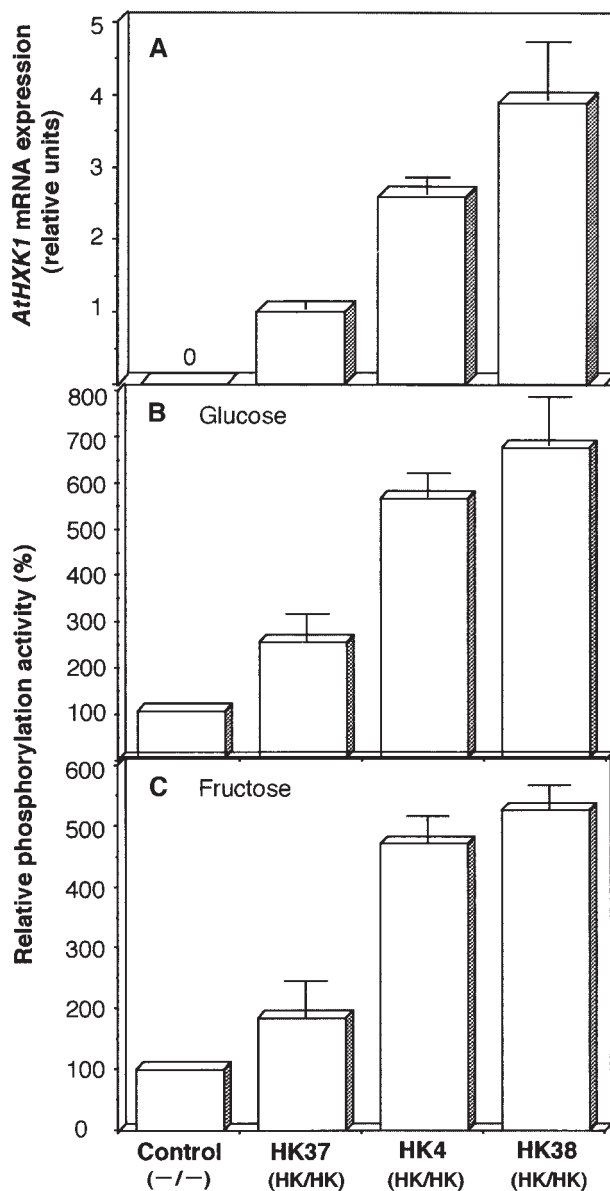


Figure 2. *AtHXK1* mRNA levels and Hexokinase Activity in Transgenic Tomato Plants.

(A) *AtHXK1* mRNA expression in HK4 and HK38 plants is higher than that in HK37 plants. Control wild-type plants, which did not express *AtHXK1*, did not cross-hybridize with the *AtHXK1* probe under stringent conditions and showed no signal.

(B) and (C) Hexokinase activity measured in crude protein extracts prepared from mature leaves. Hexokinase activity of control wild-type plants (1.8 and 7 nmol/mg protein/min for glucose and fructose, respectively), which is a result of endogenous native hexose phosphorylating enzymes, was determined as 100%.

Error bars represent standard deviations. HK/HK, plants homozygous for *AtHXK1*; -/-, control wild-type plants.

expression and activity. To verify that growth inhibition was related to the increased activity of hexokinase, we germinated seeds of HK4 plants on half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) in the presence of mannoheptulose, which is a competitive inhibitor of hexokinase. Growth inhibition was partially attenuated in the presence of 10 mM mannoheptulose and was further attenuated in the presence of 50 mM mannoheptulose (Figure 6), supporting the hypothesis that the increased hexokinase activity was responsible for the growth inhibition.

High Expression of *AtHXK1* Does Not Affect the Shoot/Root Ratio

AtHXK1 in the transgenic plants was under the control of the cauliflower mosaic virus 35S promoter, which is expressed

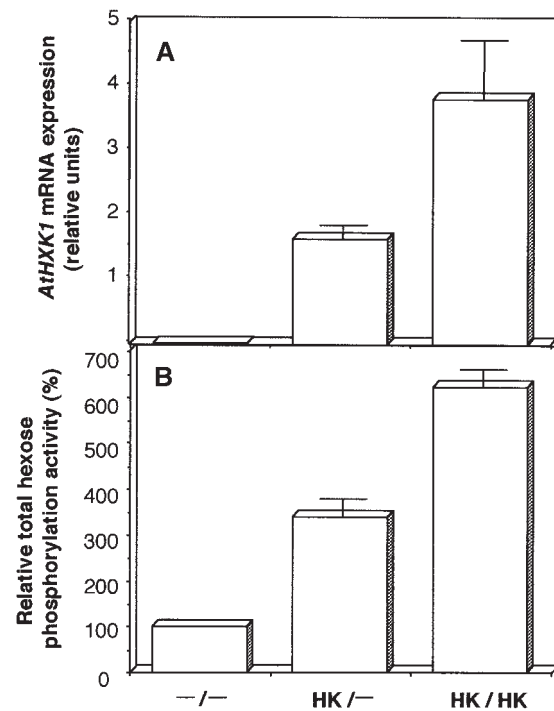


Figure 3. Expression of *AtHXK1* and Hexokinase Activity in Segregating HK38 Plants.

(A) *AtHXK1* mRNA expression in HK38 plants heterozygous and homozygous for *AtHXK1*. *AtHXK1* mRNA expression is relative to HK37. (See the legend to Figure 2A.)

(B) Total relative hexose phosphorylation activity is the mean of the relative phosphorylation activities with glucose and fructose. (See the legend to Figure 2B.)

