Sugar status in preexisting leaves determines systemic stomatal development within newly developing leaves

PNAS RESEARCH ARTICLE | PLANT BIOLOGY

Sugar status in preexisting leaves determines systemic stomatal development within newly developing leaves

Qin-Xin Bao[a,1], Xin-Rong Mu[a,1], Chen Tong[a], Cong Li[b], Wen-Zhe Tao[a], Sheng-Ting Zhao[a], Yu-xin Liu[a], Wan-Ni Wang[a], Yu-ting Wei[a], Fu-Huan Yu[a], Jing-wen Wang[a], Zhi-Lan Sun[a], Bing-Ling Fan[a], Jia Sun[a], Chen Wang[c,2], Gary J. Loake[d,e,2], and Lai-Sheng Meng[a,2]

Edited by David Baulcombe, University of Cambridge, Cambridge, United Kingdom; received February 23, 2023; accepted May 11, 2023

Stomata are pores found in the epidermis of stems or leaves that modulate both plant gas exchange and water/nutrient uptake. The development and function of plant stomata are regulated by a diverse range of environmental cues. However, how carbohydrate status in preexisting leaves might determine systemic stomatal formation within newly developing leaves has remained obscure. The glucose (Glc) sensor HEXOKINASE1 (HXK1) has been reported to decrease the stability of an ethylene/Glc signaling transcriptional regulator, EIN3 (ETHYLENE INSENSITIVE3). EIN3 in turn directly represses the expression of *SUC2* (*sucrose transporter 2*), encoding a master transporter of sucrose (Suc). Further, KIN10, a nuclear regulator involved in energy homeostasis, has been reported to repress the transcription factor SPCH (SPEECHLESS), a master regulator of stomatal development. Here, we demonstrate that the Glc status of preexisting leaves determines systemic stomatal development within newly developing leaves by the HXK1—¦EIN3—¦SUC2 module. Further, increasing Glc levels in preexisting leaves results in a HXK1-dependent decrease of EIN3 and increase of SUC2, triggering the perception, amplification and relay of HXK1-dependent Glc signaling and thereby triggering Suc transport from mature to newly developing leaves. The HXK1—¦EIN3—¦SUC2 molecular module thereby drives systemic Suc transport from preexisting leaves to newly developing leaves. Subsequently, increasing Suc levels within newly developing leaves promotes stomatal formation through the established KIN10⟶ SPCH module. Our findings thus show how a carbohydrate signal in preexisting leaves is sensed, amplified and relayed to determine the extent of systemic stomatal development within newly developing leaves.

ethylene-insensitive3 (EIN3) | sucrose (Suc) | sucrose transporter 2 (SUC2) | stomatal development | sucrose phloem transport

Significance

Stomata control both plant water/nutrient uptake and gas exchange. The development and function of these plant pores are regulated via a diverse range of environmental cues. However, a potential role for leaf carbohydrate status in determining systemic stomatal development has remained unknown. Our findings now identify a HXK1—¦EIN3—¦SUC2 (HEXOKINASE1—¦ETHYLENE INSENSITIVE3—¦sucrose transporter 2) signaling module that links the glucose status of preexisting leaves to the formation of stomata within distal, newly developing, systemic leaves. These data provide novel insights into the biochemical control of stomatal formation at a distance, potentially enabling crop design strategies to help increase the resilience of crop plants within a changing environment.

Stomata, comprised of two adjacent guard cells (GCs), are central to plant gas exchange and also both water and nutrient uptake through roots as a consequence of transpiration (1, 2). The modulation of stomatal development is mediated through a complex network of signaling pathways that control diverse environmental and endogenous cues (2).

In leaves, the cell lineage that gives rise to GCs is initiated in the postembryonic epidermis, in which some cells go through asymmetric division in the postembryo meristemoids (3). Stomata are generated through a specialized epidermal lineage that undergoes an orchestrated unidirectional successive division and associated distinct cell states. Protodermal cells form MMC (meristemoid mother cells), which divide asymmetrically to generate a M (meristemoid) and a larger stomatal lineage ground cell. M cells have stem-cell–like properties and undergo amplifying divisions providing the major source of both stomata and epidermal pavement cells. Ultimately, M cells transition into a GMC (guard mother cell), which symmetrically divides, subsequently forming two GCs, following a further state transition (4).

In seed embryos, Suc is a main soluble sugar, and it is also a major sugar transported in *Arabidopsis* (5). Long-distance Suc transport is mediated by the sieve tubes [comprised of SEs (sieve elements)] of phloem (6, 7). Ses go through limited autolysis during maturation, with organelles becoming degraded, being subsequently dependent on CCs (companion cells) for metabolic support (8). Suc transporters (SUTs/SUCs) have a key function in the efflux of photosynthetically fixed carbon from leaves. In this context, SUTs facilitate the uptake of Suc into the Ses and CCs (9). In addition, SUTs, most evidently SUT4 from potato (*Solanum tuberosum*), are integral to other physiological processes, including: shade avoidance, tuberization and flowering, independently of Suc efflux (10).

Arabidopsis SUC2 (sucrosetransporter 2) has a key function in Suc phloem loading and is indispensable to high-performance Suc transport from source to sink tissues (5). The loss-of-function mutant, *suc2*, presented delayed development, stunted growth and sterility.

Author contributions: L.-S.M. designed research; Q.-X.B., X.-R.M., C.T., C.L., W.-Z.T., S.-T.Z., Y.-x.L., W.-N.W., Y.-t.W., F.-H.Y., J.-w.W., Z.-L.S., B.-L.F., J.S., C.W., and L.-S.M. performed research; C.W. and L.-S.M. contributed new reagents/analytic tools; Q.-X.B., X.-R.M., C.T., and L.-S.M. analyzed data; C.W. provide fund for this work; and G.J.L. and L.-S.M. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

[1]Q.-X.B. and X.-R.M. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: wangchen@njau.edu.cn, gloake@ed.ac.uk, or menglsh@jsnu.edu.cn.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2302854120/-/DCSupplemental.

Published June 5, 2023.

Other *SUTs*, termed *SWEET11* and *SWEET12*, are expressed in the phloem parenchyma cells of minor veins and their cognate proteins are localized on the phloem plasma membrane (5). Suc efflux from phloem parenchyma cells into the apoplast prior to Suc uptake is mediated by these SUTs (5). The single mutant of *sweet11/sweet12* presents no abnormal phenotypes, likely because of functional redundancy. However, *sweet11/sweet12* double mutant plants show seriously delayed growth and large starch/sugar accumulation in the leaf blades relative to wildtype (5).

Sugars promote stomatal development (11). Target of rapamycin and SnRK1 (Snf1-related protein kinase 1) finely modulate the transcript and protein abundance of SPCH (SPEECHLESS), a master regulator of stomatal development, to regulate stomatal number in response to sugars (11, 12). Moreover, some previous reports have revealed that glucose (Glc)-antagonism of ethylene signaling occurs under excess Glc levels (13–15). It has been further shown that Glc signaling is uncoupled from ethylene signal transduction under low Glc availability (16). However, it is unclear how the interaction of sugar and ethylene signaling regulates stomatal development by modulating Suc transport.

Here we demonstrated that the sugar status of mature leaves determines epidermal cell fate in young leaves. Further, we demonstrate that under "standard" conditions, with advancing age, increasing Glc in mature leaves drives a (HEXOKINASE1) HXK1-dependent decrease in (ETHYLENEINSENSITIVE3) EIN3 levels and an associated increase in SUC2 levels. This triggers the sensing, amplification, and relay of Glc signaling and associated Suc phloem transport from mature leaves to young leaves, driving stomatal development within these young leaves. However, under disadvantageous growth conditions (such as excess photosynthetic products or high CO_2 levels), Glc-antagonism of ethylene signaling triggers a decrease in Suc transport from mature leaves and an associated reduction of stomatal formation within newly developing leaves. Our results thus uncover a molecular mechanism whereby metabolic status in mature leaves controls epidermal cell fate decisions in young developing leaves.

Results

Glc-Mediated Stomatal Development Is Partially Dependent on *HXK1*. It has previously been reported that sugars may promote stomatal development (11, 12). To confirm and extend these findings, we explored the impact of Suc on the SI (stomatal index) [=(number of stomata)/(number of pavement cells + number of stomata) ×100%] of the abaxial epidermis of mature cotyledons in the 12-d-old *Arabidopsis* seedlings (17). In the presented micrographs (for example, Fig. 1*A*), stomata are delineated in yellow. Pavement cells are indicated as puzzle-shaped cells in the epidermis (Fig. 1*A*).

We employed an *Arabidopsis* transgenic line expressing a GREEN FLUORESCENT PROTEIN (GFP) marker fused to the bHLH (basic helix–loop–helix) transcription factors, SPCH or MUTE, with the resulting transgene driven by the endogenous *SPCH* promoter (*SPCHpro::SPCH-GFP*) or *MUTE* promoter (*MUTEpro::MUTE-GFP*), respectively. *SPCHpro::SPCH-GFP* was only detected in the MMCs and meristemoids (Ms), whereas *MUTEpro::MUTE-GFP* was expressed in the GMCs (3, 18). Our findings confirmed that Glc promotes stomatal development and changes cell fate by using SI and % of SPCH-GFP and MUTE-GFP marked cells (Fig. 1 *A* and *C* and *SI Appendix*, Fig. S1), concurring with previous data (11, 19). Further, our findings indicated that SIs in 12-d-old wildtype (Col-0) cotyledons increased when seedlings were grown in low availability (0 to 3% Glc) (Fig. 1 *A* and *C*). Conversely, SIs decreased when seedlings were grown in the presence of 5% Glc. However, *hxk1-3* seedlings, which are compromised in hexokinase 1 (HXK1) function, a Glc sensor (20), were largely insensitive to these Glc concentrations (Fig. 1 *A* and *C*). Thus, suggesting the impact of Glc on stomatal development may be partially dependent on *HXK1*.

To confirm and extend these findings, we determined stomatal development in the *SPCHpro::SPCH-GFP* line. Our findings indicated that with 4-d-old seedlings, the number of cells marked by GFP expression was enhanced in wildtype plants relative to the *hxk1-3* line in immature mature cotyledons (Fig. 1 *B* and *D*).

Together, these data show that Glc promotes stomatal development and changes cell fate, and Glc-mediated stomatal development is partially dependent on *HXK1* function.

