The Pitfalls of Transgenic Selection and New Roles of *AtHXK1*: A High Level of *AtHXK1* Expression Uncouples Hexokinase1-Dependent Sugar Signaling from Exogenous Sugar[1][W]

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Arabidopsis (*Arabidopsis thaliana*) hexokinase (*AtHXK1*) encodes a dual-function enzyme that mediates sugar sensing in addition to its involvement in hexose phosphorylation activity, thereby coordinating sugar availability with plant physiology and development (Moore et al., 2003; Rolland et al., 2006). Sugars such as Glc are essential metabolic nutrients and structural components and are important regulatory molecules that control gene expression, metabolism, physiology, the cell cycle, and development in prokaryotes and eukaryotes. Hexokinase (HXK), which catalyzes the essential step of Glc phosphorylation, is an evolutionarily conserved Glc sensor (Wilson, 2003; Rolland et al., 2006; Karve et al., 2010). Most studies of HXK in plants have involved *AtHXK1*. The *AtHXK1* loss-of-function mutant *glucose-insensitive2* (*gin2*) was isolated through a mutant screen based on a high-Glc (6%) repression assay, in which wild-type plants display inhibited cotyledon expansion, chlorophyll accumulation, and shoot growth at the early stage of seedling development (Moore et al., 2003). *gin2* mutants are insensitive to Glc, whereas overexpression of *AtHXK1* accelerates the inhibition of cotyledon expansion, chlorophyll accumulation, and seedling development and reduces the expression of photosynthetic genes (Jang et al., 1997; Xiao et al., 2000). These effects have been observed only in the presence of 3% to 6% Glc, limiting the experiments to sterile synthetic medium, usually agar supplemented with one-half-strength Murashige and Skoog (1/2MS) nutrient additive (Murashige and Skoog, 1962) and 3% to 6% Glc. As a consequence, these experiments (Jang et al., 1997; Xiao et al., 2000) have mostly been limited to germinating seeds and seedlings.

The use of a high concentration of Glc has raised concerns about the physiological relevance of these assays (León and Sheen, 2003; Rook and Bevan, 2003). For this reason, Cho et al. (2010) eliminated nitrate from the medium in order to minimize the nonphysiological conditions imposed by a high concentration of Glc. Because Glc responses are antagonized by nitrate (Stitt, 1999; Stitt and Krapp, 1999; Moore et al., 2003), growing the plants on medium without nitrate (without Murashige and Skoog additive) allowed the Glc concentration to be reduced to 2% while still obtaining *AtHXK1*-dependent sugar signaling effects (Cho et al., 2010). However, the experiments were still limited to sterile medium and the early seedling developmental stages.

Unlike Arabidopsis, tomato (*Solanum lycopersicum*) plants that overexpress *AtHXK1* exhibit inhibited growth, reduced photosynthesis, and accelerated leaf senescence when grown in soil under regular growth conditions in the absence of exogenous Glc (Dai et al., 1999). Accordingly, studies with tomato plants that overexpress *AtHXK1* have not been limited to the early developmental stages but have involved mature plants and both vegetative and reproductive tissues, including young and mature fruits (Dai et al., 1999; Menu et al., 2003; Roessner-Tunali et al., 2003; Moing et al., 2004).

Studies have also been conducted using potato (*Solanum tuberosum*) and rice (*Oryza sativa*) plants that overexpress HXK. Overexpression of *StHK1*, the potato homolog of *AtHXK1*, had no effect on the development of potato plants (Veramendi et al., 1999, 2002). Similarly, overexpression of the rice homologs *OsHXK5* and *OsHXK6* had no effect on the growth or photosynthetic gene expression of rice plants, except when they were grown on Glc (Cho et al., 2009). It seems that tomato is the only plant species tested so far in which a high level of *AtHXK1* expression causes developmental, physiological, and molecular sugar-sensing effects in the absence of exogenous sugar (Dai et al., 1999).

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We hypothesized that due to the inhibition effects of *AtHXK1*, transgenic plants were discriminated against high expression levels of *AtHXK1* throughout the selection procedure, in favor of plants with low expression levels. To test this hypothesis, we retransformed Arabidopsis (Columbia ecotype) with *AtHXK1* under the control of the cauliflower mosaic virus 35S promoter and searched for kanamycin-resistant individuals that exhibit poor growth on selective medium. Out of 50 resistant T1 plants, we isolated 16 plants that exhibited growth retardation in soil. Independent homozygous T2 lines were then identified, and the *AtHXK1* effects of three lines were characterized. These lines exhibited growth inhibition and accelerated leaf senescence in soil under normal growing conditions (Fig. 1A). The *AtHXK1* expression levels of these lines were about 12 times higher than those observed in wild-type plants (Fig. 2A).