Error bars represent standard deviations. HK/HK, plants homozygous for *AtHXK1*; HK/-, heterozygous plants; -/-, control wild-type plants without *AtHXK1*.

in most tissues, including roots (Narvaez Vasquez et al., 1993). Therefore, the inhibited growth of these plants could result from the effects of *AtHXK1* overexpression on root development. To analyze the effect of *AtHXK1* on the roots, we grew transgenic tomato plants in an aeroponic system to allow continuous examination of root development without root disruption. Throughout 2 months of observation, the roots of HK4 and HK38 plants were found to be shorter than those of control plants. However, there was no visual evidence for inappropriate development of the roots (data not shown). In addition, whereas the total dry weight of 7-week-old transgenic plants was inversely correlated with hexokinase activity (Figure 7A), there was no significant difference between the shoot/root ratio in the transgenic plants and that in the control plants (Figure 7B), leaving open the issue of which part of the plant is primarily affected by *AtHXK1* overexpression.

Growth Inhibition Occurs When *AtHXK1* Is Expressed in Photosynthetic Tissues

To analyze further in which part of the plant *AtHXK1* overexpression mediates growth inhibition, we performed reciprocal grafting experiments. Shoots from HK4 plants were grafted onto wild-type roots, and wild-type shoots were grafted onto roots of HK4 plants. Whereas wild-type shoots grew normally on transgenic roots, indicating that the expression of *AtHXK1* in the roots had no inhibitory effect, transgenic shoots on wild-type roots exhibited stunted growth (Figure 8), suggesting that the repressing effects of *AtHXK1* are exhibited when *AtHXK1* is expressed in the photosynthetic tissues. Moreover, wild-type shoots that emerged from wild-type roots on which HK4 shoots were grafted grew normally (Figure 8). In addition, HK4 roots on which wild-type shoots were grafted did not appear different from roots of wild-type plants. These results indicate that the suppressive effect was restricted to photosynthetic tissues expressing *AtHXK1* and that no transmissible compounds were involved in the growth inhibition.

Overexpression of Hexokinase Reduces Photosynthesis

It had been reported previously that overexpression of Arabidopsis hexokinase in transgenic Arabidopsis plants inhibited expression of the sugar-repressible photosynthetic gene *CAB1* (Jang et al., 1997). Because the growth inhibition in the transgenic tomato plants is associated with the expression of *AtHXK1* in photosynthetic tissues, we analyzed photosynthetic activities in the transgenic plants. CO₂ uptake rates of mature leaves were found to be inversely related to the expression and *AtHXK1* activity. As shown in Figure 9A, HK37 plants had significantly lower rates of photosynthesis than did wild-type plants, and HK4 and HK38 plants had even lower rates. The expression of *AtHXK1* in

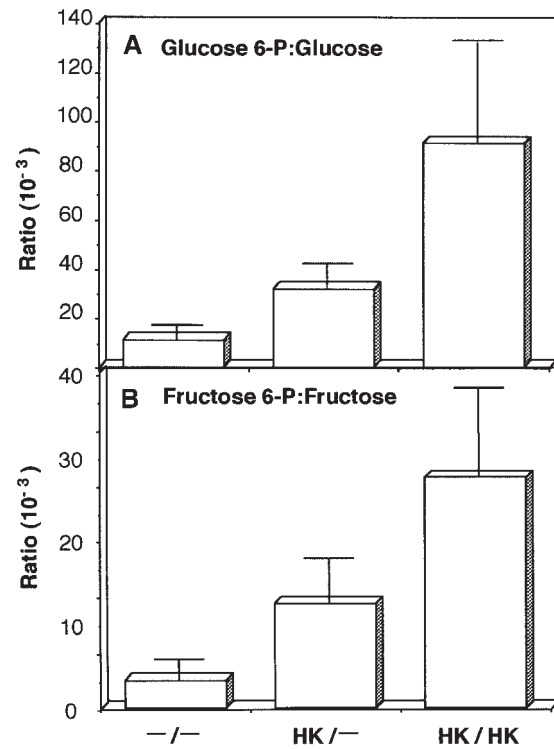


Figure 4. Increased Level of Phosphorylated Sugars in Segregating Plants.

(A) Ratio of glucose 6-phosphate to glucose.

(B) Ratio of fructose 6-phosphate to fructose.

The ratio of phosphorylated sugars to nonphosphorylated sugars was measured in mature leaves of HK4 plants. Error bars represent standard deviations. HK/HK, plants homozygous for *AtHXK1*; HK/-, heterozygous plants; -/-, control wild-type plants without *AtHXK1*; P, phosphate.

tomato plants also resulted in reduced chlorophyll content. Figure 9B shows that the chlorophyll *a* and *b* content (per unit leaf area) were also inversely related to *AtHXK1* expression and activity. However, the reduced rate of CO₂ uptake could not be solely attributed to the reduction in chlorophyll content. In HK37 plants, the reduction in chlorophyll content was negligible compared with the substantial (~32%) reduction in the rate of photosynthesis. In HK4 and HK38 plants, chlorophyll *a* and *b* content was reduced by ~45%, whereas CO₂ uptake rate was reduced by ~70% (Figures 9A and 9B).

Measuring chlorophyll fluorescence at room temperature is a noninvasive approach that provides insight into the organization and functionality of photosystem II (PSII) reaction centers (Schreiber et al., 1986; Karukstis, 1992; Lavergne and Briantais, 1996). We used this approach to examine whether malfunctioning organization of the chlorophyll molecules in the