Glc-Promoted Stomatal Development Is Partially Dependent on *EIN3/EIL1*. Glc/Suc has been proposed to promote stomatal formation (Fig. 1) (11, 19). Further, HXK1 has been reported to decrease the stability of EIN3 (21). The EIN3/ELI1 transcription factors are key regulators of ethylene signaling (22). Moreover, EIN3/ELI1 is required for sugar signaling (21). We therefore determined whether EIN3/ELI1 might also function in the regulation of stomatal development mediated by Glc signaling.

We observed that with an increase of Glc concentration, mature cotyledons of 12-d-old wildtype seedlings exhibited increased SIs (Fig. 2 *A* and *C*). However, in the *ein3/eil1* lines, an increasing Glc concentration did not significantly increase the SI relative to wildtype plants (Fig. 2 *A* and *C*). Therefore, the *ein3/eil1* mutant already possesses a higher SI in the absence of added Glc, congruent with ethylene inhibiting meristemoid amplifying divisions (23). Consistent with these phenotypes, *EIN3* expression was observed in pavement cells, in addition to stomatal lineage cells, in transgenic plants marked by expression of a β-GLUCURONIDASE (GUS) reporter enzyme under the control of the *EIN3* promoter (*EIN3pro-GUS*) (*SI Appendix*, Fig. S2*B*).

To confirm these findings, we determined the stomatal development phenotype in response to Glc in the stomatal cell-type–specific marker line, *SPCHpro::SPCH-GFP*. Our findings indicated that with 4-d-old seedlings, the number of cells marked by GFP in immature cotyledons was greater in the *ein3/eil1* seedlings relative to wildtype (Col-0) (Fig. 2 *B* and *D*). Collectively, our data indicated that Glc-promoted stomatal development was partially dependent on *EIN3/EIL1*.

***EIN3* Acts Upstream of *SUC2* but Downstream of *HXK1*, in the Regulation of Stomatal Development.** The loss-of-function mutant of *HXK1*, *hxk1-3*, in the Col-0 background, showed reduced SI (Fig. 3 *A*, *B*, and *H*). By contrast, mature cotyledons in *ein3/eil1* plants exhibited increased SI (Fig. 3 *A*, *C*, and *H*), relative to wildtype. Further, *hxk1/ein3/eil1* cotyledons also exhibited increased SI (Fig. 3 *A*, *D*, and *H*), relative to wildtype, indicating that *ein3/eil1* mature cotyledons had an epistatic phenotype to *hxk1-3*.

Recently, we demonstrated that EIN3 directly regulates the function of SUC2 by inhibiting the expression of this transporter (24). We therefore determined if *EIN3* acts in a similar fashion with respect to the regulation of stomatal development. In this context, *suc2-5*/+ mutant cotyledons showed reduced SI (Fig. 3 *A*, *F*, and *H*). In addition, we analyzed a previously generated transgenic line where *SUC2* was overexpressed from the CaMV*35S* promoter (*35S:SUC2*) (25). The cotyledons of the *35S:SUC2* line exhibited increased SI (Fig. 3 *A*, *E*, and *H*), relative to wildtype. Further, *ein3/eil1/suc2-5*/+ cotyledons exhibited decreased SI

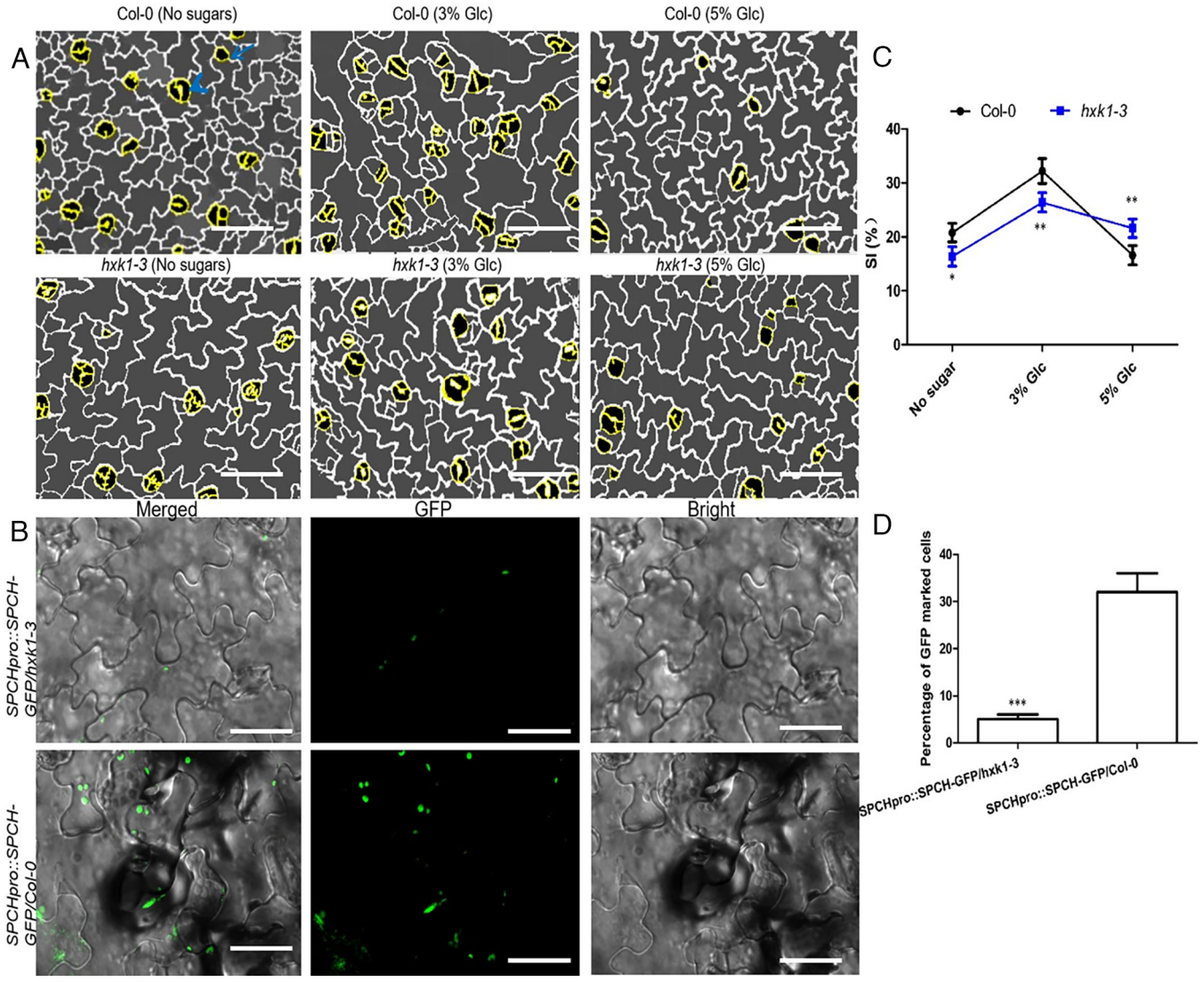

Fig. 1. Glc signaling/metabolism promoting stomatal development is partially dependent of HXK1. (*A*) Images illustrating abaxial epidermal stomata on mature cotyledons of 12-d-old wildtype (Col-0) and *hxk1-3* seedlings grown on solid 1/2 MS medium with 3%, 5% Glc or without under same 16/8 light/dark cycle. (Bars, 50 μm.) Raw stomatal micrographs were processed by using ImageJ [BAR(A collection of Broadly Applicable Routines)] (https://imagej.net/software/fiji/downloads). Arrow indicates meristemoid (black triangle), whereas an arrowhead indicates stomata outlined in yellow. (*B*) Images illustrating SPCHpro::SPCH-GFP fluorescence detection in immature cotyledons of 4-d-old wildtype (Col-0) and *hxk1-3* transgenic seedlings expressing a *SPCHpro::SPCH-GFP* transgene. These seedlings were grown on solid 1/2 MS medium with 1% Glc for 4 d under same 16/8 light/dark cycle. (Bar, 50 μm.) (*C*) Bar graph illustrating abaxial epidermal stomata in *A*. (*D*) Bar graph illustrating quantification of the percentage of GFP-expressing cells per total epidermal cells in *B*. Student's *t* test (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$). Error bars represent SD (n = 3).

(Fig. 3 *A*, *G*, and *H*), relative to wildtype, indicating that *suc2-5*/+ cotyledons had an epistatic phenotype to *ein3/eil1*.

Collectively, our data suggest that *EIN3*, being downstream of *HXK1*, acts upstream of *SUC2*. Thus, we identified a HXK1—¦EIN3—¦SUC2 signaling module for regulating stomatal development.

With Increasing Endogenous Suc, *EIN3*, and *SUC2* Show Distinct Spatiotemporal Expression Patterns. With increasing age, endogenous Suc/Glc content increased (Fig. 4 *A* and *B*), consistent with previous data (26). By performing qPCR analysis, we determined that with increasing age, *EIN3* mRNA accumulation gradually decreased. Conversely, *SUC2* mRNA gradually increased in developing cotyledons of juvenile seedlings (Fig. 4*C*). In addition to stability (21), *EIN3* expression is also regulated by Glc/Suc (Fig. 4 *A* and *B*). This appears to be an important effect and regulatory mechanism. Further, we observed that with increasing endogenous Suc, EIN3 levels were gradually reduced, but in contrast, SUC2 accumulation was increased (Fig. 4 *D* and *E*). Further, endogenous or exogenous Suc/Glc promotes HXK1-dependent EIN3 degradation (21, 27). Recently, we showed that EIN3 directly regulates the function of SUC2 by repressing the expression of *SUC2* (24). Our current findings combined with previous data indicate that with increasing age, accumulating endogenous Glc/Suc (or photosynthetic product) facilitates EIN3 degradation and thereby results in enhanced *SUC2* mRNA and SUC2 accumulation.

Our *EIN3* expression data were also supported by others, showing *EIN3* was strongly expressed in the stem cells of young leaf primordia at the shoot apical meristem (28). Moreover, it has also been demonstrated that *EIN3* expression increases within aged tissues (29). Therefore, our findings, combined with previous reports, indicate that with increasing age, *EIN3* mRNA levels are decreasing within young leaves and gradually increasing in ageing leaves.

