Sugar status in pre-existing leaves determines systemic stomatal development within newly developing leaves

Sugar Status in Pre-existing Leaves Determines Systemic Stomatal Development within Newly Developing Leaves

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Short title: Sucrose in mature leaves decides stomatal fate in young leaves.

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Abstract

Stomata are pores found in the epidermis of stems or leaves, that modulates both plant gas exchange and water/nutrient uptake. The development and function of plant stomata is regulated by a diverse range of environmental cues. However, how carbohydrate status in pre-existing leaves determines 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, to orchestrate stomatal development. Here, we demonstrate that the Glc status of pre-existing leaves determines systemic stomatal development within newly developing leaves by the HXK1—¦EIN3—¦SUC2 module. Further, increasing Glc levels in pre-existing 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 pre-existing leaves to newly developing leaves. Subsequently, increasing Suc levels within newly developing leaves promotes stomatal formation through the known KIN10⟶SPCH module. Our findings thus establish how a carbohydrate signal in pre-existing leaves is

sensed, amplified and relayed to determine the extent of systemic stomatal development within newly developing leaves.

Significance Statement

Stomata control both plant water/nutrient uptake and gas exchange. The development and function of these plant pores is 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 signaling module that links the glucose status of pre-existing 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.

Introduction

Stomata, comprised of two adjacent guard cells, 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 though a complex network of signaling transduction pathways that control diverse environmental and endogenous cues (2).

In leaves, the cell lineage that gives rise to guard cells is initiated in the post-embryonic epidermis, in which some cells go through asymmetric division in the post-

93 embryo meristemoids (3). Stomata are generated through a specialized epidermal
94 lineage, that undergoes an orchestrated unidirectional successive division and
95 associated distinct cell-states. Protodermal cells form MMC (meristemoid mother cells),
96 which divide asymetirically to generate a M (meristemoid) and a larger SLGC
97 (stomatal-lineage ground cell). M cells have stem-cell like properties and undergo
98 amplifying divisions providing the major source of both stomata and epidermal
99 pavement cells. Ultimately, M cells transition into a GMC (guard mother cell), which
100 symmetrically divides, subsequently forming two GCs (guard cells), following a further
101 state transition (4).

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

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

Other *SUTs* genes, termed *SWEET11* and *SWEET12*, are expressed in the phloem parenchyma cells of minor veins, and their 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 wild-type (5).

Sugars promote stomatal development (14). TOR (Target of rapamycin) and SnRK1 (Snf1-related protein kinase 1) finely modulate the transcript and protein abundance of SPCH to regulate the stomatal number in response to the differently exogenous application of sugars (14, 49). Moreover, some previous reports have revealed that Glc-antagonism of ethylene signaling occurs under excess Glc levels (35—37). It has been further shown that Glc signaling is uncoupled from ethylene signal transduction under low Glc availability (50). 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 HXK1-dependent decrease in 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 the decline of Suc transport from mature leaves and the associated decrease 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 have been reported that sugars promote stomatal development (14, 49). To further investigate a potential role for sugars in the regulation of stomatal development, 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-day-old *Arabidopsis* seedlings (11). Only stomata with color lines or color pore (such as Figure 1A) were counted and considered as stomata. Pavement cells indicate puzzle-shaped cells in the epidermis (such as, black triangles in Figure 1A).

In this study, we used an *Arabidopsis* transgenic line expressing a GREEN FLUORESCENT PROTEIN (GFP) marker fused to the bHLH (basic helix-loop-helix) transcription factor, SPCH (SPEECHLESS) 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

159 detected in the MMCs and meristemoids (Ms), whereas *MUTEpro::MUTE-GFP* was
160 expressed in the GMCs (guard mother cells) (3, 12). Our findings confirmed that Glc
161 promotes stomatal development and changes cell fate by using SI and % of SPCH-GFP
162 and MUTE-GFP marked cells (Figures 1A and C; Figure S1), concurring with previous
163 data (13, 14). Further, our findings indicated that SIs in 12-day old wild-type (Col-0)
164 cotyledons increased when seedlings were grown in low availability (0 - 3% Glc)
165 (Figures 1A and C). Conversely, SIs decreased when seedlings were grown in the
166 presence of 5% Glc. However, *hxk1-3* seedlings, which are compromised in hexokinase
167 1 (HXK1) function, a Glc sensor (15), were largely insensitive to these Glc
168 concentrations (Figures 1A and C). Thus, suggesting the impact of Glc on stomatal
169 development may be partially dependent on *HXK1*.

