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Hexokinase mediates stomatal closure

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SUMMARY

Stomata, composed of two guard cells, are the gates whose controlled movement allows the plant to balance the demand for CO_2 for photosynthesis with the loss of water through transpiration. Increased guard-cell osmolarity leads to the opening of the stomata and decreased osmolarity causes the stomata to close. The role of sugars in the regulation of stomata is not yet clear. In this study, we examined the role of hexokinase (HXK), a sugar-phosphorylating enzyme involved in sugar-sensing, in guard cells and its effect on stomatal aperture. We show here that increased expression of HXK in guard cells accelerates stomatal closure. We further show that this closure is induced by sugar and is mediated by abscisic acid. These findings support the existence of a feedback-inhibition mechanism that is mediated by a product of photosynthesis, namely sucrose. When the rate of sucrose production exceeds the rate at which sucrose is loaded into the phloem, the surplus sucrose is carried toward the stomata by the transpiration stream and stimulates stomatal closure via HXK, thereby preventing the loss of precious water.

Keywords: stomata, guard cells, hexokinase, sucrose, abscisic acid, transpiration.

INTRODUCTION

Stomata are dynamic pores in the impermeable protective cuticle that coats the aerial parts of land plants. Stomata, comprising two guard cells and the pore they circumscribe, open at dawn to allow the entry of atmospheric carbon dioxide (CO_2) for photosynthesis, at the cost of extensive transpirational water loss. The stomata close when carbon fixation and utilization are less efficient, in order to reduce the loss of water via transpiration (Assmann, 1993). At the mechanistic level, stomata open in response to increases in the osmolarity of the guard cells. These increases in osmolarity are followed by the movement of water into the guard cells, which increases their volume and opens the stomata (Taiz and Zeiger, 1998). Stomatal closure occurs in the opposite manner; as the osmolarity of guard cells is reduced, their volume decreases.

About a century ago, it was proposed that sugars generated from the degradation of starch in guard cells at dawn are the primary guard-cell osmolytes stimulating stomatal opening (Lloyd, 1908). This hypothesis was later modified by the discovery that K^+ ions, together with Cl^- and malate ions, are the primary osmolytes that accumulate in guard cells and open stomata (Schroeder *et al.*, 2001; Roelfsema and Hedrich, 2005; Pandey *et al.*, 2007). However, in addition to these ions, it has been suggested that the accumulation of sucrose (Suc) in guard cells as a result of starch degradation, photosynthetic carbon fixation or the import of apoplastic (intercellular) Suc also contributes to the osmotic state of the guard cells and the opening of the stomata (Gotow *et al.*, 1988; Tallman and Zeiger, 1988; Poffenroth *et al.*, 1992; Talbott and Zeiger, 1993, 1996). The hypothesis that Suc opens stomata is based primarily on correlations between the Suc content of guard cells and stomatal aperture (Tallman and Zeiger, 1988; Talbott and Zeiger, 1996). In addition, the accumulation of Suc in guard cells with open stomata over the course of the day and a concomitant decrease in K^+ content have been observed (Amodeo *et al.*, 1996; Talbott and Zeiger, 1998). Therefore, the current hypothesis is that, from midday on, Suc replaces K^+ as the major osmolyte keeping stomata open (Talbott and Zeiger, 1998; Schroeder *et al.*, 2001; Lawson, 2009).

Sucrose, a glucose–fructose disaccharide, is produced primarily in leaf mesophyll cells and is then transported throughout the plant via specialized vascular tissue called phloem. In many plant species, Suc is exported to the intercellular space, called the apoplast, prior to being loaded into the phloem (Rennie and Turgeon, 2009). As a result, the concentration of Suc in the leaf apoplast increases as photosynthesis is being carried out and may reach 2 mM (Kang *et al.*, 2007). Some of this apoplastic Suc is carried toward the open stomata by the transpiration stream, so that the concentration of Suc in the vicinity of the guard cells may reach 150 mM (Lu *et al.*, 1995, 1997; Ewert *et al.*, 2000; Outlaw and De Vlieghere-He, 2001; Kang *et al.*, 2007). It has been suggested that this accumulation of Suc decreases stomatal apertures due to an extracellular osmotic effect, which may serve as a means of coordinating the rates of photosynthesis and transpiration (Outlaw, 2003; Kang *et al.*, 2007). However, it is still thought that apoplastic Suc enters the guard cells and contributes to their opening (Tallman and Zeiger, 1988; Ritte *et al.*, 1999; Lawson, 2009).

To be metabolized, Suc must either be cleaved by apoplastic (extracellular) invertase or enter the cells via sucrose transporters and then be cleaved by intracellular sucrose-cleaving enzymes to yield the hexoses glucose (Glc) and fructose (Fru) (Dennis and Blakeley, 2000). Glucose and Fru must be phosphorylated by hexose-phosphorylating enzymes before they can be further metabolized. In plants, hexokinase (HXK) is the only enzyme that can phosphorylate Glc and it may also phosphorylate Fru (Granot, 2007, 2008). Most studies of HXK in plants have involved Arabidopsis HXK1 (*AtHXK1*), which has been shown to be a dual-function enzyme that mediates sugar-sensing in addition to its catalytic hexose-phosphorylation activity (Moore *et al.*, 2003; Rolland *et al.*, 2006). Increased expression of *AtHXK1* in Arabidopsis and tomato plants reduces the expression of photosynthetic genes and inhibits growth (Jang *et al.*, 1997; Dai *et al.*, 1999). In a previous study, we found that high levels of *AtHXK1* expression reduce the stomatal conductance and transpiration of Arabidopsis plants (Kelly *et al.*, 2012), suggesting that HXK might be involved in the regulation of stomatal aperture. In the current study, we examined the role of HXK in the regulation of stomata using well-characterized tomato lines that express *AtHXK1* at various levels, as well as newly created tomato and Arabidopsis lines with guard cell-specific expression of *AtHXK1*.

RESULTS

Direct correlation between HXK activity, stomatal closure and reduced transpiration

To examine the effect of HXK on tomato stomata, we used tomato lines with different levels of *AtHXK1* expression (Dai *et al.*, 1999). We measured the stomatal apertures and stomatal conductance of the tomato lines HK37, HK4 and HK38, which have levels of HXK activity that are two, five and six times higher than those of wild-type (WT) plants, respectively (Dai *et al.*, 1999). The stomatal densities of the *AtHXK1*-expressing lines are similar to those of WT plants (Table S1 in Supporting Information), yet both stomatal aperture and conductance were significantly reduced, in direct correlation with the level of *AtHXK1* expression (Figure 1a,b). Furthermore, continued measurement of transpiration over the course of the day revealed that the transpiration rate per unit leaf area was significantly reduced in the *AtHXK1*-expressing lines (Figure 1c), such that the cumulative whole-plant relative daily transpiration per unit leaf area (RDT) was clearly negatively correlated with HXK activity (Figure 1d).