Further, we generated a transgenic line with the *GUS* reporter gene downstream of five tandem repeats of an EBS (*5×EBS*:

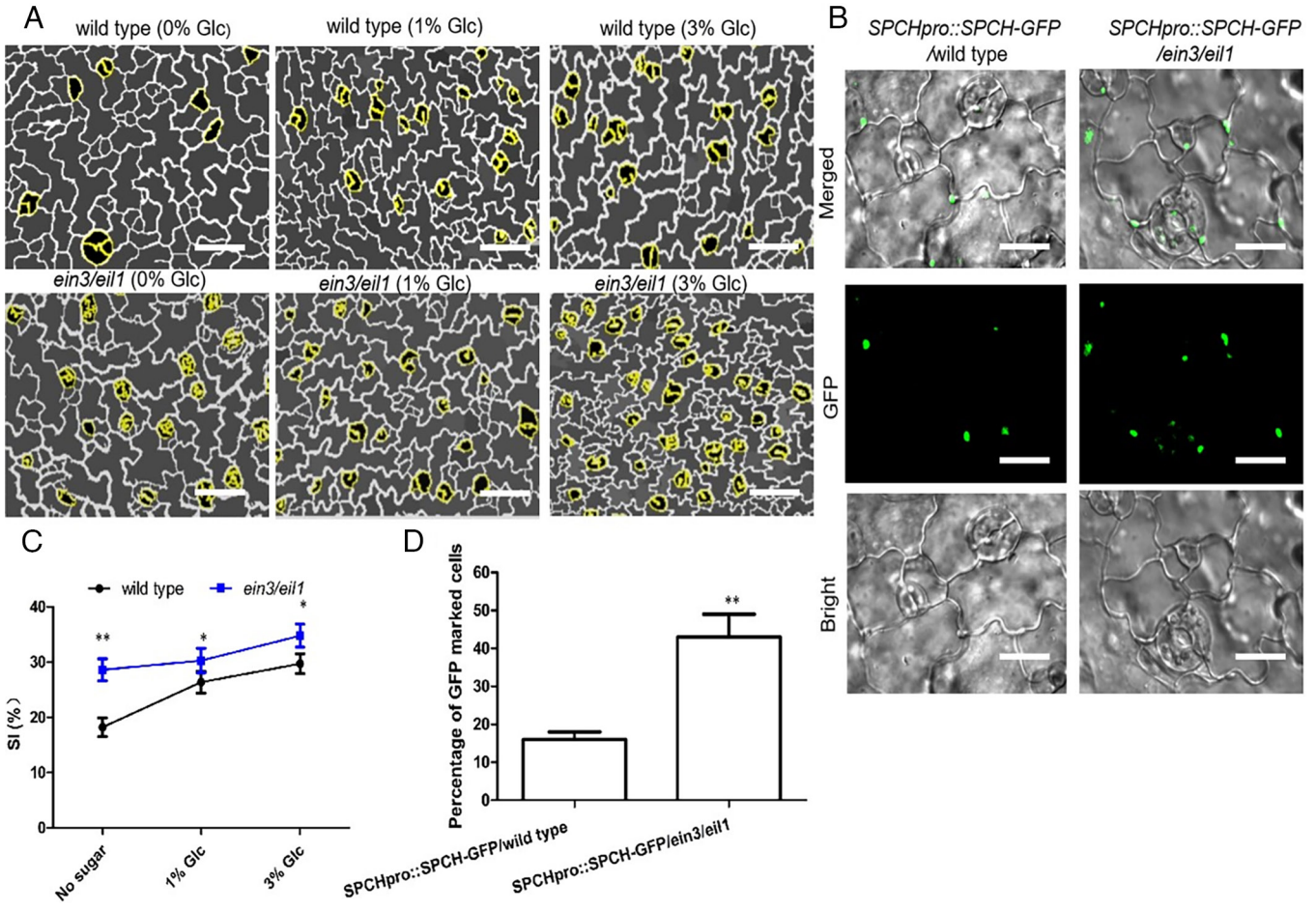

Fig. 2. Glc signaling-promoted stomatal development was partially dependent of EIN3/EIL1. (*A*) Images illustrating abaxial epidermal stomata of mature cotyledons of 12-d-old wildtype and *ein3/eil1* seedlings grown on solid 1/2 MS medium with or without 1% and 3% Glc. (Bar, 50 μm.) (*B*) Images illustrating SPCH-GFP fluorescence detection in immature cotyledons of 4-d-old wildtype (Col-0) and *ein3/eil1* transgenic seedlings expressing a *SPCHpro::SPCH-GFP* transgene. (Bar, 40 μm.) (*C*) Bar graph illustrating quantification of abaxial stomata from *A*. (*D*) Bar graph illustrating quantification of the percentage of GFP-expressing cells per total epidermal cells in *B*. Student's *t* test (**P* < 0.05; ***P* < 0.01). Error bars represent SD (n = 3).

GUS). This line was used to detect the binding activity of the transcription factor EIN3 (29). Consistent with both qPCR and western blotting data, GUS expression of EBS was strongest in young leaf primordia. In contrast, *SUC2* expression, determined by GUS driven by the *SUC2* promoter (*SUC2pro-GUS*) was observed in older leaf blades (mature cotyledons) in 22-d-old seedlings (Fig. 4 *F–H*). Furthermore, GUS activity in *5×EBS*, *EIN3pro-GUS* and *SUC2pro-GUS* lines was enhanced on the tip section of transition leaves, while being decreased at the base (Fig. 4 *F–H*), suggesting the expression of *EIN3* and *SUC2* is closely coupled with Suc accumulation, associated transport and the capacity of a growing leaf blade to import photoassimilate (30).

Collectively, these findings indicate that with increasing Suc, *EIN3* and *SUC2* exhibit a contrasting spatiotemporal expression pattern.

SUC2 Function in Mature Cotyledons Is Sufficient to Promote Stomatal Development within Newly Developing Leaves. Our data imply that both EIN3 and SUC2 may regulate the export of nutrients out of mature leaves. We therefore next determined whether *SUC2* expression in mature cotyledons is sufficient to promote stomatal development within newly developing leaves, by driving transport of Suc from mature cotyledons to newly developing leaves. It should be noted, however, that the incongruity between source and sink leaves is not absolute (31). In this study, mature cotyledons were utilized as source tissues, whereas the 3rd/4th leaves were utilized as sink tissues.

To achieve this, we employed an estradiol-inducible promoter to drive FLAG-tagged SUC2 expression in a *suc2-5* mutant background (*ER::SUC2-3×FLAG/suc2-5* (*iER*)). This approach will delineate two different possibilities: 1) whether Suc transported from mature cotyledons promotes stomatal development within newly developing leaves, 2) whether the local Suc content within newly developing leaves influences local *SUC2* expression, triggering stomatal development within newly developing leaves.

Therefore, following induction of *SUC2* expression specifically within cotyledons (but not other organs) following the application of 20 μM estradiol for 1 h, the 3rd and 4th leaves of 16-, 18-, 20-d-old inducible *ER::SUC2-3×FLAG/suc2-5* seedlings were all found to exhibit similar growth to the mock treated control lines (Fig. 5*A*).

Both *SUC2* mRNA and SUC2 accumulation in cotyledons of inducible 16-, 18-, 20-d-old *ER::SUC2-3×FLAG/suc2-5* seedlings gradually increased with increasing age (Fig. 5 *C* and *E*). Accordingly, the Suc content in cotyledon phloem exudates from inducible *ER::SUC2-3×FLAG/suc2-5* seedlings was greater relative to those from mock treated lines and again increased with age (Fig. 5*F*). The resulting Suc content in the 3rd and 4th leaves of inducible *ER::SUC2-3×FLAG/suc2-5* seedlings was higher than that in corresponding mock treated leaves of these seedlings (Fig. 5*G*). As shown above by both our findings and previous

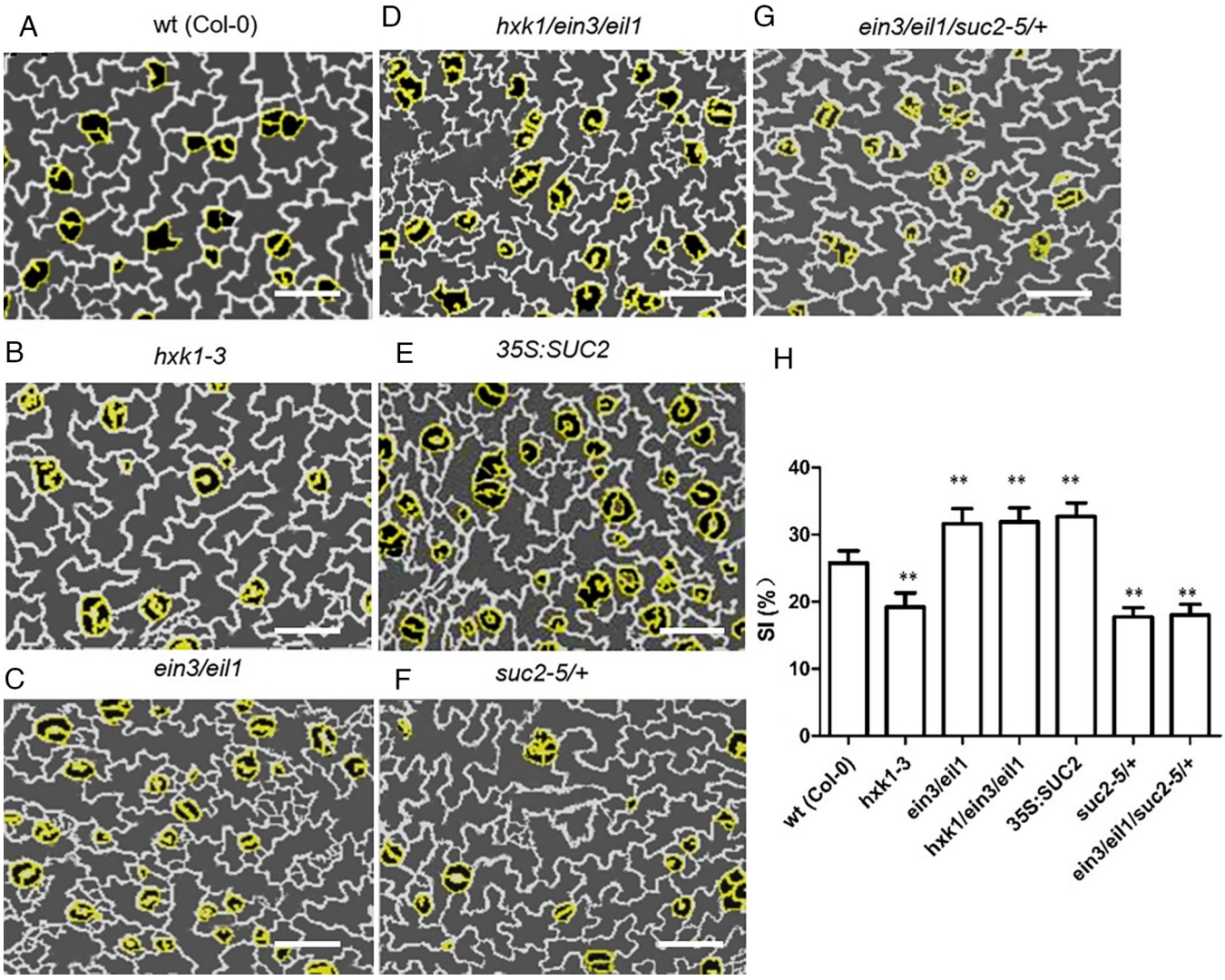

Fig. 3. *EIN3* acts downstream of *HXK1* and upstream of *SUC2* in the regulation of stomatal development. (*A*–*G*) Images illustrating abaxial epidermal stomata of mature cotyledons of 12-d-old wildtype and the indicated mutant or transgenic lines grown on solid 1/2 MS medium with 2% Glc. (Bar, 50 μm for *A* to *G*.) (*H*) Bar graph illustrating quantification of abaxial epidermal stomata in *A*–*G*. Student's *t* test (**$P < 0.01$). Error bars represent SD (n = 3).