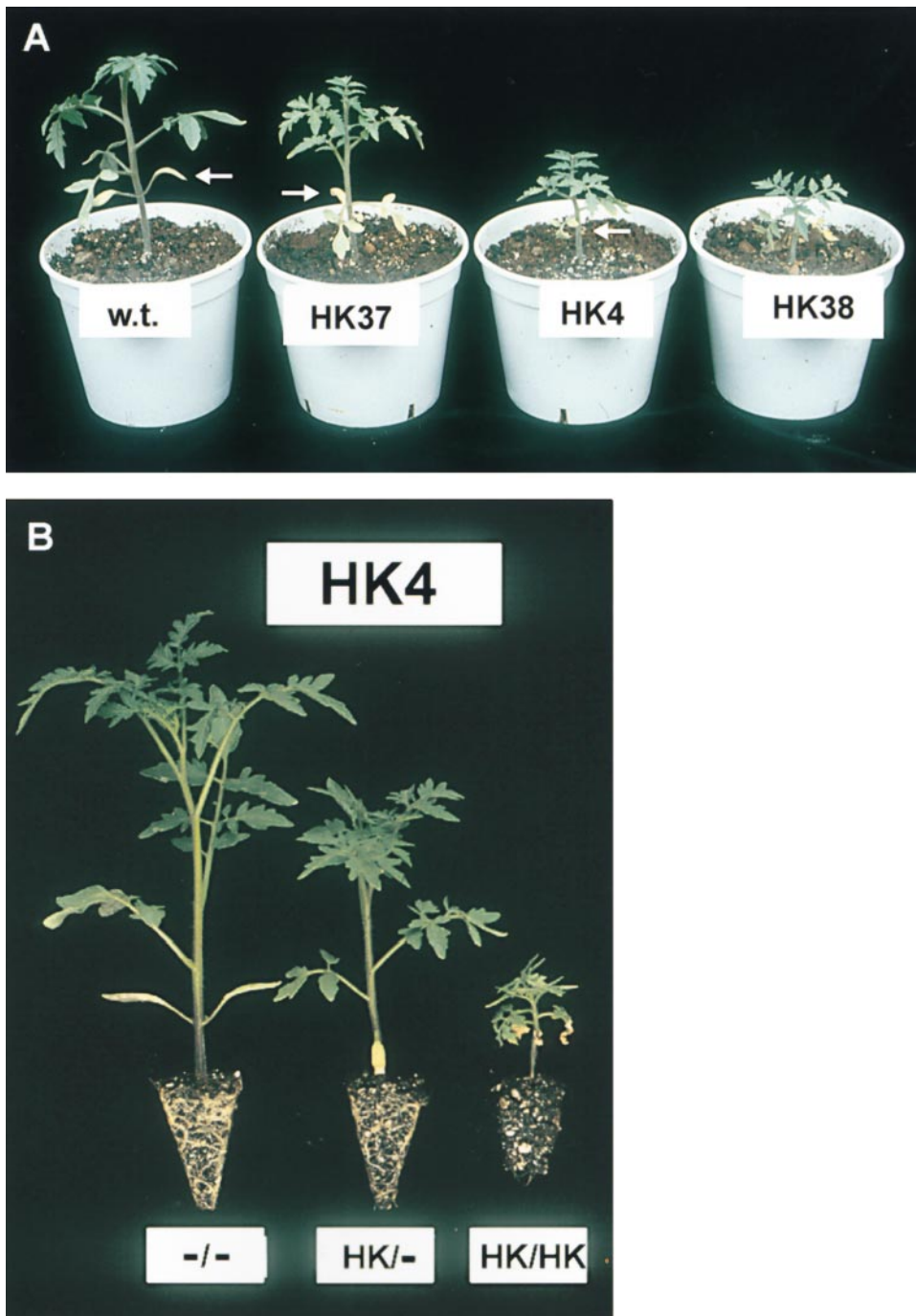


Figure 5. Growth Inhibition of *AtHXX1* Transgenic Plants.

(A) The wild-type (w.t.) plant and HK37, HK4, and HK38 plants homozygous for *AtHXX1* grown in regular soil under normal growth conditions. The arrows indicate senescing cotyledons. Yellowing of old leaves is also evident for HK37, HK4, and HK38.

(B) Growth inhibition in segregating HK4 plants. HK/HK, plants homozygous for *AtHXX1*; HK/-, heterozygous plants; -/-, control plants without *AtHXX1*.

reaction centers could account for our results. The ratio of variable to maximal chlorophyll fluorescence (F_v/F_m) is indicative of the photochemical quantum efficiency of PSII reaction centers, and it reflects the photoreduction efficiency of the primary electron acceptor Q_A . The ratio is determined by illuminating dark-adapted leaves with a saturating light pulse. The ratio between the contributions of phase I (also named F_{PI} in the literature) and F_v (i.e., the F_i/F_v ratio) is thought to represent the fraction of Q_B nonreducing or closed nonfunctional centers (Karukstis, 1992; Lavergne and Briantais, 1996); it is derived from fluorescence induction curves obtained by illumination of dark-adapted leaves with continuous actinic light. As shown in Figures 10A and 10B, HK37 leaves were identical to those of the wild type for both fluorescence parameters. However, in HK38, which has the highest expression of *AtHXK1*, F_v/F_m values were found to be very low, and F_i/F_v values were very high. These results suggest that increased levels of *AtHXK1*, such as in HK37 and HK4, cause inhibition of photosynthesis at the level of secondary electron transport (beyond PSII reaction centers) and/or at the level of the enzymatic reactions of CO_2 fixation and do not affect the functionality of the PSII reaction centers. Higher expression of *AtHXK1* as in HK38 leads to dis-

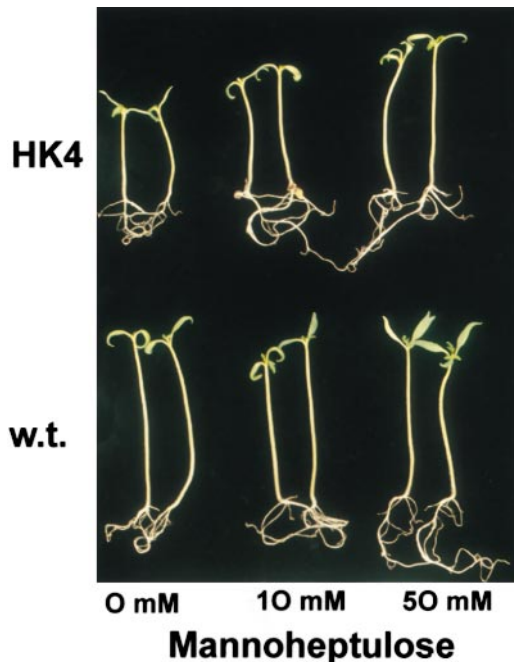


Figure 6. Mannoheptulose, a Hexokinase Inhibitor, Releases Growth Inhibition.