The new *35S*::*HXK* transgenic plants were also tested on 1/2MS agar medium without sugar. Although nitrate antagonizes Glc and *AtHXK1* responses (Stitt, 1999; Stitt and Krapp, 1999; Moore et al., 2003; Cho et al., 2010), inhibition of both shoot and root growth was clearly visible on 1/2MS medium (Fig. 1B). In comparison with wild-type leaves, *35S*::*HXK* leaves were smaller and rounded (Figs. 1B and 2B). The roots of the *35S*::*HXK* plants were shorter, with only secondary lateral roots, whereas wild-type plants developed secondary and tertiary lateral roots (Fig. 1B). Hence, in both soil and synthetic medium, the effects of *AtHXK1* are clearly evident in the presence of nitrate and are independent of exogenous Glc.

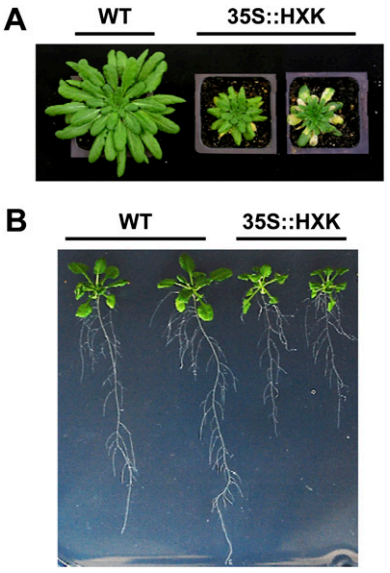

Figure 1. Overexpression of *AtHXK1* inhibits growth and accelerates leaf senescence under normal growth conditions. A, Plants grown in soil under regular growth conditions in the absence of any exogenous sugar. B, Plants grown on 1/2MS agar medium without sugar. Two independent transgenic lines are shown. WT, Wild type.