data, Suc levels are positively linked to the associated SI (11) (Fig. 1). Accordingly, the SIs of the 3rd and 4th leaves of these seedlings whose cotyledons were treated with estradiol were higher than those of corresponding leaves from mock treated seedlings (Fig. 5 *B* and *H*).

It is well established that estradiole is membrane permeable and thus treated cotyledons might leak estradiol into the developing primordia of the 3rd/4th leaf. Hence, cotyledons (but not other organs) were induced by the application of 20 μM estradiol for 1 h, to reduce any possible leakage. Further, we tested the 3rd/4th leaves following cotyledons treated by estradiol and our findings indicate that SUC2 accumulation was not detected within these 3rd/4th leaves (Fig. 5*D*). Therefore, we can largely exclude possible estradiol leakage.

Together, our findings indicate that *SUC2* expression in mature cotyledons is sufficient to promote stomatal formation within newly developing leaves.

The EIN3—¦SUC2 Module Triggers the Sensing, Amplification and Relay of HXK1-Dependent Glc Signaling and thereby Triggers Suc Transport and Associated Stomatal Development. The transcriptional regulator EIN3 is integral to numerous sugar signaling pathways (21, 22). We therefore employed both *EIN3pro::EIN3-GFP* and *ER::SUC2-3×FLAG* transgenic lines to demonstrate if the sensing, amplification and relay of HXK1-dependent Glc signaling and associated Suc transport are mediated by an EIN3-SUC2 module.

We determined the content of both EIN3 and SUC2 by western blotting in response to increasing exogenously supplied Glc. Our findings indicated that whereas the content of SUC2 was elevated, in contrast, EIN3 accumulation decreased in young leaves (Fig. 6 *B* and *C*). In this context, Suc or Glc has previously been shown to promote HXK1-dependent EIN3 degradation (21, 27). Further, with increasing age, both endogenous Glc and Suc content gradually increased (Fig. 4 *A* and *B*) (26), which in turn promoted both a decrease in EIN3 levels and an associated increase in SUC2 (Fig. 4 *D* and *E*). Therefore, with increasing age, increasing Glc promotes a HXK1-dependent reduction in EIN3 and in contrast, an associated increase in SUC2.

Accordingly, with increasing exogenously supplied Glc, the amount of endogenous Suc in wildtype cotyledon phloem exudates gradually increased (Fig. 6*E*). However, the level of endogenous Suc in seedlings expressing either an *EIN3pro::EIN3-GFP* transgene, to score EIN3 accumulation or an *ER::SUC2-3×FLAG* transgene to

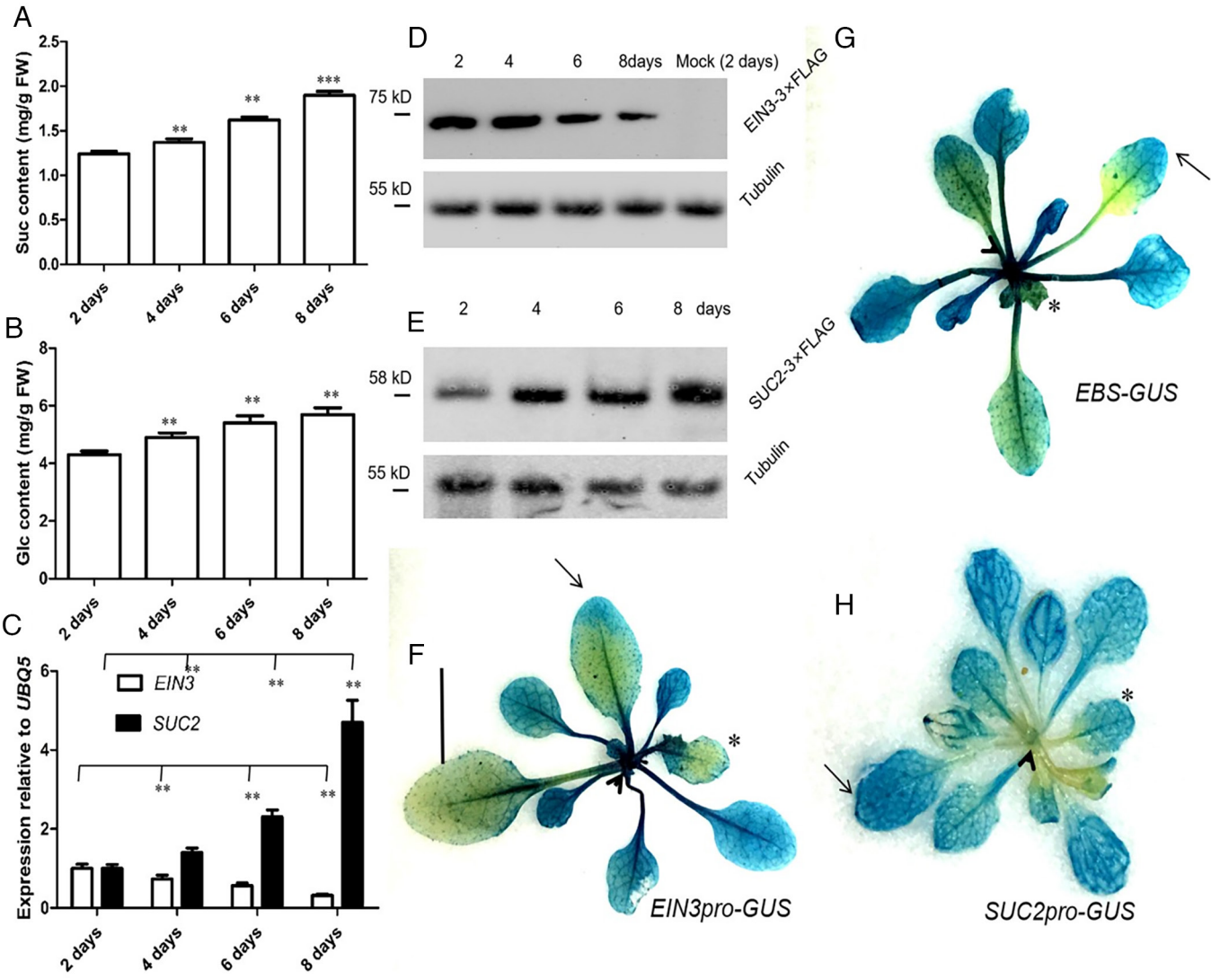

Fig. 4. Contrasting expression of *EIN3* and *SUC2* genes. (*A* and *B*) Bar graph illustrating Suc and Glc content in 2, 4, 6, and 8 day postemergence (DAE) cotyledons of wildtype seedlings. (*C*) Bar graph illustrating differential expression of *EIN3* and *SUC2* in *A*. 2-, 4-, 6-, 8-DAE cotyledons of wildtype seedlings were gathered and total RNA was extracted from different leaf blades and qPCR was performed. Quantification of 2 DAE was set as 1 in qPCR. Quantifications were normalized to the expression of *UBQ5*. (*D*) Images illustrating the abundance of EIN3 in cotyledons expressed from a *ER::EIN3-3×FLAG* (iER) inducible transgene at 2-, 4-, 6-, 8-DAE detected by an anti-FLAG antibody. A Tubulin was employed as a loading control. (*E*) Images illustrating the abundance of SUC2 in cotyledons expressing a *ER::SUC2-3×FLAG* transgene at 2-, 4-, 6-, 8-DAE detected by an anti-GFP antibody. Tubulin was utilized as a loading control. (*F–H*) Images illustrating β-glucuronidase (GUS) expression in transgenic plants driven by a *EIN3pro-GUS* transgene (*F*), a *EBSpro-GUS* transgene (*G*) and a *SUC2pro-GUS* transgene (*H*) in 22-d-old seedlings. Arrows indicate the source-to-sink transition leaves, and stars indicate the oldest leaves/cotyledons and arrowheads indicate young leaves. (Bar, 1.0 cm for *F–H*.) GUS staining patterns were consistent in at least 15 independent transgenic lines. Student's *t* test (***P* < 0.01; ****P* < 0.001). Error bars represent SD (n = 3).

enable conditional SUC2 expression, both showed a greater increase in Suc within cotyledon phloem exudates, relative to wildtype (Fig. 6*F*). As a result, whereas the Suc content within the 3rd and 4th leaves from wildtype seedlings gradually increased (Fig. 6*F*), the Suc content in the 3rd and 4th leaves from seedlings transgenic for either *EIN3pro::EIN3-GFP* or *ER::SUC2-3×FLAG* increased more strongly (Fig. 6*F*). Finally, with increasing exogenously supplied Glc, the SI of wildtype 3rd and 4th leaves gradually increased (Fig. 6 *A* and *G*). However, the corresponding leaves of seedlings transgenic for either *EIN3pro::EIN3-GFP* or *ER::SUC2-3×FLAG* exhibited a greater increase in SI (Fig. 6 *A* and *G*).

Further, we tested the 3rd/4th leaves following cotyledons treated by estradiol, and our findings indicated that SUC2 accumulation was not detected within these 3rd/4th leaves (Fig. 6*D*). Therefore, we can exclude the possibility of leakage.