To rule out the possibility that the observed decrease in transpiration was the result of inhibitory effects of *AtHXK1* on root water uptake or stem water transport, reciprocal grafting experiments were performed. HK4 shoots were grafted onto WT roots and WT shoots were grafted onto HK4 roots (Figure 2a). Continued measurements of the transpiration rates and cumulative whole-plant relative daily transpiration per unit leaf area of the grafted plants indicated that decreased transpiration was generally associated with HK4 shoots, with the roots having only minor influence (Figure 2b,c). To further examine the effect of HK4 stems on transpiration, we made triple-grafted plants in which HK4 interstock replaced a portion of the stem of WT plants (Figure 2d). The HK4 interstock had no effect on RDT (Figure 2e), indicating that the decreased transpiration of *AtHXK1*-expressing plants was the result of reduced transpiration by the leaves and not reduced water uptake by the roots or attenuated transport through the stem. This effect of *AtHXK1* on leaf transpiration suggests that HXK controls stomatal behavior that affects whole-plant transpiration.

Guard cell-specific expression of *AtHXK1* induces stomatal closure and reduces transpiration in tomato and Arabidopsis plants

To examine the role of HXK specifically in guard cells, we created tomato and Arabidopsis plants that express *AtHXK1* under the *KST1* guard cell-specific promoter (Muller-Rober *et al.*, 1995). The specific expression of the *KST1* promoter in tomato and Arabidopsis guard cells was verified by expression of GFP under the *KST1* promoter (GCGFP lines, Figure 3). In both tomato and Arabidopsis plants, expression of the *KST1* promoter was specific to guard cells and was not detected in organs that do not have stomata, such as roots (Figure 3d).

Unlike the expression of *AtHXK1* under the 35S promoter (Dai *et al.*, 2002; Kelly *et al.*, 2012), the expression of *AtHXK1* under the guard-cell specific *KST1* promoter

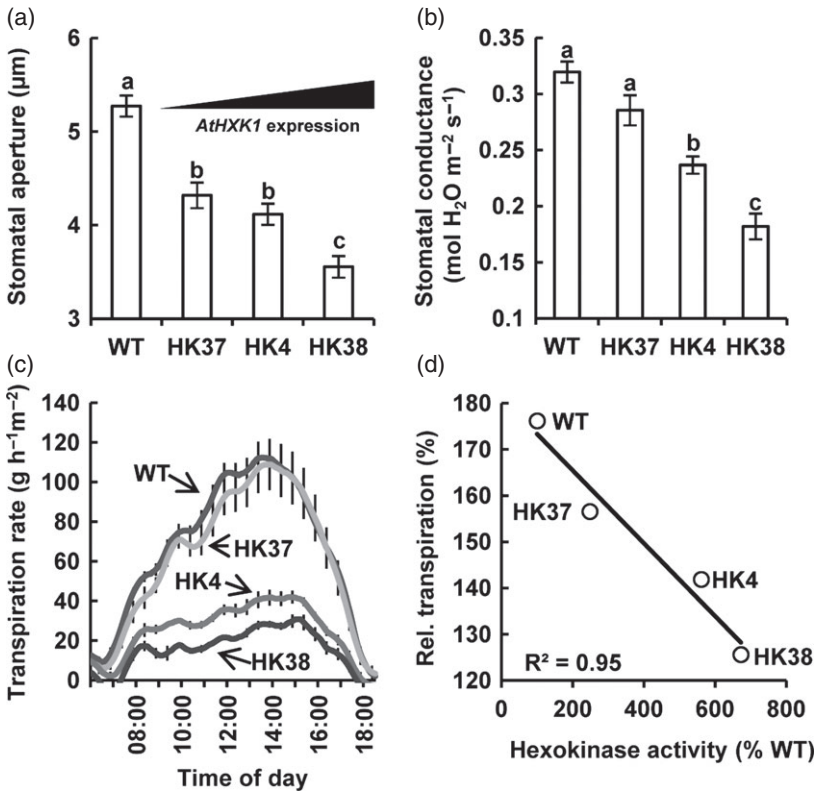

Figure 1. Elevated expression of hexokinase (HXK) enhances stomatal closure and decreases transpiration.
Stomatal aperture (a) and stomatal conductance (b) were determined for control (WT) and transgenic plants expressing different levels of *AtHXK1* (HK38 > HK4 > HK37) (Dai *et al.*, 1999). Aperture data are means of 200 stomata from four independent repeats ±SE. Stomatal conductance data are means of six independent repeats ±SE. Different letters indicate a significant difference (*t*-test, $P < 0.05$).
(c) The rate of transpiration normalized to the total leaf area was monitored simultaneously and continuously throughout the day and the data are given as the means ± SE for each tenth sampling point ($n = 6$).
(d) A negative correlation was observed between whole-plant relative daily transpiration and relative hexokinase-phosphorylation activity. The transpiration data were normalized to the total leaf area and the amount of water taken up by the neighboring submerged fixed-size wick each day, which was set to 100%. Wild-type hexokinase activity was set to 100%.

(GCHXK lines) did not inhibit the growth of tomato or Arabidopsis plants (Figures 4a and 5a). Yet, expression of *AtHXK1* under the *KST1* promoter reduced both stomatal conductance and transpiration in both tomato and Arabidopsis plants (Figures 4b,c and 5b,c). These results suggest that HXK plays a specific role in guard cells, influencing stomatal closure.

The role of HXK in stomatal closure was further examined using the Arabidopsis *AtHXK1*-knockout *gin2-1* mutant (Moore *et al.*, 2003). The Arabidopsis *gin2-1* mutant had higher stomatal conductance, a higher transpiration rate (Figure 5b,c) and a 23% increase in stomatal aperture (*t*-test, $P < 0.05$), as compared with the WT control plants, supporting the hypothesis that HXK affects stomatal closure.

Sucrose stimulates stomatal closure via HXK

Given that HXK phosphorylates hexose sugars, we hypothesized that the control of stomatal closure by HXK is mediated by sugar. Sucrose is the primary sugar produced in photosynthetic tissues of many plant species, including tomato and Arabidopsis. It is exported to the apoplast prior to being loaded into the phloem and may enter the symplastically isolated guard cells via sucrose transporters located in the plasma membranes of guard cells (Meyer *et al.*, 2004; Weise *et al.*, 2008; Bates *et al.*, 2012) and then be cleaved to yield hexoses that are substrates of HXK. The apoplastic Suc may also be cleaved by apoplastic invertases to yield the hexoses Glc and Fru, which may enter the guard cells via hexose transporters, also found in the guard-cell plasma membrane (Stadler *et al.*, 2003; Bates *et al.*, 2012). In both cases, the hexose Glc must be phosphorylated by HXK, which may also phosphorylate Fru. HXK is expressed in guard cells (Bates *et al.*, 2012). Accordingly, we hypothesized that HXK may stimulate stomatal closure in response to extracellular accumulation of naturally transported apoplastic Suc.