HK4 (homozygous for *AtHXK1*) seeds were germinated on half-strength Murashige and Skoog medium with or without 10 or 50 mM mannoheptulose. Mannitol was used to adjust the osmotic pressure. As a control, seeds of wild-type (w.t.) plants germinated in the same medium are shown.

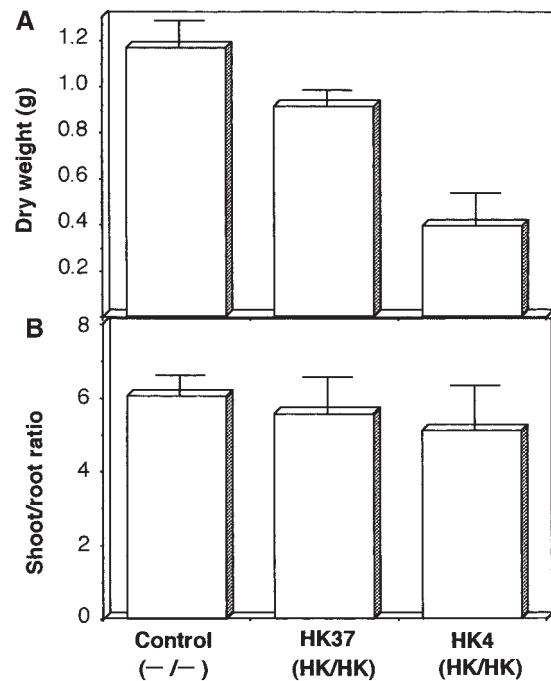


Figure 7. Total Dry Weight and Shoot/Root Ratio in Different Transgenic Plants.

(A) Total dry weight of 7-week-old transgenic and control (wild-type) plants.

(B) Shoot/root ratio of the same plants given in (A).

The dry weights of shoots and roots of five individual plants of each line were measured. Error bars represent standard deviations. HK/HK, plants homozygous for *AtHXK1*; -/-, control wild-type plants.

organization of the reaction centers. Therefore, there might be a threshold level of hexokinase activity beyond which PSII reaction centers are disrupted.

Overexpression of *AtHXK1* Decreases Fruit Yield

The reduced rate of photosynthesis and growth inhibition in plants overexpressing hexokinase could have affected the fruit yield of the transgenic tomato plants. Therefore, we analyzed the effects of *AtHXK1* overexpression on the fruits. Fruit weight, starch accumulation in young fruits, and total soluble solids (TSS) in mature fruits were found to decrease as a function of hexokinase activity (Figures 11A to 11C). HK37 plants, whose hexokinase activity was approximately two to three times greater than the endogenous activity in wild-type plants (Figure 2), exhibited a nonsignificant yield reduction, whereas HK4 and HK38 plants, which had hexokinase activities approximately six times that in wild-type plants, displayed significant yield reduction. Similar results were obtained with segregating HK4 plants. HK4 heterozygous plants showed only two to three times the increase in

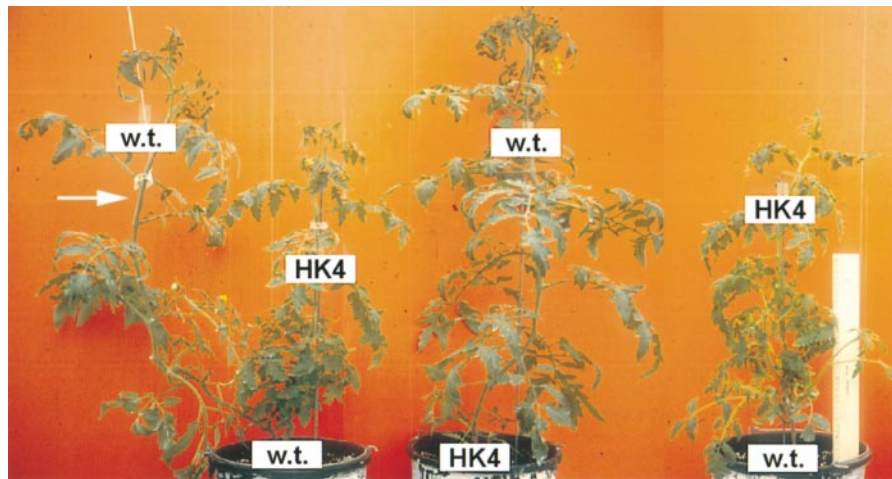


Figure 8. Reciprocal Grafting between HK4 and Wild-Type Plants.

Pots at right and left contain plants with HK4 shoots grafted onto wild-type (w.t.) roots; the pot at center contains a plant with a wild-type shoot grafted onto HK4 roots. The arrow indicates a wild-type shoot that emerged from the wild-type roots on which HK4 shoots were grafted.

hexokinase activity compared with wild-type plants (data not presented), and accordingly, their fruit weight, starch content, and TSS were higher than those of homozygous HK4 plants and were similar to those of HK37 (Figure 11). Thus, yield was inversely correlated with hexokinase activity.

***AtHXX1* Overexpression Induces Rapid Senescence**

In addition to growth inhibition, transgenic plants displayed rapid senescence of mature leaves, indicated by leaf yellowing proceeding from the margins toward the centers of the leaves (Figure 12). Rapid senescence was observed only in mature leaves and was more evident in older leaves than in younger leaves, thereby exhibiting a normal developmental regulation of leaf senescence. Accelerated senescence was already evident in HK37 plants, and HK4 and HK38 plants, which have higher hexokinase activity, senesced even faster (Figure 12). The differences in rates of senescence were also clear in the cotyledons' senescence (Figure 5). Whereas cotyledons of wild-type plants were still green, those of HK37 plants were yellow and those of HK4 and HK38 plants were already dry. These results indicate that *AtHXX1*-induced senescence correlated with hexokinase activity, suggesting that hexokinase plays a role in the regulation of senescence. In addition, rapid senescence was observed in HK4 shoots grafted onto wild-type stems and not in wild-type shoots grafted onto HK4 stems. Also, the rapid senescence was not evident in wild-type shoots that emerged from wild-type stems onto which shoots from HK4 plants were grafted. These observations indicate that accelerated

senescence is dependent on expression of *AtHXX1* in photosynthetic tissues.