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

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

177

178 **Glc promoted stomatal development is partially dependent on *EIN3/EIL1***

179 Glc/Suc has been exhibited to promote stomatal formation (Figure 1) (13, 14). HXK1
180 has been reported to decrease the stability of EIN3 (16). The EIN3/ELI1 transcription

181 factors are key regulators of ethylene signaling pathway (17). Moreover, EIN3/ELI1 is
182 required for sugar signaling (16). We therefore determined if EIN3/ELI1 might also be
183 participated in the regulation of Glc signaling-mediated stomatal development.
184 We observed that with an increase of Glc concentration, mature cotyledons of 12-
185 day-old wild-type seedlings exhibited increased SIs (Figures 2A and C). However, in
186 the *ein3/eil1* lines, an increasing Glc concentration did not significantly increase the SI
187 relative to wild-type plants (Figures 2A and C). Therefore, the *ein3/eil1* mutant already
188 possesses a higher SI in the absence of added Glc, congruent with ethylene inhibiting
189 meristemoid amplifying division (18). Consistent with these phenotypes, *EIN3*
190 expression was observed in pavement cells, in addition to stomatal lineage cells, in
191 transgenic plants marked by expression of a β-GLUCURONIDASE (GUS) reporter
192 enzyme under the control of the *EIN3* promoter (*EIN3pro-GUS*) (Figure S2B).
193 To confirm these findings, we determined the stomatal development phenotype in
194 response to Glc in the stomatal cell-type-specific marker line, *SPCHpro::SPCH-GFP*.
195 Our findings indicated that with 4-day-old seedlings, the number of cells marked by
196 GFP in immature cotyledons was greater in the *ein3/eil1* seedlings relative to wild-type
197 (Col-0) (Figures 2B and D). Collectively, our data indicated that Glc promoted stomatal
198 development was partially dependent on *EIN3/EIL1*.
199
200 ***EIN3* acts upstream of *SUC2* but downstream of *HXK1*, in the regulation of**
201 **stomatal development**

The loss-of-function mutant of *HXK1*, *hxk1-3*, in the Col-0 background, showed reduced SI (Figures 3A, B and H). By contrast, mature cotyledons in *ein3/eil1* plants exhibited increased SI (Figures 3A, C, and H), relative to wild-type. Further, *hxk1/ein3/eil1* cotyledons also exhibited increased SI (Figures 3A, D, and H), relative to wild-type, indicating that *ein3/eil1* mature cotyledons had an epistatic phenotype to *hxk1-3*.

Recently, we demonstrated that EIN3 directly regulates the function of *SUC2* to inhibit the expression of *SUC2* (19). 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 (Figures 3A, F, and H). In addition, we analyzed a previously generated transgenic line where *SUC2* was overexpressed from the CaMV*35S* promoter (*35S:SUC2*) (20). The cotyledons of the *35S:SUC2* line exhibited increased SI (Figures 3A, E, and H), relative to wild-type. Further, *ein3/eil1/suc2-5/+* cotyledons exhibited decreased SI (Figures 3A, G, and H), relative to wild-type, indicating that *suc2-5/+* cotyledons had an epistatic phenotype to *ein3/eil1*.

Collectively, our data suggests 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 spatio-temporal expression patterns

With increasing age, endogenous Suc/Glc content increased (Figure 4A and B), consistent with previous data (21). 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 (Figure 4C). In addition to stability (16), *EIN3* expression is also regulated by Glc/Suc (Figure 4A 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 (Figures 4D and E). Further, endogenous or exogenous Suc/Glc promotes HXK1-dependent EIN3 degradation (16, 22). Recently, we showed that EIN3 directly regulates the function of *SUC2* to repress the expression of *SUC2* (19). Our current findings combined with previous data indicates 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 was also supported by other work, showing *EIN3* was strongly expressed in stem cells of young leaf primordia at the shoot apical meristem (23). Moreover, it has also been demonstrated that *EIN3* expression increases within aged tissues (24). 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*:*GUS*). This line was used to detect the binding

activity of the transcription factor EIN3 (24). Consistent with both qPCR and western blotting data, GUS expression of EBS was strongest on 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-day-old seedlings (Figures 4F—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 (Figures 4F—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 (25).