To test this hypothesis, we examined the effect of extracellular Suc on stomatal aperture. We immersed intact WT tomato leaflets in artificial apoplastic solutions (Wilkinson and Davies, 1997) containing either 100 mM Suc or 100 mM sorbitol, a non-metabolic sugar used as an osmotic control, and measured stomatal aperture. Sucrose decreased stomatal aperture size by 29% relative to sorbitol (Figure 6a,b). Cleavage of Suc by cell wall (apoplastic) invertases may yield Glc and Fru in equal proportions (Granot, 2007), which may result in extracellular osmolarities that approach 200 mOsm L^{-1}, as compared with the 100 mOsm L^{-1} of the original Suc added. We, therefore, compared the effects of 100 mM sucrose, 100 mM Glc + 100 mM Fru and 200 mM Glc or Fru with the effect of 200 mM mannitol, which was used as an additional osmotic control. All of the sugar combinations decreased stomatal apertures compared to the effect of 200 mM mannitol

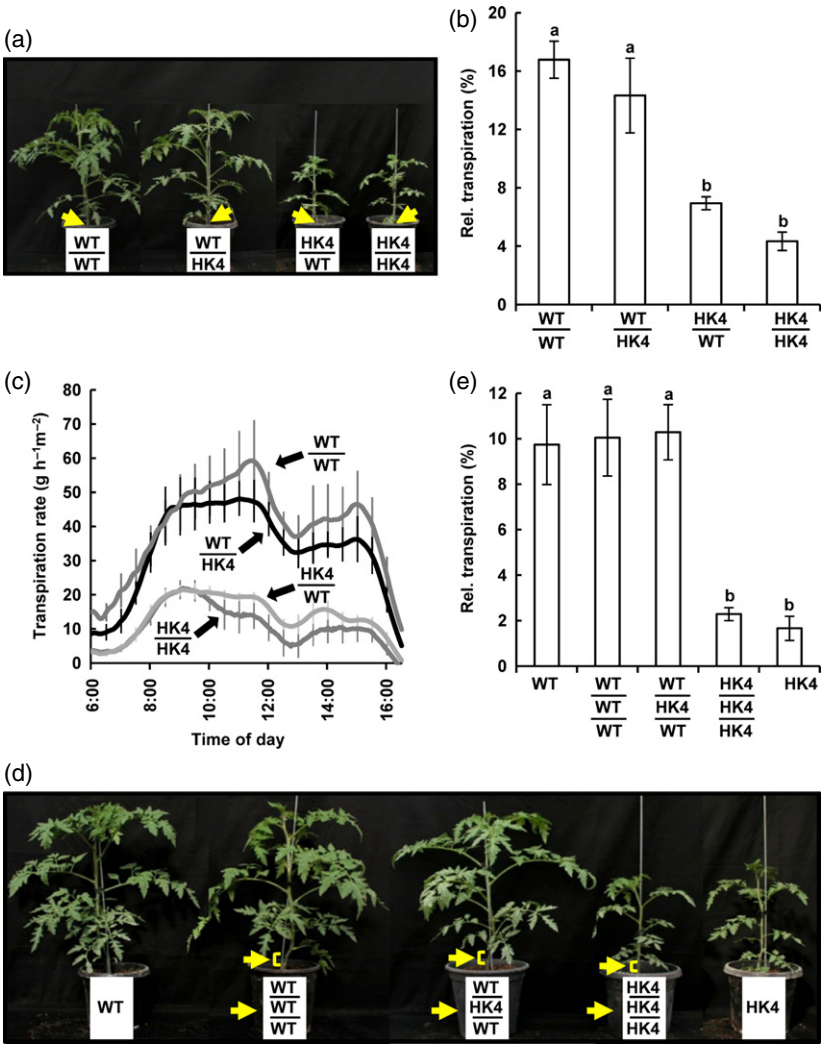

Figure 2. *AtHXK1* reduces transpiration primarily when expressed in leaves.
Reciprocal grafting (a) and triple-grafting (d) procedures were performed at the seedling stage and plants were photographed and used for transpiration measurements about 4 weeks after grafting. The yellow arrows and brackets indicate the location of the grafts.
(b) Whole-plant relative daily transpiration of reciprocal-grafted plants. Data were normalized to the total leaf area and the amount of water taken up by the neighboring submerged fixed-size wick each day, which was set to 100%. Data are given as means of four independent repeats ±SE. Different letters indicate a significant difference (*t*-test, $P < 0.05$).
(c) Transpiration rate normalized to the total leaf area of reciprocal-grafted plants was monitored simultaneously and continuously throughout the day. The data are given as the means ± SE for each 10th sampling point ($n = 4$).
(e) Relative daily transpiration of whole triple-grafted plants calculated as in (b).

(Figure 6c), supporting an osmotic-independent role for sugars in the regulation of stomatal closure. To examine whether Suc stimulates stomatal closure via HXK, we tested the effect of Suc in the presence of *N*-acetyl glucosamine (NAG), an efficient inhibitor of HXK activity (Hofmann and Roitsch, 2000). *N*-acetyl glucosamine almost completely abolished the effect of Suc and prevented stomatal closure, suggesting that HXK mediates stomatal closure (Figure 6b).

To further explore whether HXK mediates stomatal closure, we examined the effect of Suc on the stomata of *AtHXK1*-expressing tomato plants (Dai *et al.*, 1999). The stomatal aperture of *AtHXK1*-expressing plants (the HK4 line, which has a level of HXK activity that is five times higher than that of WT plants) was reduced by 21% relative to the control WT plants even under the control conditions (100 mM sorbitol) (Figure 6b). The addition of Suc caused the stomata of HK4 plants to close even further (Figure 6b) and the HXK inhibitor NAG abolished the closing effect of Suc, further indicating that HXK stimulates stomatal closure (Figure 6b).

HXK mediates stomatal closure independent of downstream metabolism of the phosphorylated sugars

It has previously been shown that AtHXK1 mediates sugar effects independent of the downstream metabolism of the phosphorylated sugar (Moore *et al.*, 2003). To examine whether the mediation of stomatal closure by HXK is independent of downstream metabolism of the phosphorylated sugars, we examined the effects of mannose (a glucose epimer at the second carbon atom) and 2-dexoxyglucose (2-dG, a glucose analog). Both of these sugars are phosphorylated by HXK, but 2-dG is not metabolized and mannose is metabolized poorly (Klein and Stitt, 1998; Pego *et al.*, 1999). Both mannose and 2-dG reduced stomatal aperture, with mannose having an even stronger effect (Figure 7) in line with previous observations that mannose is more potent than glucose with regard to HXK-mediated sugar effects