Together, our findings show that with enhancing age, increasing Glc levels promote a HXK1-dependent decrease in EIN3 and in contrast, an associated increase in SUC2. As a result, this HXK1-dependent contrasting change in EIN3 and SUC2 levels triggers the sensing, amplification and relay of HXK1-dependent Glc signaling, and thereby triggers Suc transport from mature to newly developing leaves, thereby promoting stomatal development within these leaves.

HXK1 Signaling Activity Is within a EIN3—¦SUC2 Module Integral to the Regulation of Stomatal Development. We next determined whether HXK1 has signaling or metabolism functions in the regulation of EIN3—¦SUC2-mediated stomatal development. Here, the switch from Col-0 to L*er* was performed, because the availability of *HXK* mutants with uncoupled metabolic and signaling activity is within the L*er* background. We first analyzed the expression of *EIN3* and *SUC2* by qPCR in response to Glc. Low exogenous Glc levels reduced *EIN3* expression but enhanced *SUC2* expression in wildtype (L*er*) seedlings, but this was not

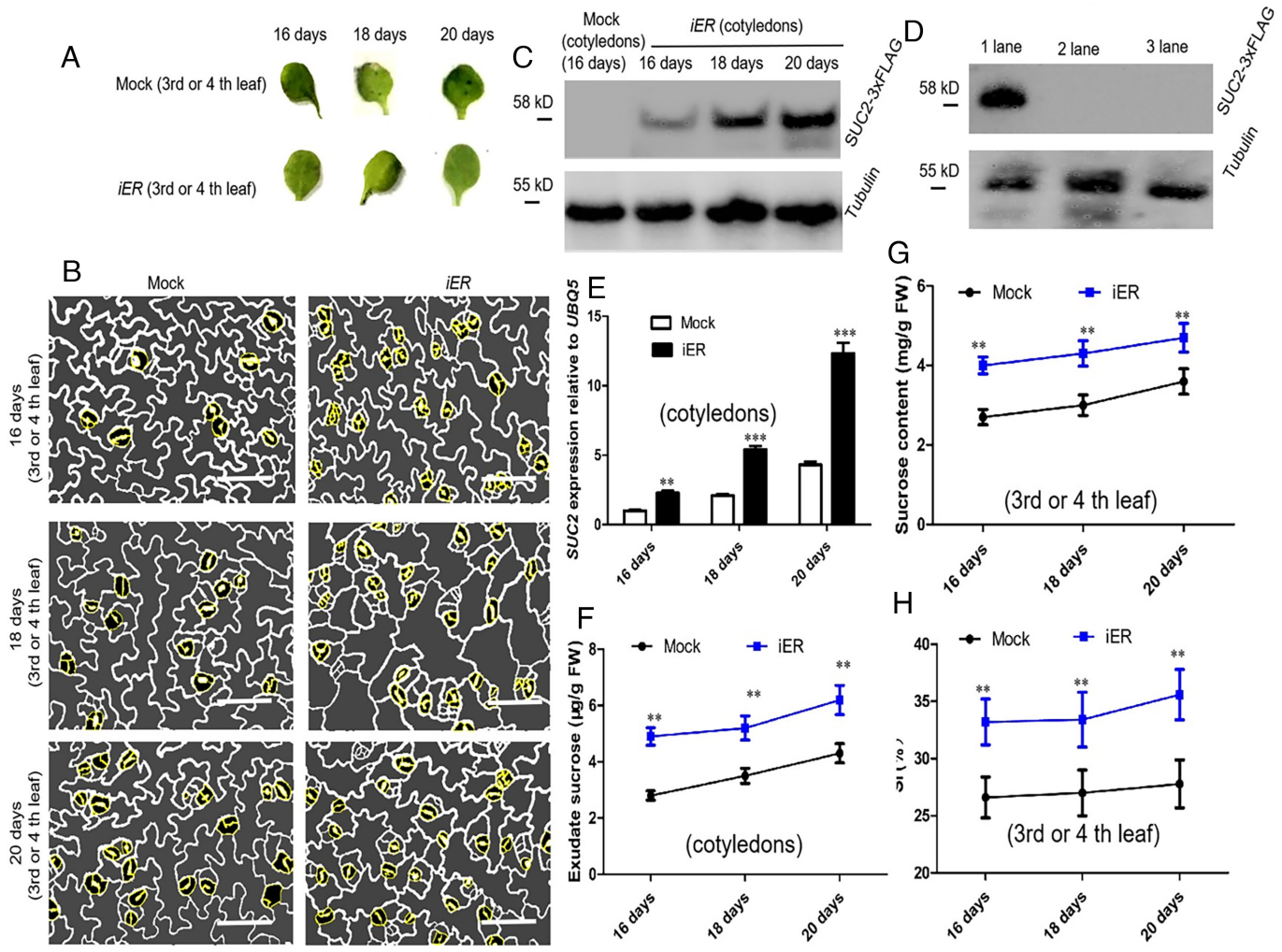

Fig. 5. *SUC2* expression in mature cotyledons is sufficient to promote stomatal formation in young leaves. (*A*) Images illustrating the 3rd or 4th leaves (as sink leaves) harvested from either 16-, 18- or 20-d-old *ER::SUC2- 3×FLAG/suc2-5* seedlings. Mature cotyledons (as source leaves) (but not other organs) of 15-d-old *ER::SUC2-3×FLAG/suc2-5* seedlings were treated with 20 μM estradiol for 1 h or mock treated. Subsequently, the 3rd or 4th leaves of these seedlings were photographed. The size of these 3rd or 4th leaves was not visibly different between the estradiol or mock treated lines. *iER* represents an inducible transgene *ER::SUC2- 3×FLAG/suc2-5*. (*B*) Images illustrating abaxial epidermal stomata of the given leaves in *A*. (Bars, 50 μm.) (*C*) Images illustrating the abundance of SUC2 in mature cotyledons of either 16-, 18- or 20-d-old *ER::SUC2-3×FLAG/suc2-5* seedlings. SUC2 accumulation was detected using an anti-FLAG antibody. Tubulin was used as a loading control. Similar findings were found in at least three biological repeats. (*D*) Images illustrating the abundance of SUC2 in16-d-old *ER::SUC2-3×FLAG/suc2-5* seedlings. 1 lane represents cotyledons treated by estradiol (positive control), 2 lane represents the 3rd/4th leaf following cotyledons treated by estradiol, and 3 lane represents the 3rd/4th leaf following cotyledons treated by mock (negative control). SUC2 accumulation was detected using an anti-FLAG antibody. Tubulin was used as a loading control. Similar findings were found in at least three biological repeats. (*E*) Bar graph illustrating differential expression of *SUC2* in cotyledons of either 16-, 18- or 20-d-old *ER::SUC2-3×FLAG/suc2-5* seedlings. Quantification at 16 d following treatment was set as 1 in qPCR. (*F*) Bar graph illustrating Suc content in phloem exudates from cotyledons of either 16-, 18- or 20-d-old *ER::SUC2-3×FLAG/suc2-5* seedlings. (*G*) Bar graph illustrating the given Suc contents in *A*. (*H*) Bar graph illustrating the quantification of the given abaxial stomata in *B*. Student's *t* test (**$P < 0.01$; ***$P < 0.001$). Error bars represent SD (n = 3).

recapitulated in a *gin2-1* line (*SI Appendix*, Fig. S3 *F* and *G*). Therefore, HXK1 contributes to the reduced level of *EIN3* transcripts and the elevated level of *SUC2* transcripts during stomatal development.

As HXK1 presents both enzymatic and signaling functions(20, 27), we thereby determined if HXK1 signaling or enzymatic activity is associated with the EIN3—¦SUC2 module that regulates stomatal development. Serine177 is essential for HXK1 catalytic activity but not for its Glc signaling activity (20). Thus, *gin2-1*-expressing a serine (S) to alanine (A) mutation at residue 177 $HXK1^{S177A}$ (*S177A/gin2-1*) was utilized to discern between signaling or metabolic roles for HXK1 (20, 32). Analysis of *HXK1/gin2-1* and *S177A/gin2-1* lines displayed an expression feature for these two genes similar to that detected in L*er* seedlings (*SI Appendix*, Fig. S3 *A–E*). Therefore, HXK1 enzymatic function is not essential for the changes in the expression of both *EIN3* and *SUC2*. Concurring with these results, the *gin2-1* phenotype of stomatal development was completely restored by expression of either a wildtype *HXK1* transgene or a transgene containing the *S177A* mutation (*SI Appendix*, Fig. S3 *A–E*).

Together, our findings indicate that HXK1 signaling activity is required in addition to the EIN3—¦SUC2 module in the regulation of stomatal development.

phyB Function Is not Linked to Glc-Regulated Stomatal Development. Sucrose and Glc are the major products of photosynthesis, and photoreceptors are required for mediating photosynthesis to increased light (33). Further, *phyB* encoding the apoprotein of the major red/far-red photoreceptor, is essential for the systemic regulation of stomatal development (33). Thus, we explored if changes in SIs might result from photoreceptor activity rather than changes to Glc levels. We found that mature cotyledons