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

SUC2 function in mature cotyledons is sufficient to promote stomatal development within newly developing leaves

Figure 4 implies that both EIN3 and SUC2 may regulate the export of nutrients out of mature leaves. We next determined if *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. Source and sink leaves were not absolute (47). In this study, mature cotyledons were used as source tissues, whereas the 3rd/4th leaves were used 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 hour, the 3rd and 4th leaves of 16-, 18-, 20-day-old inducible *ER::SUC2-3×FLAG/suc2-5* seedlings were all found to exhibit similar growth to the mock treated control lines (Figure 5A).

Both *SUC2* mRNA and SUC2 accumulation in cotyledons of inducible 16-, 18-, 20-day-old *ER::SUC2-3×FLAG/suc2-5* seedlings gradually increased with increasing age (Figures 5C 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 (Figure 5F). 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 leaves of these seedlings treated with mock in cotyledons (Figure 5G). As shown above by both our findings and previous data, Suc levels are positively linked to the associated SI (14) (Figure 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 (Figures 5B and H).

It is well-known that estradiole is membrane permeable, and inducible cotyledons might leak estradiol into the developing primordia of the 3rd/4th leaf. In this study, cotyledons (but not other organs) were induced by the application of 20 μM estradiol for 1 hour, to lessen the 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 (Figure 5D). Therefore, we can exclude the probability of 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 (16, 17). In this experiment, we 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 declined in young leaves (Figures 6B and C). In this context, Suc or Glc has previously been shown to

promote HXK1-dependent EIN3 degradation (16, 22). Further, with increasing age, both endogenous Glc and Suc content gradually increased (Figures 4A and B) (21), which in turn promoted both a decrease of EIN3 levels and an associated increase of SUC2 (Figures 4D 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 wild-type cotyledon phloem exudates gradually increased (Figure 6E). 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 enable conditional SUC2 expression, both showed a more increase in Suc within cotyledon phloem exudates, relative to wild-type (Figure 6F). As a result, whereas the Suc content within the 3rd and 4th leaves from wild-type seedlings gradually increased (Figure 6F), 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 (Figure 6F). Finally, with increasing exogenously supplied Glc, the SI of wild-type 3rd and 4th leaves gradually increased (Figures 6A 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 (Figures 6A and G).

Further, we tested the the 3rd/4th leaves following cotyledons treated by estradiol, and our findings indicate that SUC2 accumulation was not detected within these 3rd/4th leaves (Figure 6D). Therefore, we can exclude the probability 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 newly developing 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 under 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 wild-type (L*er*) seedlings, but this was not recapitulated in a *gin2-1* line (Figures S3F 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 (15, 22), 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 (15). Thus, *gin2-1*-expressing a

353 serine (S) to alanine (A) mutation at residue 177 *HXK1*^{S177A} (*S177A/gin2-1*) was utilized
354 to discern between signaling or metabolic roles for HXK1 (15, 26). Analysis of
355 *HXK1/gin2-1* and *S177A/gin2-1* lines displayed an expression feature for these two
356 genes similar to that detected in L*er* seedlings (Figures S3A—E). Therefore, HXK1
357 enzymatic function is not essential for the changes in the expression of both *EIN3* and
358 *SUC2*. Concurring with these results, the *gin2-1* phenotype of stomatal development
359 was completely restored by expression of either a wild-type *HXK1* transgene or a
360 transgene containing the *S177A* mutation (Figures S3A—E).
361 Together, our findings indicate that HXK1 signaling activity is required in addition
362 to the EIN3—¦SUC2 module in the regulation of stomatal development.
363
364 **phyB function is not linked to Glc regulated stomatal development**
365 Sucrose and Glucose are the major products of photosynthesis, and photoreceptors are
366 required for mediating photosynthesis to increased light (27). Further, *phyB* encoding
367 the apoprotein of the major red/far-red photoreceptor, is essential for the systemic
368 regulation of stomatal development (27). So, we must exclude the change of SIs is due
369 to photoreceptor activity and not linked to Glc levels. We found that mature cotyledons
370 of the phytochrome photoreceptor mutant, *phyB-9*, exhibited reduced SI compared with
371 wild-type plants grown on solid medium with 2% Glc, but not with 2% mannose
372 (Figures S4).
373 Thus, suggesting phyB function was not linked to Glc regulated stomatal
374 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 (14) (Figure 7).