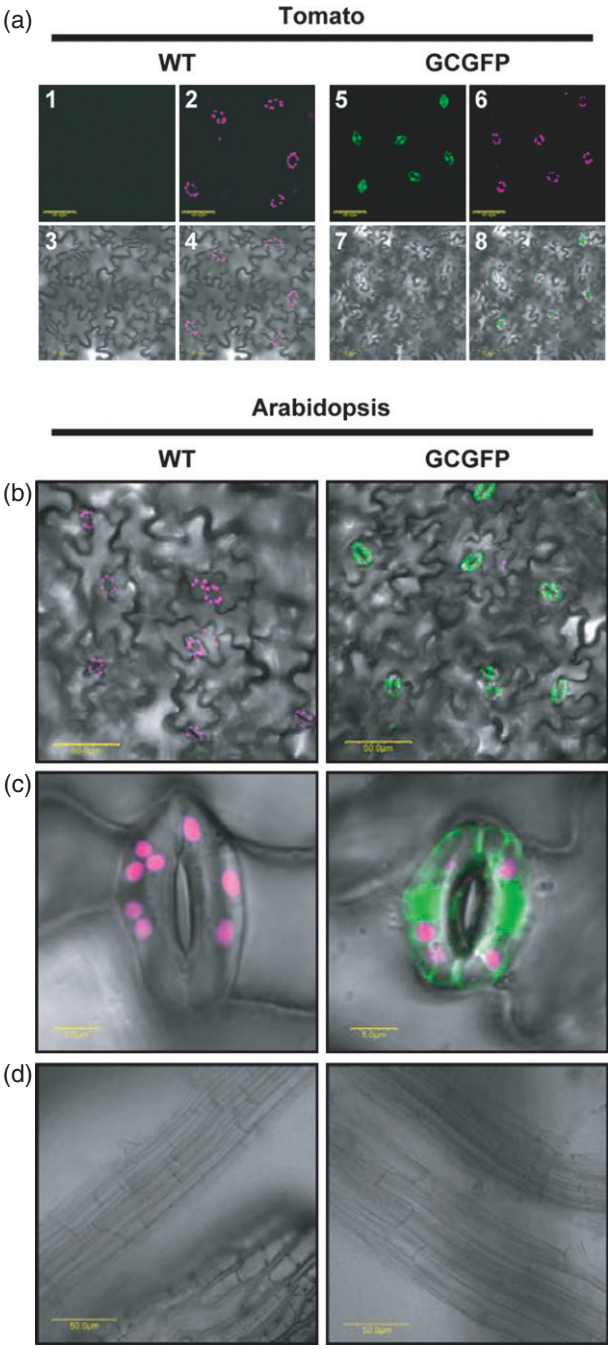

Figure 3. Expression of GFP under the control of the *KST1* promoter is specific to guard cells.
(a) Confocal images of wild-type (WT) (panels 1–4) and transgenic tomato leaves (panels 5–8) of plants with guard-cell specific expression of GFP (designated GCGFP) under the control of the *KST1* promoter. Panels 1 and 5 are images of GFP fluorescence (stained green), panels 2 and 6 are chlorophyll autofluorescence (stained magenta), panels 3 and 7 are white light images and panels 4 and 8 are merged images.
(b–d) Confocal images of WT (left) and transgenic Arabidopsis GCGFP plants (right). Images were taken from leaves (b and c, bars = 50 and 5 μm, respectively), and roots (d, bar = 50 μm). All panels are merged images of white light, chlorophyll autofluorescence (magenta) and GFP fluorescence (green).

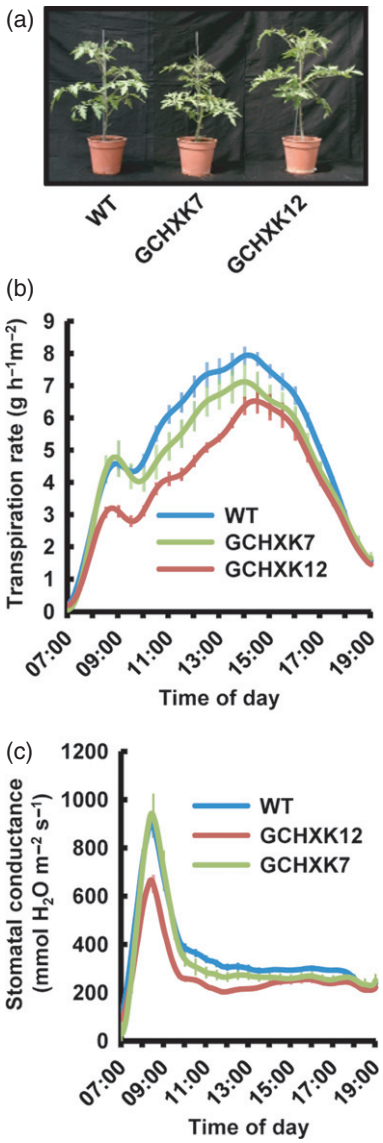

Figure 4. Guard cell-specific expression of *AtHXK1* induces stomatal closure and reduces transpiration of tomato plants.
(a) Representative images of wild-type (WT) and two independent transgenic tomato lines expressing *AtHXK1* specifically in guard cells (GCHXK7 and 12).
The rates of transpiration (b) and stomatal conductance (c) were monitored simultaneously and continuously throughout the day in WT plants (blue line) and two independent transgenic tomato lines expressing *AtHXK1* specifically in guard cells (GCHXK7, green line, and GCHXK12, red line).
(b) The rate of transpiration was normalized to the total leaf area and the amount of water taken up by the neighboring submerged fixed-size wick each day, which was set to 100%.
(c) Stomatal conductance was calculated as described in the Experimental Procedures.
Data points in (b) and (c) are given as means ± SE for each tenth sampling point (n = 12, 10 and 8 for WT, GCHXK12 and GCHXK7 respectively). When not seen, SE is smaller than the symbol.

(Jang and Sheen, 1994; Pego *et al.*, 1999). These results imply that HXK mediates stomatal closure independent of downstream metabolism of the phosphorylated sugars.

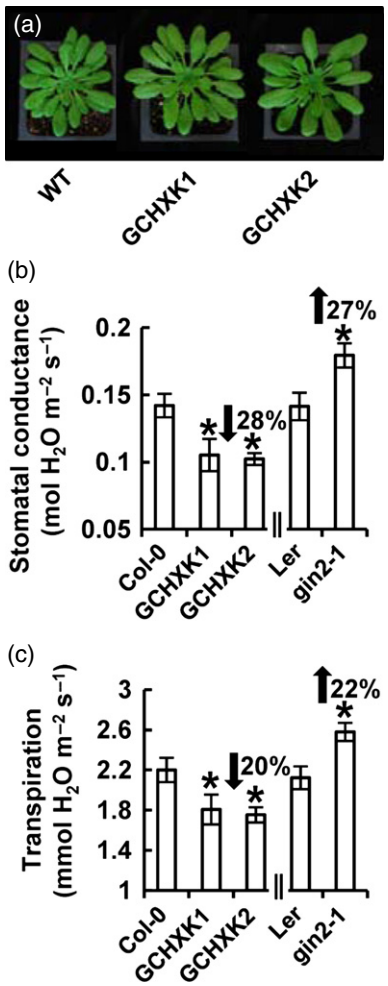

Figure 5. Guard cell-specific expression of *AtHXK1* induces stomatal closure and reduces transpiration of Arabidopsis plants.
(a) Representative images of wild-type (WT) Arabidopsis (Col. ecotype) and two independent transgenic lines expressing *AtHXK1* specifically in guard cells (GCHXK1 and 2).
(b), (c) Stomatal conductance and transpiration measurements of WT, two independent transgenic Arabidopsis lines, GCHXK1 and GCHXK2 (Col. ecotype), and of the *gin 2-1* (*AtHXK1* null mutant, L*er.* ecotype). Arrows indicate increased or decreased conductance and transpiration relative to the WT. Data are given as means (±SE) of 8 and 12 independent repeats for the GCHXK and *gin2-1* lines, respectively. Asterisks denote significant differences relative to the WT (*t*-test, $P < 0.05$).