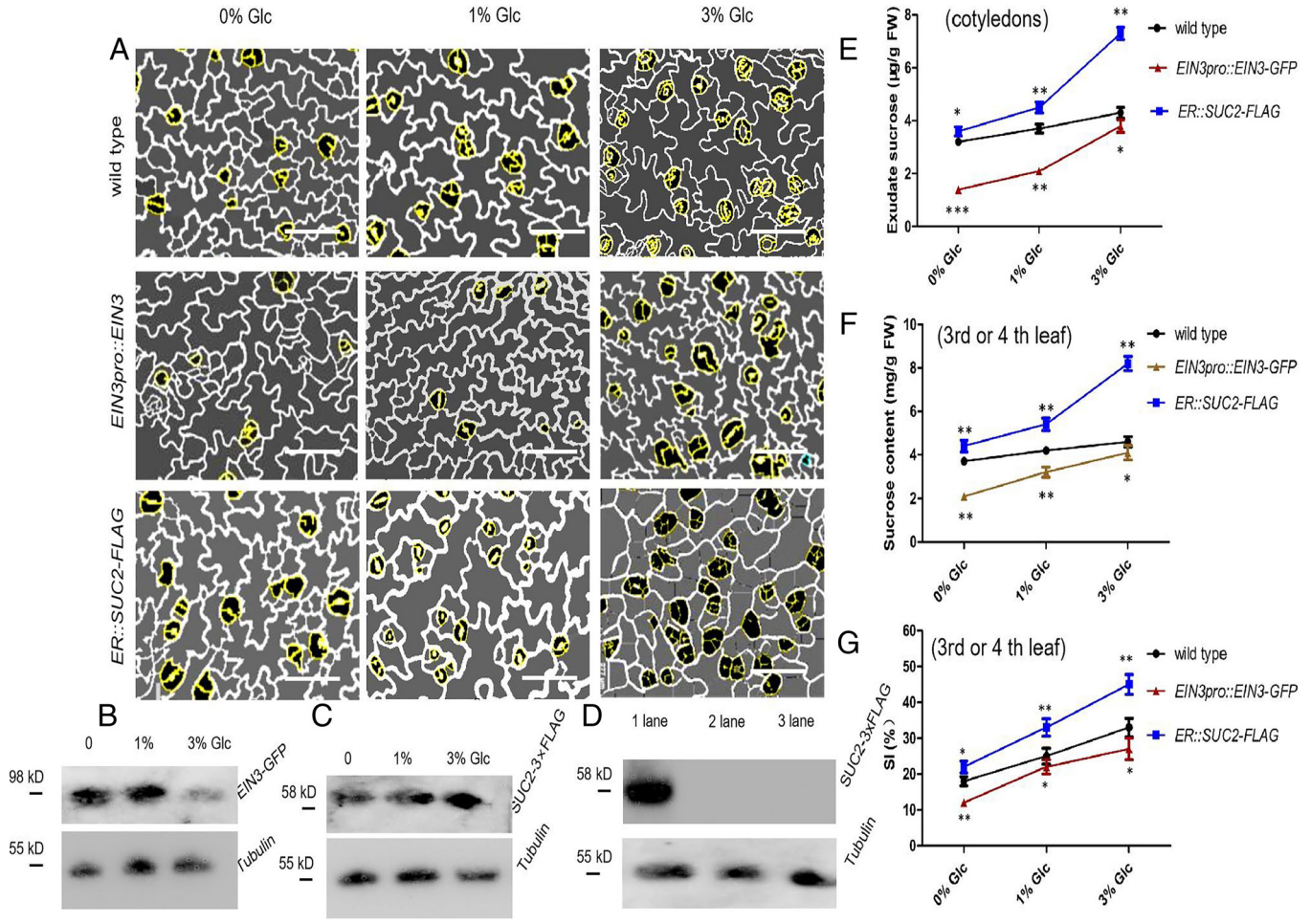

Fig. 6. Sensing, amplification and relay of a HXK1-dependent Glc signaling and associated Suc transport mediated by the EIN3-SUC2 module. (*A*) Images illustrating the abaxial epidermal stomata of the 3rd or 4th leaves (as sink leaves) of either 16-d-old wildtype (Col-0), transgenic plants containing an *EIN3pro::EIN3-GFP* transgene or transgenic plants containing an *ER::SUC2-3×FLAG* transgenein the wildtype (Col-0) background, grown on solid 1/2 MS medium with either 1% or 3% Glc, in conjunction with a mock treated control. (Bar, 50 μm.) (*B* and *C*) Images illustrating the abundance of EIN3 (*B*) and SUC2 (*C*) in the 3rd or 4th leaves of 16-d-old seedlings containing either an *EIN3pro::EIN3-GFP* or *ER::SUC2-3×FLAG* transgene in the wildtype (Col-0) background. EIN3 was detected using an anti-GFP antibody and SUC2 by an anti-FLAG antibody. Tubulin was employed as a loading control. (*D*) Images illustrating the abundance of SUC2 in16-d-old *ER::SUC2-3×FLAG* in the wildtype (Col-0) background. 1 lane represents cotyledons treated by estradiol (positive control), 2 lane represents the 3rd/4th leaf following cotyledons treated by estradiol, and 3 lane represents the 3rd/4th leaf following cotyledons treated by mock (negative control). SUC2 accumulation was detected using an anti-FLAG antibody. Tubulin was used as a loading control. Similar findings were found in at least three biological repeats. (*E*) Bar graph illustrating the Suc content in phloem exudates from mature cotyledons (as source leaves) of 16-d-old wildtype (Col-0), transgenic *EIN3pro::EIN3-GFP* plants or transgenic *ER::SUC2-3×FLAG* in the wildtype (Col-0) background plants grown on solid 1/2 MS medium with either 1% or 3% Glc or mock treated. (*F*) Bar graph illustrating Suc content in the 3rd or 4th leaves of 16-d-old wildtype (Col-0), transgenic *EIN3pro::EIN3-GFP* plants or transgenic *ER::SUC2-3×FLAG* in the wildtype (Col-0) background plants grown on solid 1/2 MS medium with either 1% or 3% Glc or mock treated. (*G*) Bar graph illustrating quantification of abaxial stomata from *A*. Student's *t* test (**P* < 0.05; ***P* < 0.01). Error bars represent SD (n = 3).

of the phytochrome photoreceptor mutant, *phyB-9*, exhibited reduced SI compared with wildtype plants grown on solid medium with 2% Glc, but not with 2% mannose (*SI Appendix*, Fig. S4).

Thus, our data suggest that phyB function was not linked to Glc-regulated stomatal development.

Discussion

Sugars function as nutrient signaling molecules and have previously been exhibited to regulate many aspects of plant growth and development. The findings of this study uncover an additional role for sugar signaling in the positive regulation of stomatal development through HXK1, EIN3 and SUC2 function. In this context, HXK1 inhibits EIN3, which in turn suppresses the transcription of *SUC2* through binding to its cognate gene promoter, leading to an adjustment in stomatal number. As a result, Glc signaling promotes SUC2 activity and Suc phloem loading of mature leaves by the HXK1—¦EIN3—¦SUC2 signaling module. Therefore, under "standard" light conditions, via the HXK1—¦EIN3—¦SUC2 module, the photosynthetic product Glc promotes Suc transport activities within mature leaves increasing Suc retrieval for the transport phloem system. Thus, Suc transport from mature leaves to young leaf is promoted. Finally, the Suc transported into young leaf induces the accumulation of the protein kinase energy sensor, KIN10, which in turn phosphorylates and stabilizes SPCH to facilitate stomatal development (11) (Fig. 7).

In young leaves, transported Suc stimulates formation of stomata (Fig. 7), whereas increased CO_2 levels in young leaves reduces formation of these pores for gaseous exchange, apparently as a negative feedback system (34). However, the underpinning mechanism is complex, because growth at increased CO_2 levels not only promotes the accumulation of photosynthetic products but also typically decreases the stomatal number (35). The accumulation of excess photosynthetic products (such as, Suc/Glc) may cause the high accumulation of Tre6P (trehalose-6-phosphate), which in turn suppresses KIN10 activity via decreasing the

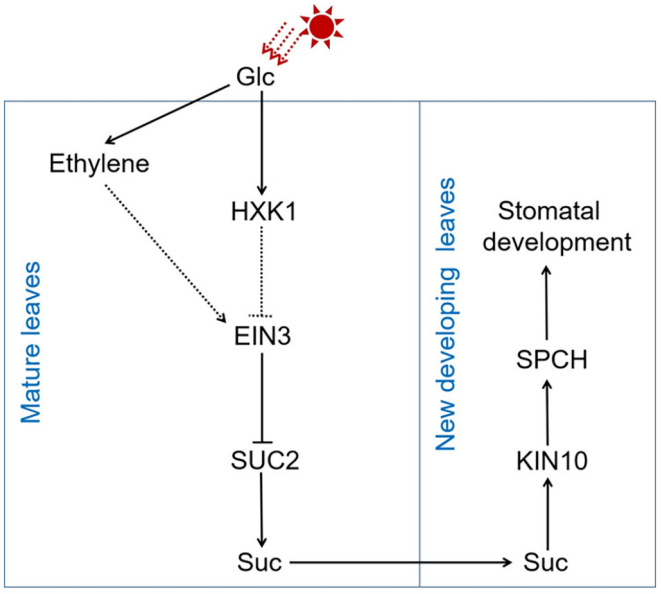

Fig. 7. Model illustrating how Suc promotes its phloem loading in mature leaves, to facilitate stomatal development within new developing leaves. Under normal light condition, Glc signaling is sensed by HXK1, which in turn represses EIN3 accumulation. In turn, depleted EIN3 levels enable increased accumulation of the key Suc transporter, SUC2, facilitating phloem transport of Suc from mature leaves to new developing leaves. Thus, a HXK1-EIN3-SUC2-Suc pathway coordinates this process. Subsequently, within newly developing leaves, transported Suc positively regulates the protein kinase, KIN10. Subsequently, KIN10 positively regulates the master regulator of stomatal development, SPCH, promoting the formation of stomata (11). Thus, a Suc-KIN10-SPCH pathway promotes stomatal development within newly developing leaves in response to transported Suc. Under disadvantageous growth conditions (high CO_2 levels or excess photosynthetic products), excess Glc induces ethylene production and Suc transport in mature leaves is decreased by the Glc-ethylene-EIN3-SUC2-Suc pathway. Thus, stomatal development is inhibited by the Suc- KIN10-SPCH pathway. Solid lines indicate direct regulation, whereas dotted lines indicate either indirect regulation or regulation in an unknown manner. Blunt arrows indicate negative regulation and pointed arrows indicate positive regulation.

interplay between SnAK1/2 and KIN10 (12), which may decrease the resulting stomatal number (12). Indeed, excess of a photosynthetic product, Glc, inhibited stomatal development (Fig. 1). Therefore, the profile of metabolic products generated at increased CO_2 levels could be different for those generated by photosynthesis under "standard" conditions.