In young leaves, transported Suc stimulates formation of stomata (Figure 7), whereas increased CO_2 levels in young leaves reduces formation of these pores for gaseous exchange, apparently as a negative feedback system (28). 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 (29). The accumulation of excess photosynthetic products (such as,

Suc/Glc) may cause the high accumulation of Tre6P (trehalose-6-phosphate), which in turn suppresses the KIN10 activity via declining the interplay between SnAK1/2 and KIN10 (49). The resulting stomatal number is decreased (49). Indeed, excess photosynthetic products, Glc inhibited stomatal development (Figure 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 guard cells 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. In details, 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 (30,31). When the rate of photosynthesis is in excess, surplus Suc/Glc produced in mature leaves stimulates the production of ethylene, a stress hormone (32-34). As a result, transported Suc from mature leaves is significantly reduced by the Glc-ethylene-EIN3-SUC2-Suc pathway (Figure 7). Accordingly, stomatal formation within newly developing leaves is blocked by the Suc-KIN10-SPCH pathway (Figures 1A and C; Figure 7). Therefore, under disadvantageous growth conditions (high CO_2 levels or

419 excess photosynthetic products), excess Glc-induced ethylene triggers the reduction of
420 Suc transport from mature leaves and the associated decrease of stomatal formation
421 within newly developing leaves, consistent with glucose-antagonizing ethylene
422 signaling under excess Glc levels (15, 35-37). Further, under other disadvantageous
423 growth conditions (low light and short-day conditions), the activation of KIN10 (a
424 energy-sensing SnRK1 kinase) phosphorylates and stabilizes SPCH to facilitate
425 stomatal formation, which in turn elevates photosynthesis ability and/or carbon
426 assimilation of plants (14). The resulting feed-forward loop is formed to assist plants
427 recover from stress. By contrast, under "normal" conditions, the Suc content of mature
428 leaves is transported by the Glc-HXK1-EIN3-SUC2-Suc pathway (Figures 1A and C;
429 Figure 7). Accordingly, stomatal formation within newly developing leaves is promoted
430 by the Suc-KIN10-SPCH pathway (Figure 7).

431 These findings provide novel insights into the biochemical control of stomatal
432 number at a distance, potentially designing crop strategies to aid in elevating the
433 resilience of crop plants under a changing environment. Globally, an elucidation of the
434 key molecular and genetic players that mediate the sugar-controlled plant stomatal
435 number response can become critical for agriculturally influences to improve crop
436 resilience.

437

438 ## Materials and Methods

439 ### Plant Materials and Growth Conditions

440 The *ER::EIN3-FLAG*, *5 × EBS-GUS* (38), *EIN3-GUS* (39), *35S:EIN3-GFP*, *ein3-1* and *ein3/eil1*
441 (40), *SUC2pro-GUS*, *ein3/eil1/suc2-5/+*, *EIN3pro:EIN3-GFP* (41), *phyb-9* (CS6217) (42), *hxk1-3*

(CS861759) (43), *gin2-1* (CS6383), *HXK1/gin2-1* and *S177A/gin2-1* (39), *35S:SUC2* and *suc2-5* (44) 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 (45).

The *ER::SUC2-3×FLAG* transgene was transformed into wild-type (Col-0) or *suc2-5* lines by using the *Agrobacterium tumefaciens*-mediated floral dip method (Meng et al., 2018). The *SPCHpro::SPCH-GFP* and *MUTEpro::MUTE-GFP* transgenes were transformed into wild-type (Col-0), *ein3/eil1*, or *hxk1-3* lines by using the *Agrobacterium tumefaciens*-mediated floral dip method (39).

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, 1.1kb); 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 microscope for GFP or YFP imaging

Confocal laser scanning microscope for GFP or YFP imaging was performed, as has been described in details (39).

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 (DIC) and inversion microscopy.

From the same position in two cotyledons or $3^{rd}/4^{th}$ leaf (such as, Figure S2A), counts were from one area (or section) per cotyledon/$3^{rd}/4^{th}$ 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 3 biological repeats (3×20=60) with similar results but only the stomatal index (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 with color pores or with color lines) (such as Figure 1A) were counted and considered as stomata. Pavement cells indicate 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—5 times with PBS buffer (80—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 6h in the dark. After staining, these seedlings were washed 3—5 times with PBS, followed via decolorization using different ethanol gradients, as has been described (46). 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. 0.1g cotyledons of the relevant age was ground into homogenate at 23℃. 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 4,000g 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 4,000g 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 minutes. 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 (47).