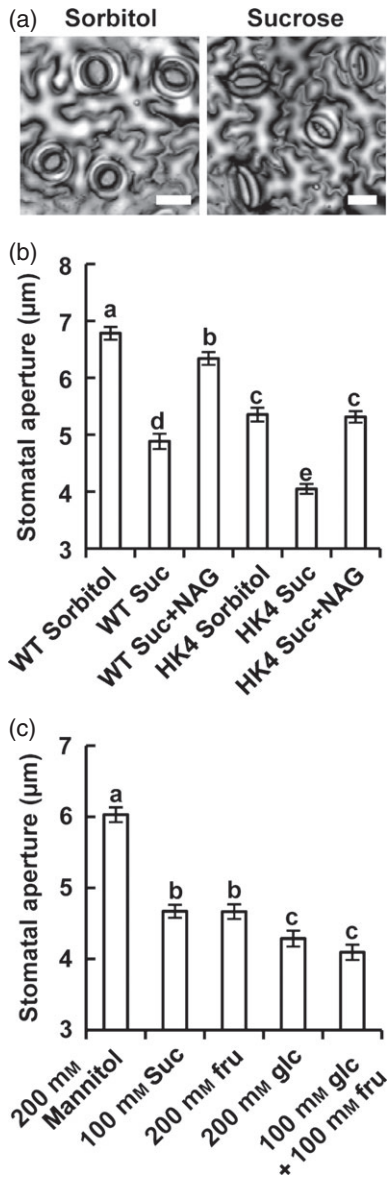

Figure 6. Sugars stimulate stomatal closure.
(a) Representative light microscopy images of stomata taken from epidermal imprints 3 h after treatment with 100 mM sorbitol or 100 mM sucrose (white bar = 20 μm).
(b) Stomatal response to sucrose in wild-type (WT) and *AtHXK1*-expressing plants (HK4) was assayed with intact leaves immersed for 3 h in artificial apoplastic sap (Wilkinson and Davies, 1997) containing 100 mM sorbitol (as an osmotic control), 100 mM sucrose (Suc) or 100 mM Suc together with 20 mM of the hexokinase inhibitor *N*-acetyl-glucoseamine (NAG). Epidermal imprints were then taken and stomatal aperture was measured.
(c) The stomatal responses of WT plants to the different sugar combinations were assayed as described in (b), with 200 mM mannitol serving as an additional osmotic control.
The data shown in (b) and (c) are means of 300 stomata from six independent biological repeats ±SE. Different letters indicate a significant difference (*t*-test, $P < 0.05$).

Sucrose stimulates the production of nitric oxide (NO) in guard cells

It has previously been shown that the sugar-signaling effects of HXK, such as the inhibition of photosynthesis and growth, are mediated by abscisic acid (ABA) [for an updated review see Rolland *et al.* (2006)], a well-known phytohormone that also induces stomatal closure. We, therefore, speculated that Suc might stimulate stomatal closure via HXK and ABA within guard cells. The effects of ABA in guard cells are mediated by the rapid production of nitric oxide (NO), which is required for ABA-induced stomatal closure and serves as an indicator of stomatal-closure stimuli (Garcia-Mata *et al.*, 2003; Neill *et al.*, 2008). To examine the effect of Suc on the ABA-signaling pathway

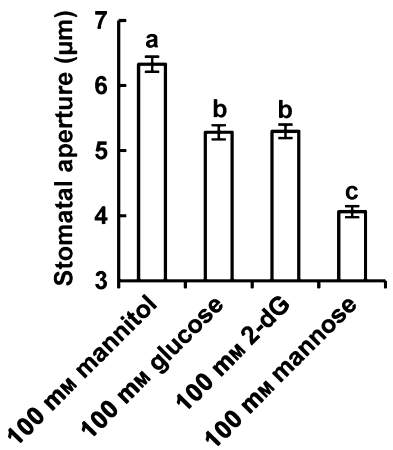

Figure 7. Glucose and sugars that have been phosphorylated, but not metabolized stimulate stomatal closure.
Stomatal responses to different sugars were assayed in intact leaves of wild-type plants. The leaves were immersed for 3 h in artificial apoplastic sap (Wilkinson and Davies, 1997) containing mannitol (as an osmotic control), glucose, 2-deoxyglucose (2-dG) or mannose. Epidermal imprints were then taken and stomatal aperture was measured. Data are given as means of 400 stomata from eight independent biological repeats ±SE. Different letters indicate a significant difference (*t*-test, $P < 0.05$).

in guard cells, we monitored NO levels within guard cells in response to applications of Suc. Epidermal peels were incubated with Suc and monitored using the fluorescent NO indicator dye diaminofluorescein diacetate (DAF-2DA). Applications of 100 mM sorbitol had no effect on NO levels in guard cells (Figure 8a). However, the application of 100 mM Suc resulted in a 3.5-fold increase in guard-cell fluorescence, indicating a rapid increase in NO levels, which was correlated with stomatal closure (Figure 8a). These results are in line with previous work showing that the application of ABA triggers a three-fold increase in the DAF-2DA fluorescence of isolated guard cells (Desikan *et al.*, 2002). The guard cells of untreated HK4 (*AtHXK1*-expressing line) epidermal peels exhibited high NO levels, similar to those of Suc-treated WT epidermal peels (Figure 8b), and the addition of Suc to the peeled HK4 epidermis led to even more intense fluorescence (Figure 8b).

To further examine the involvement of HXK in the production of NO in guard cells, we used the HXK inhibitor NAG with epidermal peels. NAG not only inhibited the effect of Suc and blocked stomatal closure (Figure 6b), it also prevented the production of NO (Figure 8c). Washing out NAG with 100 mM Suc led to the resumption of NO production within <30 min (Figure 8d,e). These results suggest that Suc elicits a guard cell-specific NO response via HXK.

Abscisic acid is essential for Suc-stimulated production of NO

To verify that ABA is indeed required for the stomatal NO response to Suc, we conducted experiments with the ABA-deficient tomato mutant *Sitiens*, which has stomata that are always open (Neill and Horgan, 1985). Unlike what was observed for the WT plants, treating *Sitiens* epidermal peels with 100 mM Suc did not result in any increase in fluorescence or stomatal closure, indicating that there was no production of NO (Figure 8f). However, treating *Sitiens* peels with externally supplied ABA did trigger the production of NO (Figure 8f) and stomatal closure. These findings indicate that *Sitiens* guard cells retain their ability to respond to externally supplied ABA by producing NO and that only the absence of ABA production in the *Sitiens* mutant prevents Suc-triggered NO production and stomatal closure. This observation confirms that ABA is a vital mediator of the stomatal response to Suc and that *Sitiens* stomata do not respond to Suc, due to this mutant's ABA deficiency. Together, these results support the hypothesis that Suc stimulates stomatal closure via HXK and ABA.

DISCUSSION

The role of HXK in specific types of cells

Hexokinase is the only enzyme that can phosphorylate glucose in plants, and therefore is probably present in most, if not all, types of cells (Granot *et al.*, 2013). Specifically, HXKs are expressed in guard cells (Arabidopsis eFP Browser http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi?dataSource=Guard_Cell, and Bates *et al.* (2012)). Yet, the sugar-sensing role of HXK might vary between different plant parts and tissues. Most studies of the role of HXK in plants have involved whole seedlings and intact plants in which the expression of HXK was enhanced or reduced by the use of the globally expressed 35S promoter or HXK-knockout plants, such as the Arabidopsis *AtHXK1* mutant *gin2-1* (Jang *et al.*, 1997; Moore *et al.*, 2003). The possibility of different roles for HXK in shoots compared with roots was examined in reciprocal grafting experiments in which Arabidopsis *AtHXK1* under the 35S promoter inhibited the growth of tomato plants only when expressed in the shoots (Dai *et al.*, 1999). A role for AtHXK1 in mesophyll cells, inhibiting the expression of photosynthetic genes, has also been demonstrated (Cho *et al.*, 2006). In this study, we show that HXK plays a role in guard cells, mediating stomatal closure in response to sugar.