Stomata are composed of two GCs which enable the plant to balance the loss of water through transpiration with the demand for CO_2 associated with photosynthesis. Plants have evolved mechanisms for both short-term control of stomatal aperture and long-term control of stomatal formation to enable adaptation to environmental change (4). Our data revealed that the Suc forward module promotes stomatal development under "standard" conditions, whereas under disadvantageous growth conditions, such as high CO_2 levels or excess photosynthetic products, a feedback module reduces stomatal production. Thus, the Suc content in mature leaves might alter as a result of the relationship between the rate of photosynthesis and the export capacity of the photosynthetic products from mature leaves by the phloem (36, 37). When the rate of photosynthesis is in excess, surplus Suc/Glc produced in mature leaves stimulates the production of ethylene, a stress hormone (38–40). As a result, transported Suc from mature leaves is significantly reduced by the Glc-ethylene-EIN3-SUC2-Suc pathway (Fig. 7). Accordingly, stomatal formation within newly developing leaves is blocked by the Suc-KIN10-SPCH pathway (Figs. 1 *A* and *C* and 7). Therefore, under disadvantageous growth conditions (high CO_2 levels or excess photosynthetic products), excess Glc-induced ethylene triggers the reduction of Suc transport from mature leaves and the associated decrease of stomatal formation within newly developing leaves, consistent with Glc-antagonizing ethylene signaling under excess Glc levels (13–15, 20). Further, under other disadvantageous growth conditions (low light and short-day conditions), the activation of KIN10 (a energy-sensing SnRK1 kinase) might phosphorylate and stabilizes SPCH to facilitate stomatal formation, which in turn elevates photosynthetic ability and/or carbon assimilation (11). The resulting feed-forward loop may assist plants to recover from stress. By contrast, under "normal" conditions, the Suc content of mature leaves is transported by the Glc-HXK1-EIN3-SUC2-Suc pathway (Figs. 1 *A* and *C* and 7). Accordingly, stomatal formation within newly developing leaves is promoted by the Suc-KIN10-SPCH pathway (Fig. 7).

These findings provide novel insights into the biochemical control of stomatal number at a distance, potentially providing future design strategies to increase crop resilience under a changing environment. To realize this potential, additional molecular components of this signaling machinery may need to be uncovered, together with a deeper understanding into their respective functions.

Materials and Methods

Plant Materials and Growth Conditions. The *ER::EIN3-FLAG*, *5 × EBS-GUS* (41), *EIN3-GUS* (32), *35S:EIN3-GFP*, *ein3-1* and *ein3/eil1* (22), *SUC2pro-GUS*, *ein3/eil1/suc2-5/+*, *EIN3pro:EIN3-GFP* (24), *phyb-9* (CS6217) (42), *hxk1-3* (CS861759) (43), *gin2-1* (CS6383), *HXK1/gin2-1* and *S177A/gin2-1* (32), *35S:SUC2* and *suc2-5* (25) were all reported or described in previously.

For simultaneous germination, seeds were subjected to at 4 °C overnight and then sown on solid 1/2 MS medium supplemented with different Glc levels (such as, 1%, 3% Glc or without sugars), pH 5.8 and 0.8% agar. Seedlings grown on agar were maintained in a growth room under 16/8 h of light/dark cycle with cool white fluorescent light at 21 ± 2 °C. Plants grown in soil were maintained in a controlled environment growth chamber under 16/8 h light/dark cycle with white light (130 μmol quanta PAR m^{-2} s^{-1}) conditions at 21 ± 2 °C. Unless otherwise stated, seedlings grown on agar were under "middle" light conditions at 21 ± 2 °C and white light (130 μmol quanta PAR m^{-2} s^{-1}) conditions.

Statistical Analysis. In all experiments, three biological replicates were performed with similar results and error bars represent SD. Student's *t* test was used to analyze the significance between two indicated samples at a significance level of 0.05 (***$P < 0.001$; **$0.001 < P < 0.01$; *$0.01 < P < 0.05$).

Generation of Mutants and Transgenic Plants. The *35S:EIN3-GFP*, *ER::EIN3-FLAG*, *ein3/eil1,* and *ein3-1* seeds were kindly provided by Prof H. W. Guo (South University of Science and Technology of China, China). *pGreen0800-LUC* vector and *5×EBS:GUS* seeds were kindly provided by Prof Ziqiang Zhu (Nanjing Normal University, Nanjing, China). The *hxk1-3* (CS861759) and *phyb-9* (CS6217) seeds were obtained from the ABRC (Ohio State University). The *35S:SUC2* and *suc2-5* seeds were kindly provided by Prof D Liu (Tsinghua University, China). The *gin2-1* (CS6383), *HXK1/gin2-1* and *S177A/gin2-1* seeds were kindly provided by Prof R Scott Poethig (University of Pennsylvania, Philadelphia, United States).

The *hxk1/ein3/eil1* mutant was obtained from F2 plants (*hxk1/ein3/eil1*) that had elongated hypocotyls on solid MS medium with 6 μM ACC (3) and had green cotyledons grown on solid MS medium with 5% Glc (20).

The *ER::SUC2-3×FLAG* transgene was transformed into wildtype (Col-0) or *suc2-5* lines by using the *Agrobacterium tumefaciens*-mediated floral dip method (44). The *SPCHpro::SPCH-GFP* and *MUTEpro::MUTE-GFP* transgenes were transformed into wildtype (Col-0), *ein3/eil1*, or *hxk1-3* lines by using the *Agrobacterium tumefaciens*-mediated floral dip method (32).

Plasmid Constructs. To construct estradiol (*ER):SUC2-3×FLAG*, the *SUC2* coding region sequence (CDS) (1.5 kb) was amplified and cloned into a pER8-derived plasmid with 3×FLAG tag (3). the primers used were: *SUC2*: F1-5′-ATC CAA TGG AGA AAG CTG CAA A-3′, R-5′-ATC CCA TAG TAG CTT TGA AGG-3′.

To construct *SPCHpro::SPCH-GFP* plasmid, used primers were F-ggg gac aag ttt gta caa aaa agc agg ct TAA TAC CGG ATT TTC TTG AAG AG, R-ggg gac cac tttg tac

aag aaa gct ggg t TTG CTG AAT TTG TTG AGC CAG TT (for cloning CDS, 1.1 kb); F-ggg gac aag ttt gta caa aaa agc agg ct aga ttt tga ttt ctg cgc aac ttg c; R-ggg gac cac tttg tac aag aaa gct ggg t ctc att tat gtt tta gat ata aat atg c (for cloning promoter sequence, 2 kb).

To construct *MUTEpro::MUTE-GFP* plasmid, used primers were F-ggg gac aag ttt gta caa aaa agc agg ct TCT CAC ATC GCT GTT GAA AGG, R-ggg gac cac tttg tac aag aaa gct ggg t TTA ATT GGT AGA GAC GAT CAC (for cloning CDS, 0.6 kb); F-ggg gac aag ttt gta caa aaa agc agg ct gag aat ctt gat caa tta agt at; R-ggg gac cac tttg tac aag aaa gct ggg t atg aga gtt cat cat gcc act cta (for cloning promoter sequence, 1.9 kb).

The above-targeted sequences were certified, and the vector was introduced into *Agrobacterium tumefaciens GV3101* via electroporation at 2.2 V using a MicroPulser (Bio-Rad).

Confocal Laser Scanning Mfor GFP or YFP Imaging. Confocal laser scanning microscope for GFP or YFP imaging was performed, as has been described (32).

Stomatal Micrographs. The abaxial epidermis of cotyledons or true leaves of different age seedlings was obtained using nail polish and were photographed using differential interference contrast and inversion microscopy.

From the same position in two cotyledons or 3rd/4th leaf (such as, *SI Appendix*, Fig. S2*A*), counts were from one area (or section) per cotyledon/3rd/4th per seedling with a total of 20 measurements (one area [section] × 2 cotyledon/2 true leaves × 10 seedlings = 20 measurements) from 10 seedlings. This was then repeated for a total of three biological repeats (3×20=60) with similar results but only the SI data appears in a Figure.

The SI was assessed on the basis of the following formula: SI=(number of stomata)/(number of stomata + number of pavement cells) ×100%. Only stomata indicated in yellow (such as Fig. 1*A*) were counted and considered as stomata. Pavement cells are indicated by puzzle-shaped cells in the epidermis. The SD and mean were estimated from these relevant data. Raw stomatal micrographs were processed by using Image J [BAR(A collection of Broadly Applicable Routines)] (https://imagej.net/software/fiji/downloads).

GUS Assays. For GUS staining, leaf blades of indicated seedlings grown on a given media were washed 3 to 5 times with PBS buffer (80 to 100 mM Na_3PO_4, pH 7.0) and then incubated in GUS staining mix buffer [1 mM X-gluc, (0.1% (v/v) Triton X-100, 0.4 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6$, and 60 mM NaPO4 buffer] at 37 °C for 6 h in the dark. After staining, these seedlings were washed three to five times with PBS, followed via decolorization using different ethanol gradients, as has been described (44). Finally, the staining of individual seedlings/leaves was observed with an inverted microscope or Zeiss microscope.

Assay of Sugar Metabolites and Suc/Glc Content in Phloem Exudates. Seedlings were grown on solid MS medium with different Suc/Glc levels, or were grown in soil for the indicated times. A Plant Suc /Glc Assay Kit (Beijing Solarbio Science & Technology Co., Ltd, Cat#BC2465; http://www.solarbio.com/goods-9298.html) was used to measure sugar metabolites. 0.1 g cotyledons of the relevant age was ground into homogenate at 23°C. 0.5 mL extraction buffer was added followed by further grinding and storage at 80 °C for 10 min. After cooling these extracts were centrifuged at 4,000 g for 10 min, the supernatant was transferred to a fresh tube and 2 mg reagent 5 was added at 80 °C for 30 min. 0.5 mL extraction buffer was subsequently added, mixed and centrifuged at 4,000 g for 10 min. The supernatant was transferred to a fresh tube as samples for visible light analysis.

Three centrifuge tubes per sample were used with 25 μL of sample. Standard product (reagent 1) and water were added, respectively. 15 μL of reagent 2 was added, mixed and then boiled at 100 °C for 5 min. 175 μL of reagent 3 and 50 μL of reagent 4 were added, respectively, followed by boiling in water for 10 min. Light absorption at 480 nm was recorded after cooling.

Assay of Suc content in phloem exudates was described by ref. 31.

Quantitative PCR. Total RNA was extracted from the tissues indicated by TRIZOL reagent (Invitrogen), as has been described by refs. (32) and 45. First-strand cDNA samples were generated from total RNA samples by reverse transcription using an AMV reverse transcriptase first-strand cDNA synthesis kit (Life Sciences, Promega; 25 and were used as templates for qPCR–based gene expression analysis. SYBR green was used to monitor the kinetics of PCR product in real-time qPCR, as has been described by refs. (32) and 45. Each sample was detected in three technical replicates, and three biological replicates were performed. Quantifications were normalized to the expression of *UBQ5*. The related expression of target genes was measured through the $2^{-\Delta\Delta Ct}$ method (12). Primer pairs of *EIN3* have been described (32, 44). Primer pairs of *SUC2* have been described (22).

Western Blotting. Western blotting was performed, as has been described previously (32, 45).