DISCUSSION

Our study presents evidence that hexokinase is involved in the regulation of photosynthesis, growth, and senescence, based on analysis of transgenic tomato plants overexpressing *AtHXX1*. These plants had reduced chlorophyll content in their leaves and low rates of photosynthesis, and their growth was inhibited compared with wild-type plants. All of these phenotypes were correlated with the expression and activity levels of *AtHXX1*. Recently, Jang et al. (1997) showed that overexpression of *AtHXX1* in Arabidopsis seedlings led to "sugar-sensing" phenomena, such as reduced expression of photosynthetic genes, inhibited hypocotyl elongation, and reduced greening of cotyledons. The phenotypes that we observed in our transgenic tomato plants suggest that *AtHXX1* retains its regulatory functions within the heterologous host. Furthermore, our results suggest that there are similar sugar signaling pathways in tomato and Arabidopsis and that regulatory functions of hexokinase are conserved in plants.

Whether an analogous native sugar-phosphorylating enzyme in tomato mediates the regulatory functions needs to be determined. Although a glucokinase (Martinez-Barajas and Randall, 1998) and fructokinases (Kanayama et al., 1998) have been isolated from tomato, no tomato hexokinase has been reported to date. However, the isolated glucokinase reported by Martinez-Barajas and Randall (1998)

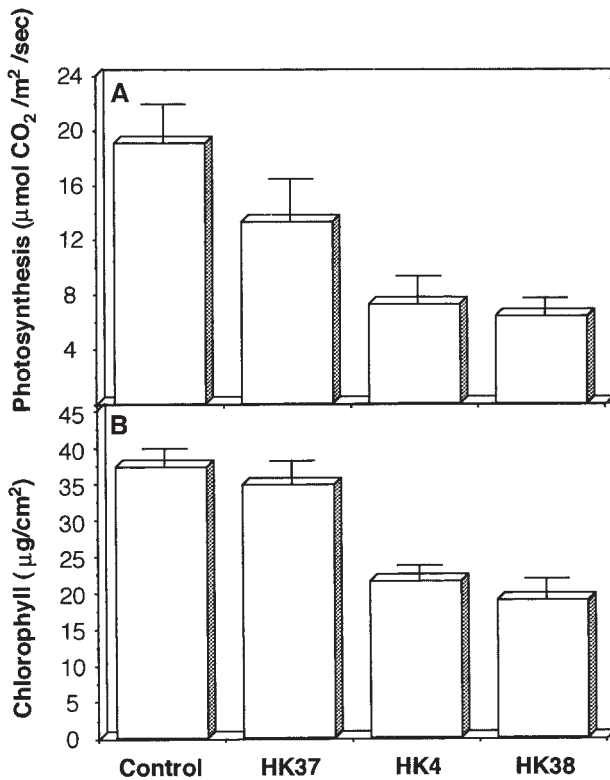


Figure 9. Photosynthesis Rate and Leaf Chlorophyll Content in Different Transgenic Plants.

(A) CO₂ uptake rates.

(B) Chlorophyll content.

Uptake rates and chlorophyll content were measured in mature leaves, as described in Methods. Eight leaves from four control and six transgenic plants were assayed. Error bars represent standard deviations.

did show "extremely low" fructose phosphorylation activity and could perhaps be defined as a hexokinase. Recently, we (N. Dai and D. Granot, unpublished data) and others (B. Ricard and C. Rothan, personal communication) have independently isolated tomato hexokinase cDNA clones. The proteins corresponding to these cDNA clones are closely related to Arabidopsis hexokinase; they are therefore the primary candidates in efforts to identify the proteins that mediate sugar regulatory functions in tomato.

Although some of the phenotypes of transgenic tomato plants overexpressing *AtHXK1* are similar to those characterized for Arabidopsis plants, there are several major differences between the two experimental systems used to obtain these results. These differences enable us to extend the conclusions drawn from studies in Arabidopsis. First, whereas the phenotypes of Arabidopsis plants were observed only in seedlings, transgenic tomato plants exhibited their phenotypes both as seedlings and as mature plants. Thus, hexokinase

appears to regulate photosynthesis and growth throughout the life cycle of the plant. Second, in addition to exhibiting inhibition of photosynthesis and growth, transgenic tomato plants underwent rapid senescence, suggesting that hexokinase is also involved in the regulation of senescence. Third, hexokinase inhibited growth and induced rapid senescence only when expressed in photosynthetic tissues. Fourth, unlike transgenic Arabidopsis plants, which expressed their phenotype only when grown on high concentrations of extracellular sugar (i.e., 2 to 6%; Jang et al., 1997), transgenic tomato plants displayed their phenotypes under normal growth conditions and without any extracellular sugar, indicating that the regulatory roles of hexokinase are expressed in response to endogenous sugar levels in tomato.

This final difference between transgenic Arabidopsis and tomato plants could be attributed to differing activity levels of *AtHXK1* in Arabidopsis and tomato. Transgenic tomato

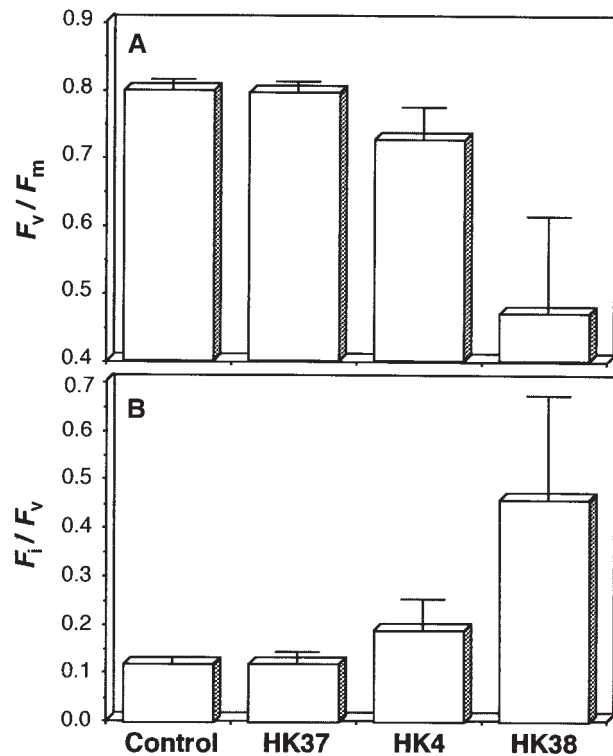


Figure 10. Chlorophyll Fluorescence Parameters F_v/F_m and F_i/F_v in Transgenic Plants.