New roles for Suc and HXK in the regulation of stomatal closure

Sucrose is the main sugar generated throughout photosynthesis and transported in the phloem of many plant species. Sucrose may also be generated within guard cells via photosynthesis or starch degradation (Vavasseur and Raghavendra, 2005; Lawson, 2009). Over the years, scientists have put much effort into identifying osmolytes that can open stomata by contributing to guard-cell osmolarity and Suc taken up from the apoplast or generated within

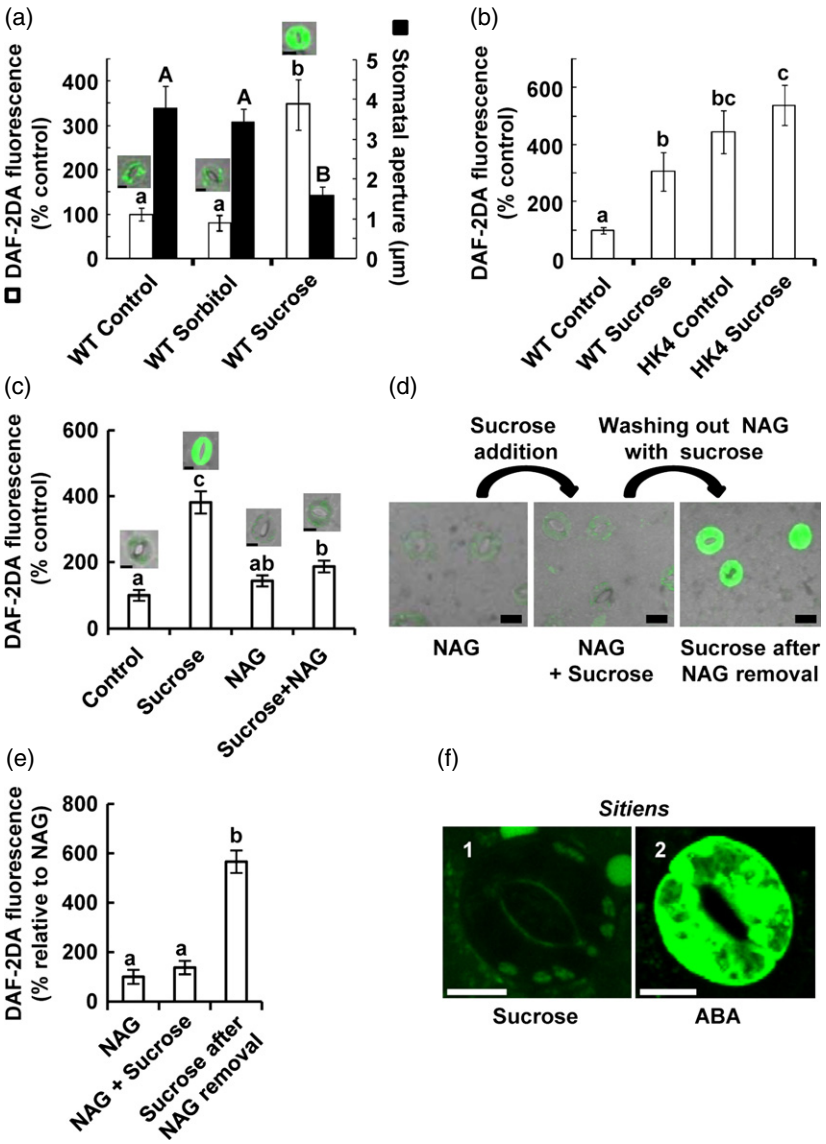

Figure 8. Sucrose (Suc) stimulates ABA-dependent NO production in guard cells that is mediated by hexokinase.
(a), (b) Nitric oxide (NO) levels were monitored in guard cells from epidermal peels of wild-type (WT) and *AtHXK1*-expressing (HK4) plants using the fluorescent NO indicator dye diaminofluorescein diacetate (DAF-2DA). Relative fluorescence levels of guard cells (white bars) and stomatal apertures (black bars) were determined after 30 min of treatment with 2-(*N*-morpholino)-ethane sulfonic acid (MES) buffer (control) or MES containing either 100 mM Suc or 100 mM sorbitol as an osmotic control. Representative fluorescence images are shown above the fluorescence columns (bar = 10 μm). Data are given as means ± SE of 90 stomata (a) or 60 stomata (b) for each treatment with three to four independent biological repeats of each treatment.
(c) Relative fluorescence levels of WT guard cells were determined after 30 min of treatment with MES buffer (control), MES containing 20 mM of the hexokinase inhibitor *N*-acetyl-glucosamine (NAG), or 100 mM Suc with or without 20 mM NAG. Representative fluorescence images are shown above the fluorescence columns (bar = 10 μm). Data are given as means of 60 stomata from three independent biological repeats per treatment ±SE.
(d) Confocal images of NO production in guard cells of epidermal peels treated with 20 mM NAG only (left), 30 min after the addition of 100 mM Suc (middle) and 30 min after the NAG was washed out with 100 mM Suc (right). The assay was conducted as the same epidermal strip was being photographed (bar = 20 μm).
(e) Relative fluorescence levels of guard cells from an epidermal strip treated as in (d). Data are given as means of 40–60 stomata ±SE.
(f) Confocal images of NO production in guard cells of epidermal peels of *Sitiens* (ABA-deficient mutants) after 30 min of treatment with MES buffer containing either 100 mM Suc (left) or 100 μM ABA (right); bar = 10 μm.
Different lower-case letters in (a)–(c) and (e) indicate a significant difference among the treatments with respect to the fluorescence data and different upper-case letters in (a) indicate a significant difference among the treatments with respect to the stomatal aperture data (*t*-test, $P < 0.05$).

the guard cells has generally been considered a trivial candidate (Talbott and Zeiger, 1998; Lawson, 2009). However, the data from the studies that do support the role of Suc as a stomata-opening osmolyte are mostly correlative; Suc levels in guard cells were measured in correlation with stomatal aperture. Otherwise, almost no functional studies on the role of Suc in guard cells have been carried out (Tallman and Zeiger, 1988; Talbott and Zeiger, 1998; Lawson, 2009). Bates *et al.* (2012) analyzed changes in Arabidopsis guard-cell transcriptomes in response to extracellular Suc. They found that Suc affects the expression of 440 genes associated with photosynthesis and the transport of sugars, water, amino acids and ions. However, no conclusive evidence regarding the role of Suc in guard cells was presented in that study (Bates *et al.*, 2012).