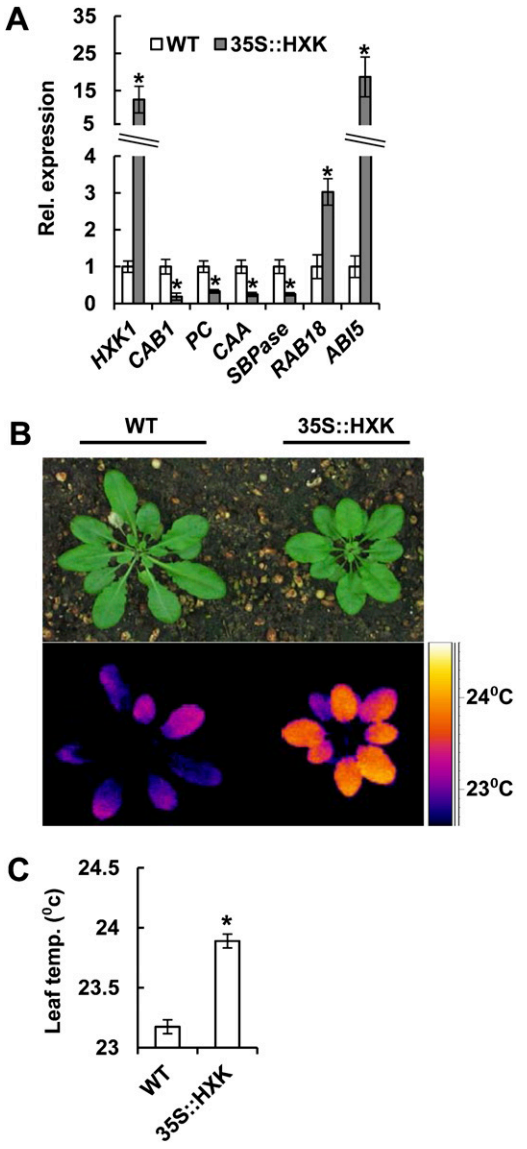

Figure 2. Gene expression analysis and thermal imaging of wild-type (WT) and *35S*::*HXK* plants. A, Quantitative real-time PCR was performed using RNA extracted from young nonsenescing leaves of the wild type and two independent transgenic lines ($n = 5$ each) grown in soil. The genes assayed were *HXK1*, *CAB1*, *PC*, *CAA*, *SBPase*, *RAB18*, and *ABI5*. *TUB2* (β-tubulin) was used for normalization. Experiments were repeated twice with similar results. Data are means ± SE; asterisks denote significant differences relative to the wild type (*t* test; $P < 0.01$). B, *AtHXK1* increases leaf temperature. Images of plants growing in soil under normal conditions were captured using a thermal camera (ThermaCAM model SC655; FLIR Systems); warm colors represent high temperatures (scale is shown at right). C, Leaf temperatures of the wild type and three independent transgenic lines were determined using ThermaCAM researcher pro 2.10 software. The experiment was repeated three times with similar results. Data are means ± SE from five biological repeats per line; four leaves were analyzed per plant. The asterisk denotes a significant difference relative to the wild type (*t* test; $P < 0.01$).

Table I. *Chlorophyll and photosynthesis measurements*

The chlorophyll contents of mature nonsenescing leaves from wild-type plants and three independent transgenic lines ($n = 10$) were measured using the SPAD-502 chlorophyll meter (Minolta). Two independent experiments were conducted with similar results. Stomatal conductance (g_s), net photosynthesis (A_N), substomatal CO_2 concentrations (C_i), and transpiration (T_r) were assayed in wild-type plants and in two independent transgenic lines using a Li-6400 portable gas-exchange system (LI-COR), as described (Flexas et al., 2007). Data are means ± SE from five biological repeats. Asterisks denote significant differences relative to the wild type (*t* test; $P < 0.01$).

Parameter	Wild Type	*35S::HXK*
Chlorophyll content (SPAD units)	28.39 ± 1.13	25.16 ± 0.33*
g_s (mol water m^{-2} s^{-1})	0.142 ± 0.009	0.069 ± 0.003*
A_N (μmol CO_2 m^{-2} s^{-1})	7.041 ± 0.386	3.635 ± 0.389*
C_i (μmol CO_2 mol^{-1})	300.00 ± 3.352	300.17 ± 8.553
T_r (mmol water m^{-2} s^{-1})	2.201 ± 0.121	1.177 ± 0.051*

To further explore the *AtHXK1* effects in the transgenic lines, we analyzed chlorophyll accumulation and the expression of genes related to photosynthesis. The chlorophyll content of the *AtHXK1* transgenic lines was significantly reduced (Table I). The expression levels of the photosynthesis-related genes *CAB1* (for chlorophyll *a*/*b*-binding protein), *PC* (for plastocyanin), *CAA* (for carbonic anhydrase), and *SBPase* (for sedoheptulose-bisphosphatase) were significantly reduced as compared with the levels observed in the control plants. In contrast, the expression levels of *RAB18*, an abscisic acid (ABA)-responsive gene, and *ABI5*, an ABA-induced transcription factor (Lång and Palva, 1992; Lopez-Molina et al., 2001; Brocard et al., 2002), were higher in the transgenic plants. These results are in agreement with previous observations that the effects of *AtHXK1* on seedling development and photosynthetic gene expression in response to exogenous Glc are dependent on ABA (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Cheng et al., 2002; Rook and Bevan, 2003). However, these results are in contrast to the observation that 2% Glc, in the absence of nitrate, represses cotyledon greening independent of *ABA2/GIN1* (an ABA biosynthesis gene; Cho et al., 2010). This difference might reflect the different experimental conditions and plant developmental stages observed in the different experiments.

Photosynthesis measurement data had never been collected for Arabidopsis seedlings grown on Glc. In our study, however, the plants grown in soil were easily amenable to measurements of photosynthetic activity. Although there was no change in intercellular CO_2 concentration in the transgenic plants, the net photosynthesis rate was diminished by half (Table I). Interestingly, stomatal conductivity and the transpiration rate were also reduced by half (Table I), indicating smaller stomatal apertures. Closed stomata and reduced transpiration increase leaf temperature (Merlot et al., 2002). Using infrared thermal imaging, we demonstrated that the leaf temperature of *AtHXK1*-expressing lines grown in soil was higher than that of the wild type (Fig. 2, B and C). These results indicate that *AtHXK1* not only inhibits photosynthesis but also induces stomatal closure and reduction in transpiration, affecting whole-plant water balance as a consequence.