(A) The F_v/F_m ratio is indicative of the photochemical quantum efficiency of PSII reaction centers.

(B) The F_i/F_v ratio is indicative of closed reaction centers.

These two chlorophyll fluorescence parameters were measured in dark-adapted leaves by using a portable pulse-amplitude modulated fluorometer (see Methods for assay details). Eight leaves for each variant from four (control) or six (transgenic) plants were assayed. Error bars represent standard deviations.

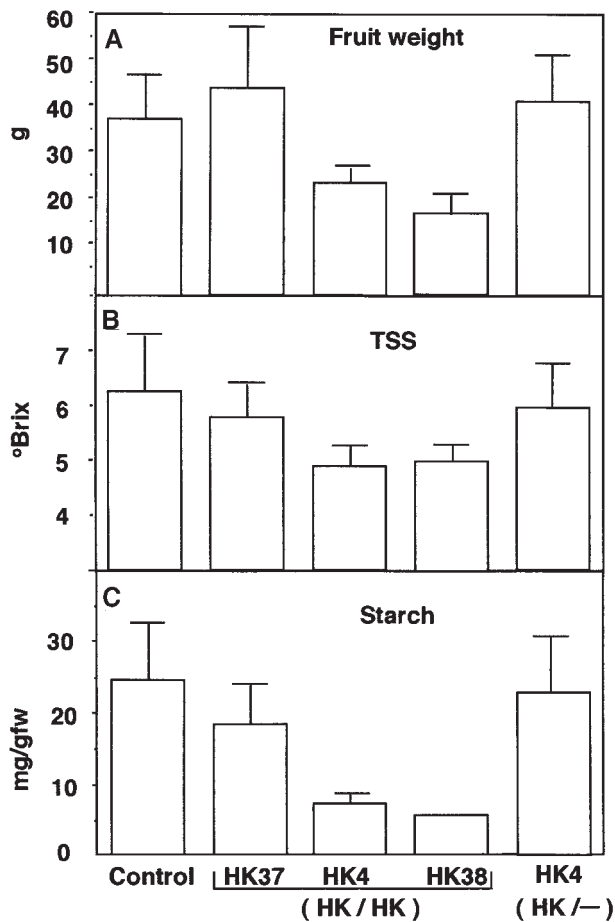


Figure 11. Fruit Yield of Transgenic Plants.

(A) and (B) Fruit weight and TSS are the means of data obtained from >25 mature red fruits from five plants of each line. (C) Starch content was determined in young (green) fruits. Control plants are wild-type plants without *AthHK1*. Error bars represent standard deviations. gfw, grams fresh weight; HK/HK, plants homozygous for *AthHK1*; HK/–, heterozygous plants.

plants could have higher hexokinase activities than do transgenic Arabidopsis plants. Higher hexokinase activities might promote the phenotypes in the absence of extracellular sugar. Indeed, most of the 64 independent transgenic tomato plants had relatively low *AthHK1* expression and activity levels and exhibited either no phenotype or only minor phenotypes, such as those described above for HK37 plants. On the other hand, a comparison between the hexokinase activities in HK38 plants and the corresponding results published for Arabidopsis plants (Jang et al., 1997) shows that both were increased by approximately five- to sixfold over the regular endogenous hexokinase activity. Therefore, it is also possible that the sensitivity to hexokinase activity of the sugar signaling pathways in Arabidopsis and tomato differ.

Similar sensitivity differences between Arabidopsis and tobacco have been observed in transgenic plants expressing a yeast-derived apoplasmic invertase, which is a sucrose-hydrolyzing enzyme (von Schaewen et al., 1990). Whereas expression of the invertase in Arabidopsis plants did not lead to drastic changes, its expression in transgenic tobacco plants elicited dramatic changes with respect to development and phenotype. Therefore, in the use of transgenic plants to study the role of hexokinase, tomato plants might be more effective tools than Arabidopsis plants.

The appearance of the phenotypes in transgenic tomato plants in the absence of extracellular sugar indicates that hexokinase is responding to normal endogenous levels of sugar. It has been reported that sugar accumulation in mature leaves is accompanied by a decrease in the rate of photosynthesis (Goldschmidt and Huber, 1992; Hensel et al., 1993; Jiang et al., 1993; Wingler et al., 1998), and perhaps an increased flux of sugar through hexokinase plays a role in photosynthesis inhibition in mature leaves. In this study, inhibition of photosynthesis in transgenic tomato plants occurred without an increase in glucose and fructose content. In fact, sugar content in the transgenic plants was reduced in mature and young leaves compared with that in wild-type plants. These results demonstrate that increased hexokinase activity is sufficient to stimulate the signaling pathway, leading to inhibition of photosynthesis and growth, without a need for an actual increase in sugar levels. In addition, mannoheptulose, a competitive inhibitor of hexokinase, suppressed the growth inhibition of HK4 plants (Figure 6), indicating that increased hexokinase activity is necessary to express the phenotypes. Hence, high activity of hexokinase is necessary and sufficient to inhibit photosynthesis and growth, suggesting that sugar regulation of photosynthesis

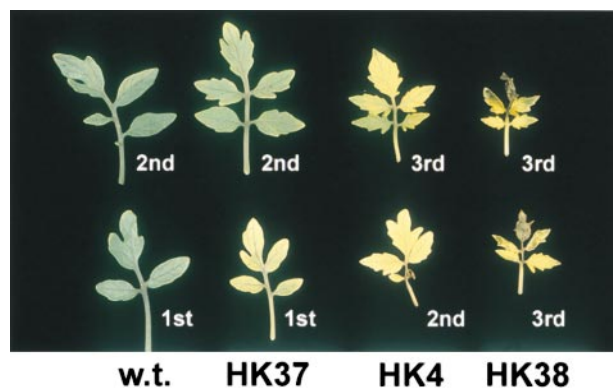


Figure 12. Rapid Senescence in Transgenic Plants.