Several studies have questioned the importance of photosynthetically produced Suc for stomatal opening (Baroli *et al.*, 2008; Lawson *et al.*, 2008). In these studies, a reduced rate of photosynthesis did not reduce stomatal opening as might be expected if Suc opens stomata. On the contrary, antisense inhibition of sedoheptulose-1, 7-bisphosphatase (SBPase), a key enzyme involved in photosynthesis and sucrose production, reduced the rate of photosynthesis, yet those plants displayed a tendency toward greater stomatal opening (Lawson *et al.*, 2008). The authors of that study suggested that their results fit nicely

with the hypothesis that lower photosynthetic rates would result in a reduced apoplastic Suc concentration in the vicinity of the guard cells and, consequently, a greater stomatal aperture, as suggested by Kang *et al.* (2007).

Our current results are in line with the findings of the studies mentioned above, which showed that Suc does not promote stomatal opening but rather stimulates stomatal closure via HXK and the ABA pathway. The observation that Suc stimulates stomatal closure via HXK suggests that Suc is being cleaved, apoplastically or within the guard cells, and that the resulting hexoses are stimulating an intracellular response via HXK that leads to stomatal closure. Bates *et al.* (2012) reported that both *AtHXK1* and *AtHXK2* are expressed in Arabidopsis guard cells, but their expression is not stimulated by Suc. This observation fits our hypothesis that HXK already present in guard cells mediates a rapid sugar-sensing response within those cells that does not require further stimulation of HXK expression. In a recent study, overexpression of the Arabidopsis trehalase (*AtTRE1*) that cleaves the Glc–Glc disaccharide trehalose into glucose monomers stimulated stomatal closure and enhanced the closure response of stomata to ABA (Van Houtte *et al.*, 2013). *AtTRE1* is also expressed in guard cells (Van Houtte *et al.*, 2013). This study (Van Houtte *et al.*, 2013) also supports our hypothesis that the hexose Glc is sensed by HXK and stimulates stomatal closure.

The reduced stomatal conductance and reduced transpiration of naturally grown, intact tomato and Arabidopsis plants that express *AtHXK1* specifically in guard cells (Figures 4 and 5) also support the hypothesis that the sugar response is mediated by HXK. We suggest that the expression of *AtHXK1* in guard cells increases the amount of HXK which enhances the HXK-mediated sugar signal and accelerates stomatal closure in response to increasing sugar levels over the course of the day. It is worth noting that the tomato plants that express *AtHXK1* in their guard cells exhibit less transpiration and lower levels of stomatal conductance mainly in the middle of the day (between 9 a.m. and 2 p.m.), when transpiration rates are high and more sucrose is supposedly being carried toward the guard cells (Figure 4b,c).

Based on our findings, we speculate that the previously observed accumulation of Suc within guard cells over the course of the day (Tallman and Zeiger, 1988; Talbott and Zeiger, 1998) might not reflect an osmotic opening role but rather the uptake of apoplastic Suc by the guard cells (Ritte *et al.*, 1999), which eventually induces the closure of the stomata. Since guard cells are capable of carrying out photosynthesis and producing sugars (Lawson, 2009), it is possible that not only extracellular sugars but also intracellular guard cell-produced sugars might have a stomatal closure effect and may stimulate the midday stomatal closure (Figure 4b,c).

Sucrose may coordinate photosynthesis with transpiration

The response of stomata to Suc may indicate a simple feedback-inhibition mechanism. The concentration of Suc in the apoplast of the guard cells might change as result of the relationship between the rate of photosynthesis and the capacity to export the photosynthetic products from the leaf via the phloem (Lu *et al.*, 1995, 1997; Ewert *et al.*, 2000; Outlaw and De Vlieghere-He, 2001; Kang *et al.*, 2007). When the rate of photosynthesis is high, any surplus Suc produced in excess of the phloem's transport capacity might be carried toward the stomata by the transpiration stream (Kang *et al.*, 2007; Nikinmaa *et al.*, 2013). At a high rate of photosynthesis the substomatal CO_2 concentration (C_i) declines and stimulates stomatal opening (Assmann, 1993). Under these conditions, the surplus of sucrose may serve as signal to close the stomata, reducing CO_2 uptake and decreasing the rate of photosynthesis.

It has been suggested that the accumulation of Suc in the apoplast of the guard cells may function as an osmotic feedback-inhibition signal that decreases stomatal apertures via an extracellular osmotic effect (Outlaw, 2003; Kang *et al.*, 2007). However, our results indicate that Suc stimulates an intracellular guard-cell response that closes stomata independent of its osmotic effect (Figures 6b,c and 7).

Hexokinase stimulates an ABA response within guard cells

The usually studied sugar-sensing effects of HXK, namely decreased expression of photosynthesis-related genes and growth inhibition, are dependent on ABA, as ABA-deficient mutants are insensitive to HXK-mediated sugar responses (Arenas-Huertero *et al.*, 2000; Leon and Sheen, 2003; Rolland *et al.*, 2006). Furthermore, it has been shown that glucose increases ABA biosynthesis and induces the expression of ABA-related genes, presumably via HXK, in as-yet unspecified tissues (Leon and Sheen, 2003). We have also shown, in a previous study, that overexpression of *AtHXK1* in Arabidopsis induces ABA-related gene expression (Kelly *et al.*, 2012). In this study, we showed that the effect of HXK in guard cells is also ABA-dependent (Figure 8f). However, unlike the unknown origin of the ABA involved in the HXK-mediated inhibition of photosynthesis and growth, it is likely that the ABA involved in the HXK-mediated guard-cell response originates from within the guard cells. This assumption is based on the fact that HXK stimulates the ABA-dependent production of NO in the guard cells of epidermal peels, which are composed primarily of epidermis and guard cells. Unlike epidermis cells, guard cells have the ability to produce ABA (Koiwai *et al.*, 2004; Melhorn *et al.*, 2008). Therefore, it is likely that HXK stimulates the production of ABA, the release of biologically active ABA from inactive ABA pools, or inhibits the degradation of ABA within the guard cells (Christmann *et al.*, 2005; Wasilewska *et al.*, 2008; Zhu *et al.*, 2011).

Closure of stomata triggered by ABA is usually associated with water stress (Comstock, 2002; Christmann *et al.*, 2005; Schachtman and Goodger, 2008; Wasilewska *et al.*, 2008). However, ABA is probably essential for daily regulation of stomata aperture independent of water stress, since the stomata of ABA-deficient mutants such as *Sitiens* and *Flacca* remain open at all times regardless of water stress (Tal and Nevo, 1973; Neill and Horgan, 1985). Our results suggest that ABA is stimulated by HXK and might have a constant diurnal role in guard cells, mediating the Suc feedback inhibition of stomatal aperture over the course of the day, thereby modulating stomatal aperture.

CONCLUSIONS

This study shows that HXK plays a role in guard cells, stimulating stomatal closure in response to sugar levels. Unlike previous studies, which relied on correlations between sucrose content and stomatal aperture, this study took a functional approach to the examination of the effects of sugar on stomatal behavior. We discovered that sugars stimulate a guard cell-specific response that is mediated by HXK and ABA and leads to stomatal closure. We assume that this response presents a natural feedback mechanism aimed at reducing transpiration and conserving water under an excess of photosynthesis, thus coordinating between photosynthesis and transpiration. We believe that this study may expand our understanding of the regulation of transpiration and may be useful in the development of crop cultivars that lose less water to transpiration.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Experiments were conducted using WT tomato (*Solanum lycopersicum* cv. MP-1), isogenic independent transgenic homozygous tomato lines expressing different levels of the Arabidopsis *AtHXK1* (*35S::AtHXK1*) [as previously described by Dai *et al.*, 1999)], isogenic transgenic homozygous lines expressing GFP or *AtHXK1* under the control of the *KST1* promoter, and the ABA-deficient mutant *Sitiens* (Neill and Horgan, 1985) (*S. lycopersicum* cv. Ailsa Craig).