A main advantage of the new *AtHXK1*-expressing plants is the fact that we can examine their phenotype at advanced developmental stages. We noticed that *AtHXK1*-expressing plants lost apical dominance, allowing the emergence of lateral buds (Supplemental Fig. S1). This effect was not evident in the tomato lines expressing *AtHXK1* (Dai et al., 1999), possibly because the tomato line used in that experiment (line MP1) generally exhibits a low level of apical dominance and persistent emergence of lateral buds. Because auxin is involved in apical dominance (Müller and Leyser, 2011), we analyzed the expression of the auxin-responsive genes *TRANSPORT INHIBITOR RESPONSE1* (*TIR1*; an auxin receptor), *PIN-FORMED1* (*PIN1*; an auxin transporter), and *INDOLE-3-ACETIC ACID INDUCIBLE5* (*IAA5*; an auxin-induced gene) in the *35S::HXK* Arabidopsis plants. The expression levels of these genes were lower in the transgenic plants than in the control plants (Fig. 3), suggesting lower levels of

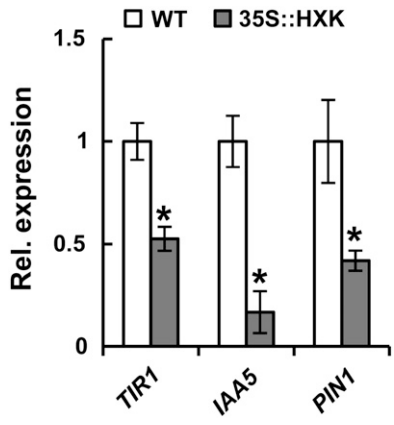

Figure 3. *AtHXK1* suppresses auxin-response genes. Relative expression levels of *TIR1*, *IAA5*, and *PIN1* were assayed as described in Figure 2A. Data are means ± SE; asterisks denote significant differences relative to the wild type (WT; *t* test; $P < 0.01$).

auxin signaling, which may explain the loss of apical dominance. These effects of *AtHXK1* on auxin-related genes differ from previously published results showing that an auxin response is essential for *AtHXK1*-mediated sugar inhibition of seedling growth on 6% Glc (Moore et al., 2003). They are also in contrast to results showing increased expression of *PIN1* in whole seedlings kept in the dark and exposed to 3% Glc for 3 h (Mishra et al., 2009). However, the observed effects are in line with the observation of reduced expression of *TIR1* in response to the same Glc treatment (Mishra et al., 2009). Hence, it appears that Glc and *AtHXK1* interact with auxin in a complicated manner that depends on growth conditions, the tissue examined, and the developmental stage of the plant. We believe that plants overexpressing *AtHXK1* under normal growth conditions might be a good system in which to dissect the interaction between *AtHXK1* and auxin in different tissues and at different developmental stages.

Transformation of Arabidopsis, as well as other plants, involves the selection of viable seedlings grown on medium containing kanamycin or another selective agent. It is very likely that the most resistant viable seedlings are selected first. However, high levels of *AtHXK1* expression have a counter growth inhibition effect, making us suspect that such transformants were most likely ignored. Being aware of this effect, we also selected poorly growing kanamycin-resistant transformants, which appear to be those with high levels of *AtHXK1* expression. Such a bias in the selection of transgenic plants may be present in many other cases, as we previously observed in an investigation of antisense suppression of *LeFRK2* (German et al., 2003). Therefore, care should be taken to avoid discrimination against the most interesting transformants. The development of both tomato and Arabidopsis plants with clear *AtHXK1* signaling effects that are manifested under normal growth conditions suggests that it might be possible to obtain similar potato and rice plants (Veramendi et al., 1999, 2002; Cho et al., 2009).

The advantage of analyzing plants under normal growth conditions is obvious. First, the use of natural physiological conditions allows for discoveries with greater biological relevance. Second, the effects of interest can be studied at all developmental stages and in all plant parts and tissues. In our case, we discovered that *AtHXK1* mediates stomatal closure, either directly via guard cells or indirectly through the effect of *AtHXK1* on photosynthesis (Mott, 2009). Nonetheless, the interesting physiological consequence of this is reduced transpiration accompanied by increased leaf temperatures, suggesting that *AtHXK1* might have a global effect on whole-plant water balance. Lastly, a new relationship between *AtHXK1*, shoot apical dominance, and auxin response has been observed. In light of these observations, we believe that these new transgenic lines will help us to broaden our understanding of the relationship between *AtHXK1*, sugar signaling, and plant physiology and development under regular physiological conditions.

Sequence data from this article can be found in the GenBank/EMBL data libraries as described in Table S1.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Lateral bud emergence in *35S::HXK* plants.

Supplemental Table S1. Quantitative real-time PCR primers used in this study.

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