Shown are leaves from transgenic and wild-type (w.t.) plants of the same age grown under normal conditions. The leaves are numbered by the order of their appearance from the bottom of the plant. The first leaves of HK4 and HK38 and the second leaf of HK38 are not shown because they were already dry.

and growth is entirely mediated via hexokinase. The high hexokinase activity in the transgenic plants is probably perceived as a sign of elevated sugar levels, which naturally inhibit photosynthesis and growth.

In other organisms, such as animals and yeast, it has been implied that hexose-phosphorylating enzymes are involved in sugar signaling. In mammalian systems, glucokinase is required in pancreatic β cells for glucose-stimulated insulin release and in liver to convert excess glucose into glycogen (Matschinsky et al., 1993; Grupe et al., 1995). In yeast, hexokinase is required for the glucose repression phenomenon (Entian, 1980). Although it is not clear how hexokinase and glucokinase transmit the signal, downstream genes involved in glucose repression in yeast are well characterized. In particular, the *SNF1* gene, which encodes a protein serine/threonine kinase, is essential for glucose repression (Celenza and Carlson, 1986). It forms a high molecular mass protein kinase complex that, upon removal of glucose, phosphorylates and thereby inactivates a DNA binding protein (Mig1) that represses transcription of glucose-repressed genes (Ostling and Ronne, 1998). Plant homologs of *SNF1*, called *SNF1*-related protein kinases 1s (SnRK1), which complement *snf1* yeast mutants, have been identified (Alderson et al., 1991; Muranaka et al., 1994; Halford and Hardie, 1998). It would be of interest to determine whether these genes are affected by hexokinase.

We do not know how hexokinase activity stimulates sugar-signaling pathways in either yeast or plants. In previous studies, it has been suggested that yeast and plant hexokinases are bifunctional enzymes with catalytic and regulatory activities (Entian and Frohlich, 1984; Jang and Sheen, 1994; Jang et al., 1997). This conclusion was based on experiments demonstrating that hexokinase activity is necessary to stimulate the regulatory functions, and that neither downstream metabolism of the sugars nor accumulation of phosphorylated sugars is required for induction of the sugar-sensing pathway (Entian and Frohlich, 1984; Jang and Sheen, 1994; Jang et al., 1997). Our results neither confirm nor negate the dual function of *AthXK1*, because we were unable to distinguish between the enzymatic activity and downstream effects of the products. The elevated levels of hexose phosphate in the transgenic plants may increase the flux toward glycolysis and respiration, and changes in intermediate metabolites could stimulate the regulatory pathway. For example, it is possible that altered ATP/ADP ratios or altered concentrations of cytosolic phosphate ions have a signaling function (Sadka et al., 1994; Halford et al., 1999). However, irrigation or spraying of HK4 and HK38 plants with phosphate did not reverse the phenotypes described above (N. Dai and D. Granot, unpublished results), suggesting that changes in the cytosolic phosphate might not be the signal.

Growth inhibition, reduced sugar levels in leaves, and decreased yield could be attributed to the inhibition of photosynthesis by *AthXK1*. Accordingly, growth inhibition was manifested only when *AthXK1* was expressed in photosynthetic tissues. Accelerated senescence might also be a con-

sequence of a decline in the rate of photosynthesis below a certain level at which senescence is initiated. However, we cannot rule out the possibility that hexokinase might have an additional role in the regulation of senescence: it is possible that a drop in the photosynthesis rate reduces the sugar flux, which might also be sensed by hexokinase. This hypothesis is supported by results obtained elsewhere with transgenic tobacco plants that express a yeast-derived invertase and that accumulate carbohydrates (Ding et al., 1993). The transgenic tobacco plants manifested the onset of early leaf senescence only when expressing an apoplastic invertase and not a cytosolic invertase. We speculate that hexokinase might sense changes in extracellular sugar flux and stimulate senescence.

Hexokinase may be involved in the regulation of additional pathways affected by sugars in addition to photosynthesis, growth, and senescence (Graham et al., 1994; Perata et al., 1997; Prata et al., 1997; Smeekens and Rook, 1997). Photosynthesis, growth, and senescence are also regulated by light and by hormones, such as auxin, cytokinin, gibberellic acid, brassinosteroids, and ethylene. It has been suggested that sugars may interact with or regulate such internal signals (DeWald et al., 1994; Thomas and Rodriguez, 1994; Sonnewald et al., 1995; Szekeres et al., 1996; Jang et al., 1997; Smeekens, 1998; Wingler et al., 1998; Zhou et al., 1998). The availability of transgenic tomato plants with differing hexokinase activities provides a means to study the role of hexokinase in other pathways and in interactions with other signaling pathways.

METHODS

Yeast Transformation and Hexokinase Complementation

The yeast (*Saccharomyces cerevisiae*) strain used was DFY632 (*MATa ura3-52 hxk1::LEU2 hxk2::LEU2 gik1::LEU2 lys1-1 leu2-1*). This strain is unable to phosphorylate glucose or fructose and therefore is unable to grow on these sugars as carbon sources. The yeast cells were transformed with *Arabidopsis* hexokinase (*AthXK1*) previously isolated from an *Arabidopsis thaliana* cDNA expression library (Dai et al., 1995) in the pFL61 shuttle vector. Yeast transformation was performed according to Ito et al. (1983). The transformants were grown on plates with galactose and without uracil and were tested on plates with glucose and without uracil and on plates with fructose and without uracil. Plates with sugar and without uracil contained 0.5% ammonium sulfate, 0.17% yeast nitrogen base without amino acids (Difco), 0.2% casamino acids (Difco), 0.004% adenine (Sigma), 0.008% tryptophan (Sigma), and 110 mM galactose, glucose, or fructose.