Arabidopsis (Columbia, Col.) and tomato (MP-1) lines that express GFP or *AtHXK1* specifically in guard cells (GCGFP and GCHXK lines, respectively) were generated following transformation with GFP or *AtHXK1* expressed under the *KST1* promoter (Muller-Rober *et al.*, 1995). Independent transgenic homozygous lines for each construct were then identified. The tomato plants were grown in a temperature-controlled greenhouse under natural growth conditions and the Arabidopsis plants were grown in a walk-in growth chamber kept at 22°C, with an 8-h light/16-h dark photoperiod.

Stomatal measurements

Stomatal aperture and density were determined using the rapid imprinting technique described by Geisler and Sack (2002). This approach allowed us to reliably score hundreds of stomata from each experiment, each of which was sampled at the same time. In brief, light-bodied vinylpolysiloxane dental resin (Heraeus-Kulzer, http://heraeus-dental.com/) was attached to the abaxial leaf side and then removed as soon as it had dried (1 min). The resin epidermal imprints were covered with nail polish, which was removed once it had dried out and served as a mirror image of the resin imprint. The nail-polish imprints were put on microscope slides and photographed under a bright-field inverted microscope (1M7100; Zeiss, http://www.zeiss.com/) on which a Hitachi HV-D30 CCD camera (Hitachi, http://www.hitachi.com/) was mounted. Stomatal images were later analyzed to determine aperture size using the ImageJ software (http://rsb.info.nih.gov/ij/) fit-ellipse tool. A microscopic ruler (Olympus, http://www.olympus-global.com/) was used for the size calibration.

To asses stomatal response, leaflets were cut and immediately immersed in artificial xylem sap solution (AXS) (Wilkinson and Davies, 1997) containing sucrose (Duchefa Biochemie, http://www.duchefa-biochemie.nl/) with or without NAG (Sigma-Aldrich, http://www.sigmaaldrich.com/), glucose (Duchefa Biochemie), fructose (Sigma-Aldrich), 2-deoxyglucose (Sigma-Aldrich), mannose (Sigma-Aldrich), sorbitol (Sigma-Aldrich) or mannitol (Duchefa Biochemie). The sorbitol and mannitol treatments served as non-metabolic osmotic controls. Imprints were taken 3 h after immersion and stomatal aperture was analyzed.

Stomatal conductance and transpiration analyses

Tomato stomatal conductance (g_s) was measured using a leaf conductance steady-state porometer LI-1600 (LI-COR, http://www.licor.com/) (Figure 1b). Arabidopsis g_s and transpiration were measured using a portable gas-exchange system (LI-COR), as described by (Flexas *et al.*, 2007).

Whole-plant transpiration and continuous stomatal conductance measurements

Whole-plant RDT and transpiration rates were determined using lysimeters, as described in detail in Sade *et al.* (2010). Wild-type plants and either HK37, HK4 and HK38, GCHXK7, GCHXK12 transgenic plants or grafted plants were planted in 3.9-L pots and grown under controlled conditions. Each pot was placed on a temperature-compensated load cell with digital output and was sealed to prevent evaporation from the surface of the growth medium. A wet vertical wick made of 0.14-m^2 cotton fibers partially submerged in a 1-L water tank was placed on a similar load cell and used as a reference for the temporal variations in the potential transpiration rate. The output of the load cells was monitored every 10 sec and the average readings over 3 min were logged in a data logger for further analysis. The whole-plant transpiration was calculated by a numerical derivative of the load cell output following a data-smoothing process (Sade *et al.*, 2010). The plant's daily transpiration rate was normalized to the total leaf area (measure by a LI-COR area meter model Li-3100) and the data for a neighboring submerged wick and these figures were averaged for a given line over all plants (amount taken up by the wick daily = 100%). Continuous stomatal conductance of whole canopy (g_{sc}; mmol sec^{-1} m^{-2}) was calculated by dividing the whole-plant transpiration rate, E (i.e. the whole-plant transpiration normalized to the plant leaf area; mmol sec^{-1} m^{-2}) by VPD using equation (1), in which P_{atm} is the atmospheric pressure (101.3 kPa).

$$g_{sc} = \frac{E * P_{atm}}{VPD} \quad (1)$$

The vapor pressure deficit (VPD) is the difference (in kPa) between the vapor pressure of the saturated air and the vapor pressure of the ambient air. In plants, this refers to the difference

between the pressure in the substomatal cavities and the atmospheric pressure. In equation (2), T is the air temperature (°C), RH is relative humidity (0–1), 0.611 is the saturation vapor pressure at 0°C and 17.502 and 240.97 are constants (Buck, 1981). The temperature and relative humidity in the greenhouse were monitored using sensors (HC2-S3-L; Rotronic, http://www.rotronic.com/).

$$\mathrm{VPD} = (1 - \mathrm{RH})0.611\ \exp\left(\frac{17.502 - T}{240.97 + T}\right) \quad (2)$$

Hexokinase activity assay

Hexokinase activity was measured as described by Dai *et al.*, (1999).

Monitoring NO production in guard cells

Detection of NO levels in stomata was performed as follows. Epidermal peels were prepared and incubated in MES buffer [25 mM MES-KOH, pH = 6.15 and 10 mM KCl (MES, 2-(*N*-morpholino)-ethane sulfonic acid; Sigma-Aldrich)] with or without 20 mM NAG, for 2.5 h under steady light, and then loaded with 60 μM NO indicator dye, DAF-2DA (4,5-diaminofluorescein diacetate; Sigma-Aldrich), diluted in MES buffer with or without NAG, and left for an additional 50 min. Then the peels were washed with MES three times and incubated for 30 min in the buffer (control, set as 100% fluorescence) or in 100 mM sorbitol, 100 mM sucrose and 20 mM NAG, as described for each experiment. The peels were then photographed under a microscope (see Confocal microscopy imaging). Three to four biological repeats containing 20–30 stomata each were included in each experiment and each experiment was repeated several times. Images were analyzed using the ImageJ software histogram tool to evaluate fluorescence intensity and the fit-ellipse tool was used to determine stomatal aperture.

Confocal microscopy imaging

Images were acquired using the Olympus IX 81 inverted laser scanning confocal microscope (Fluoview 500) equipped with a 488-nm argon ion laser and a 60 × 1.0 numerical aperture PlanApo water immersion objective. The NO fluorescence was excited by 488-nm light and the emission was collected using a BA 505–525 filter. Green fluorescent protein was excited by 488-nm light and the emission was collected using a BA 505–525 filter. A BA 660 IF emission filter was used to observe chlorophyll autofluorescence. Confocal optical sections were obtained at 0.5-μm increments. The images were color-coded green for GFP and magenta for chlorophyll autofluorescence.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Stomatal density in wild-type and *AtHXK1*-expressing plants.

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