Quantitative PCR

Total RNA was extracted from the tissues indicated by TRIZOL reagent (Invitrogen), as has been described by (39, 48). 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; 44) 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 (39, 48). 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 (49). Primer pairs of *EIN3* have been described (39, 46). Primer pairs of *SUC2* have been described (40).

Western Blotting

Western blotting was performed, as has been described previously (39, 48).

Accession Numbers

Sequence data from this article can be found in the *Arabidopsis* Genome Initiative or GenBank/EMBL databases under the following accession numbers: AT3G20770 (EIN3), AT4G29130 (HXK1), and AT1G22710 (SUC2).

Supplemental Data

Supplemental figure 1. Glc promotes stomatal development and changes cell fate.

Supplemental figure 2. Promoter Activities of *EIN3* gene on epidermal cells of leaves.

Supplemental figure 3. The signaling role of HXK1 is integral to Glc-mediated stomatal development.

Supplemental figure 4. *phyB* positively effects stomatal development independent of sugar signaling.

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Figure legends

Figure 1. Glc signaling/metabolism promoting stomatal development is partially dependent of HXK1.

A, Images illustrating abaxial epidermal stomata on mature cotyledons of 12 day-old wild-type (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 Image J [BAR(A collection of Broadly Applicable Routines)] (https://imagej.net/software/fiji/downloads). Arrow indicates meristemoid (black triangle), whereas arrowhead indicates stomata with color pores or with color lines.

B, Images illustrating SPCHpro::SPCH-GFP fluorescence detection in immature cotyledons of 4- day-old wild-type (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 days 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).

Figure 2. Glc signaling promoted-stomatal development was partially dependent of EIN3/EIL1.

A, Images illustrating abaxial epidermal stomata of mature cotyledons of 12-day-old wild-type 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-day-old wild-type (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).

Figure 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-day-old wild-type 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).

Figure 4. Contrasting expression of *EIN3* and *SUC2* genes.

A and **B,** Bar graph illustrating Suc and Glc content in 2-, 4-, 6- 8- day post emergence (DAE) cotyledons of wild-type seedlings.

C, Bar graph illustrating differential expression of *EIN3* and *SUC2* in A. 2-, 4-, 6- 8-DAE-cotyledons of wild-type seedlings were gathered and total RNA was extracted from cotyledons 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-day-old seedlings. Arrows indicate the source-to-sink transition leaves, and stars indicate 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).

Figure 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-day-old *ER::SUC2- 3×FLAG/suc2-5* seedlings. Mature cotyledons (as source leaves) (but not other organs) of 15-day-old *ER::SUC2-3×FLAG/suc2-5* seedlings were treated with 20 μM estradiol for 1h 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-day-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-day-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-day- old *ER::SUC2-3×FLAG/suc2-5* seedlings. Quantification at 16 days 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-day- 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)**.**

Figure 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-day-old wild-type (Col-0), transgenic plants containing an *EIN3pro::EIN3-GFP* transgene or transgenic plants containing an *ER::SUC2-3×FLAG* transgenein the wild-type (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-day-old seedlings containing either an *EIN3pro::EIN3-GFP* or *ER::SUC2-3×FLAG* transgene in the wild-type (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-day-old *ER::SUC2-3×FLAG* in the wild-type (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-day- old wild-type (Col-0), transgenic *EIN3pro::EIN3-GFP* plants or transgenic *ER::SUC2-3×FLAG* in the wild-type (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-day-old wild-type (Col-0), transgenic *EIN3pro::EIN3-GFP* plants or transgenic *ER::SUC2-3×FLAG* in the wild-type (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).

Figure 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 (14). Thus, a Suc-KIN10-SPCH pathway promotes stomatal development within newly developing leaves in response to transported Suc within newly developing leaves.

Under disadvantageous growth conditions (high CO_2 levels or excess photosynthetic products),
excess Glc induces ethylene production, and Suc transport in mature leaves is declined by the Glc-ethylene-EIN3-SUC2-Suc pathway, and 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.