Data, Materials, and Software Availability. Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AT3G20770 (EIN3) (46), AT4G29130 (HXK1) (47), and AT1G22710 (SUC2) (48). All study data are included in the article and/or *SI Appendix*.

ACKNOWLEDGMENTS. This study was supported by grants from the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Author affiliations: [a]School of Life Science, Jiangsu Normal University, Xuzhou, Jiangsu 221116, People's Republic of China; [b]Public Technical Service Center, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, People's Republic of China; [c]School of Horticulture, Nanjing Agricultural University, Nanjing, Jiangsu 210095, People's Republic of China; [d]Jiangsu Normal University–Edinburgh University, Centre for Transformative Biotechnology of Medicinal and Food Plants, Jiangsu Normal University, Xuzhou, Jiangsu 221116, People's Republic of China; and [e]Institute of Molecular Plant Sciences, School of Biological Sciences, Edinburgh University, Edinburgh EH9 3BF, United Kingdom

1. J. Sheen *et al.*, Sugars as signaling molecules. *Curr. Opin. Plant Biol.* **2**, 410–418 (1999).
2. K. U. Torii, Mix-and-match: Ligand-receptor pairs in stomatal development and beyond. *Trends Plant Sci.* **17**, 711–719 (2012).
3. L. J. Pillitteri *et al.*, Termination of asymmetric cell division and differentiation of stomata. *Nature* **445**, 501–505 (2007).
4. S. A. Casson, A. M. Hetherington, Environmental regulation of stomatal development. *Curr. Opin. Plant Biol.* **13**, 90–95 (2010).
5. L. Q. Chen *et al.*, Sucrose efflux mediated by SWEET proteins as a key step for phloem transport. *Science* **335**, 207–211 (2012).
6. M. Zimmermann, H. Ziegler, List of sugars and sugar alcohols in sieve-tube exudates. *Encycl. Plant Physiol.* **1**, 24 (1975).
7. T. L. Slewinski, D. M. Braun, Current perspectives on the regulation of whole-plant carbohydrate partitioning. *Plant Sci.* **178**, 341–349 (2010).
8. K. Esau, *Anatomy of Seed Plants* (Wiley, New York, NY, 1977).
9. Q. Xu, S. Chen, R. Yunjuan, S. Chen, J. Liesche, Regulation of sucrose transporters and phloem loading in response to environmental cues. *Plant Physiol.* **176**, 930–945 (2018).
10. I. A. Chincinska *et al.*, Sucrose transporter StSUT4 from potato affects flowering, tuberization, and shade avoidance response. *Plant Physiol.* **146**, 515–528 (2008).
11. C. Han *et al.*, KIN10 promotes stomatal development through stabilization of the SPEECHLESS transcription factor. *Nat. Commun.* **11**, 4214 (2020).
12. C. Han *et al.*, TOR and SnRK1 fine tune SPEECHLESS transcription and protein stability to optimize stomatal development in response to exogenously supplied sugar. *New Phytol.* **234**, 107–121 (2022).
13. J. C. Jang, P. León, L. Zhou, J. Sheen, Hexokinase as a sugar sensor in higher plants. *Plant Cell* **9**, 5–19 (1997).
14. L. Zhou, J. C. Jang, T. L. Jones, J. Sheen, Glucose and ethylene signal transduction crosstalk revealed by an *Arabidopsis* glucose-insensitive mutant. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 10294–10299 (1998).
15. A. Karve, X. Xia, B. Moore, Arabidopsis hexokinase-like1 and hexokinase1 form a critical node in mediating plant glucose and ethylene responses. *Plant Physiol.* **158**, 1965–1975 (2012).
16. Y. H. Cho, J. Sheen, S. D. Yoo, Low glucose uncouples HXK1-dependent sugar signaling from stress and defense hormone ABA and C2H4 responses in Arabidopsis. *Plant Physiol.* **152**, 1180–1182 (2010).
17. C. Y. Kang *et al.*, Cryptochromes, phytochromes, and COP1 regulate light-controlled stomatal development in *Arabidopsis*. *Plant Cell* **21**, 2624–2641 (2009).
18. C. A. MacAlister, K. Ohashi-Ito, D. C. Bergmann, Transcription factor control of asymmetric cell divisions that establish the stomatal lineage. *Nature* **445**, 537–540 (2007).
19. K. Akita, S. Hasezawa, T. Higaki, Breaking of plant stomatal one-cell-spacing rule by sugar solution immersion. *PLoS One* **8**, e72456 (2013), 10.1371/journal.pone.0072456.
20. B. Moore *et al.*, Role of the *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signalling. *Science* **300**, 332–336 (2003).
21. S. Yanagisawa, S. D. Yoo, J. Sheen, Differential regulation of EIN3 stability by glucose and ethylene signaling in plants. *Nature* **425**, 521–525 (2003).
22. F. An *et al.*, Ethylene-induced stabilization of ETHYLENE INSENSITIVE3 and EIN3-LIKE1 is mediated by proteasomal degradation of EIN3 binding F-box 1 and 2 that requires EIN2 in *Arabidopsis*. *Plant Cell* **22**, 2384–2401 (2010).
23. Y. Gong *et al.*, Tuning self-renewal in the Arabidopsis stomatal lineage by hormone and nutrient regulation of asymmetric cell division. *Elife* **10**, e63335 (2021).
24. X. R. Mu *et al.*, Feed-back loop promotes sucrose accumulation in cotyledons to facilitate sugar-ethylene signaling-mediated, etiolated-seedling greening. *Cell Rep.* **38**, 110529 (2022).

25. M. Lei *et al.*, Genetic and genomic evidence that sucrose is a global regulator of plant responses to phosphate starvation in *Arabidopsis*. *Plant Physiol.* **156**, 1116–1130 (2011).
26. S. Yu *et al.*, Sugar is an endogenous cue for juvenile-to-adult phase transition in plants. *Elife* **2**, e00269 (2013).
27. Y. H. Cho, S. D. Yoo, J. Sheen, Regulatory functions of nuclear hexokinase1 complex in glucose signaling. *Cell* **127**, 579–589 (2006).
28. J. Zeng *et al.*, Endogenous stress-related signal directs shoot stem cell fate in *Arabidopsis thaliana*. *Nat. Plants* **7**, 1276–1287 (2021).
29. Z. Li, J. Peng, X. Wen, H. Guo, *ETHYLENE-insensitive3* is a senescence-associated gene that accelerates age-dependent leaf senescence by directly repressing *miR164* transcription in *Arabidopsis*. *Plant Cell* **25**, 3311–3328 (2013).
30. R. Turgeon, J. A. Webb, Leaf development and phloem transport in *Cucurbita pepo*: Transition from import to export. *Planta* **113**, 179–191 (1973).
31. S. C. Fan *et al.*, The *Arabidopsis* nitrate transporter NRT1.7, expressed in phloem, is responsible for source-to-sink remobilization of nitrate. *Plant Cell* **21**, 2750–2761 (2009).
32. L. S. Meng *et al.*, Cytosolic invertase-mediated root growth is feedback regulated by a glucose-dependent signaling loop. *Plant Physiol.* **184**, 895–908 (2020).
33. S. A. Casson, A. M. Hetherington, phytochrome B is required for light-mediated systemic control of stomatal development. *Curr. Biol.* **24**, 1216–1221 (2014).
34. C. B. Engineer *et al.*, Carbonic anhydrases, *EPF2* and a novel protease mediate CO_2 control of stomatal development. *Nature* **513**, 246–250 (2014).
35. S. A. Coupe *et al.*, Systemic signalling of environmental cues in *Arabidopsis* leaves. *J. Exp. Bot.* **57**, 329–341 (2006).
36. M. S. Ewert, W. H. Outlaw, S. Zhang, K. Aghoram, K. A. Riddle, Accumulation of an apoplastic solute in the guard-cell wall is sufficient to exert a significant effect on transpiration in *Vicia faba* leaflets. *Plant Cell Environ.* **23**, 195–203 (2000).
37. Y. Kang, W. H. Outlaw Jr., P. C. Andersen, G. B. Fiore, Guard-cell apoplastic sucrose concentration–a link between leaf photosynthesis and stomatal aperture size in the apoplastic phloem loader *Vicia faba* L. *Plant Cell Environ.* **30**, 551–558 (2007).
38. S. Philosoph-Hadas, S. Meir, N. Aharoni, Carbohydrates stimulate ethylene production in tobacco leaf discs. II. Sites of stimulation in the ethylene biosynthesis pathway. *Plant Physiol.* **78**, 139–143 (1985).
39. H. Kobayashi, H. Saka, Relationship between ethylene evolution and sucrose content in excised leaf blades of rice. *Plant Prod. Sci.* **3**, 398–403 (2000).
40. S. W. Jeong *et al.*, Ethylene suppression of sugar-induced anthocyanin pigmentation in Arabidopsis. *Plant Physiol.* **154**, 1514–1531 (2010).
41. Z. Li *et al.*, Ethylene-insensitive3 is a senescence-associated gene that accelerates age-dependent leaf senescence by directly repressing miR164 transcription in Arabidopsis. *Plant Cell* **25**, 3311–3328 (2013).
42. R. Jutta *et al.*, Arabidopsis fhl/fhy1 double mutant reveals a distinct cytoplasmic action of phytochrome A. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 10737–10742 (2007).
43. J. P. Huang *et al.*, Cooperative control between AtRGS1 and AtHXK1 in a WD40-repeat protein pathway in Arabidopsis. *Front. Plant Sci.* **6**, 851 (2015).
44. L. S. Meng *et al.*, Sucrose signaling regulates anthocyanin biosynthesis through a MAPK cascade in *Arabidopsis thaliana*. *Genetics* **210**, 607–619 (2018).
45. M. K. Udvardi, T. Czechowski, W. R. Scheible, Elevengolden rules of quantitative RT-PCR. *Plant Cell* **20**, 1736–1737 (2008).
46. X. R. Mu, *et al.*., EIN3 information. Arabidopsis Information Resource. https://www.arabidopsis.org/servlets/TairObject?id=38697&type=locus. Deposited 11 May 2023.
47. X. R. Mu, *et al.*, HXK1 information. Arabidopsis Information Resource. https://www.arabidopsis.org/servlets/TairObject?id=127303&type=locus. Deposited 11 May 2023.
48. X. R. Mu, *et al.*., SUC2 information. Arabidopsis Information Resource. https://www.arabidopsis.org/servlets/TairObject?id=136949&type=locus. Deposited 11 May 2023.