Protein Extraction from Yeast and Plants

DFY632 yeast cells, transformed with either pFL61 or pFL61-*AthXK1*, were grown in 40 mL of liquid medium containing galactose and

without uracil for 72 hr to $\sim 5 \times 10^7$ cells per mL. Cells were spun down for 5 min at 6000 rpm, washed twice with water, and resuspended in 0.5 mL of water. A 250- μ L sample of the cells was extracted twice with 500 μ L of extraction buffer (50 mM HEPES, pH 7.6, 1 mM EDTA, 15 mM KCl, 1 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride) by vortexing with 250 μ L of glass beads. After vortexing three times for 30 sec, the mixture was centrifuged for 5 min at 12,000g at 4°C, and the supernatant was brought to 80% ammonium sulfate saturation. After centrifugation at 12,000g at 4°C, the pellet was resuspended in 0.5 mL of washing buffer (50 mM HEPES, pH 7.5, 1 mM EDTA, and 1 mM DTT), desalted on a G-25 Sephadex column (55 \times 11 mm), and used as a crude enzyme extract for subsequent enzymatic analysis.

Protein extraction from plants was performed in a similar way, with 1 to 2 g of plant tissue homogenized in 4 volumes of extraction buffer (50 mM HEPES, pH 7.6, 1 mM EDTA, 15 mM KCl, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 3 mM diethyldithiocarbamic acid, and 0.2% PVP). The mixture was centrifuged for 25 min at 16,000g at 4°C, and the supernatant was treated as described above.

Analysis of Hexokinase Activity

Hexokinase activity was measured by enzyme-linked assay according to Schaffer and Petreikov (1997). The assays contained a total volume of 1 mL of 30 mM HEPES-NaOH, pH 7.5, 2 mM $MgCl_2$, 0.6 mM EDTA, 9 mM KCl, 1 mM NAD, 1 mM ATP, and 1 unit of NAD-dependent glucose-6-phosphate dehydrogenase (G6PDH from *Leuconostoc mesenteroides*; Sigma). To assay glucose phosphorylation, the reaction was initiated with 2 mM glucose. To assay fructose phosphorylation, 1 unit of phosphoglucosomerase was added, and the reaction was initiated with fructose. Reactions were conducted at 37°C, and absorption at 340 nm was monitored continuously.

Tomato Transformation and Analysis of *AtHXK1* mRNA Expression in Transgenic Plants

AtHXK1 (Dai et al., 1995) was introduced under the control of the cauliflower mosaic virus 35S promoter into binary vector pGA643 containing the neomycin phosphotransferase II gene as a selectable marker. Transformation was performed with MP-1, a tomato (*Lycopersicon esculentum*) line known for its high transformation efficiency (Barg et al., 1997). Essentially, the transformation procedure was as described by McCormick (1991). T_0 and T_1 independent transgenic plants were analyzed by polymerase chain reaction and DNA gel blotting for the presence and copy number of the *AtHXK1* transgene. Most plants had only one copy of *AtHXK1*. Heterozygous and homozygous plants were identified after segregation analysis of *AtHXK1* within T_1 seeds.

Expression of *AtHXK1* was analyzed by RNA gel blotting using *AtHXK1* as a probe. *AtHXK1* mRNA was analyzed in the same amount of total RNA from each plant. The relative amount of *AtHXK1* mRNA in each transgenic plant was calculated by using the National Institutes of Health Image software after data were obtained using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Aerohydroponic Growth and Shoot/Root Ratio Analyses

Four-week-old transgenic plants of the HK37, HK4, and HK38 lines were transferred and grown in aerohydroponic pots according to

Bar-Tal et al. (1993). The shoot/root ratio was measured on 7-week-old plants grown in soil. The soil was carefully washed off the roots, and the plants were dried at 80°C. Total dry weight was the sum of shoot and root weights.

Total Soluble Solids, Starch, and Sugar Determinations

Portions of a whole harvested fruit were used for determination of total soluble solids (TSS), using an N-1 hand-held refractometer (Atago, Tokyo, Japan). Portions (\sim 500 mg fresh weight) were immediately placed in 80% ethanol for sugar and starch determination. Soluble sugars were extracted three times in hot ethanol, and starch content was measured in the insoluble fraction after treatment with amyloglucosidase, as described previously (Schaffer et al., 1987).

The content of soluble sugars in mature leaves was determined after extraction with ethanol and separation by HPLC, as described by Miron and Schaffer (1991).

Glucose 6-phosphate and fructose 6-phosphate were extracted and assayed spectrophotometrically on 1 g fresh weight of mature leaves, according to Tobias et al. (1992).

Photosynthetic Activity and Chlorophyll Content

The CO_2 uptake rate was measured with a portable photosynthesis measuring system (model CI-301; CID, Vancouver, WA) in mature leaves under greenhouse natural saturating light (600 to 800 $mmol\ m^{-2}\ sec^{-1}$ photosynthetically active photon flux density) in the morning (10:00 to 11:00 AM).

Chlorophyll fluorescence was measured with a portable pulse-amplitude modulated fluorometer (model PAM 2000; Heinz Walz GmbH, Effeltrich, Germany) in the same leaves. Dark adaptation was applied by dark-clips at 15 to 20 min before each measurement. The ratio of variable to maximal chlorophyll fluorescence (F_v/F_m) was measured directly by the "run 2" mode of the PAM 2000, whereas the ratio of phase I contributions to F_v (F_v/F_v) was determined from the induction curves obtained by "run 6" at the high modulation frequency (Schreiber et al., 1986).

Chlorophyll content was determined by acetone extraction according to Zhang and Kirkham (1996).

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