The role of HEXOKINASE1 in Arabidopsis Leaf Growth

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J.V.D. performed experiments, analyzed data and is the main author; M.V. and L.D.M. assisted experiments; S.H. performed the BiFC experiments; J.V.L, N.D.W. and G.D.J. performed the TAP experiments; F.R., N.G., S.D. and J.V.L. were involved in discussions throughout the project; N.G., S.D., F.R. and D.I. contributed to the writing of the manuscript. G.D.J. and D.I. supervised the project.

Key Message

Here, we used a *hxk1* mutant in the Col-0 background. We demonstrated that HXK1 regulates cell proliferation and expansion early during leaf development, and that HXK1 is involved in sucrose-induced leaf growth stimulation independent of *GPT2*. Furthermore, we identified KINγ as a novel HXK1-interacting protein possibly regulating HXK1 function in plant growth.

Abstract

In the last decade, extensive efforts have been made to unravel the underlying mechanisms of plant growth control through sugar availability. Signaling by the conserved glucose sensor HEXOKINASE1 (HXK1) has been shown to exert both growth-promoting and growth-inhibitory effects depending on the sugar levels, the environmental conditions and the plant species. Here, we used a *hxk1* mutant in the Col-0 background to investigate the role of HXK1 during leaf growth in more detail and show that it is affected in both cell proliferation and cell expansion early during leaf development. Furthermore, the *hxk1* mutant is less sensitive to sucrose-induced cell proliferation with no significant increase in final leaf growth after transfer to sucrose. Early during leaf development, transfer to sucrose stimulates expression of *GLUCOSE-6-PHOSPHATE/PHOSPHATE TRANSPORTER2* (*GPT2*) and represses chloroplast differentiation. However, also in the *hxk1* mutant *GPT2* expression was upregulated although chloroplast differentiation was not affected, suggesting that GPT2 is not involved in HXK1-dependent regulation of leaf growth. Finally, using tandem affinity purification of protein complexes from cell cultures, we identified KINγ, a protein containing four cystathionine β-synthase domains, as an interacting protein and possible regulator of HXK1.

Keywords HEXOKINASE1, Leaf growth, Sucrose, Sink-source

Electronic supplementary material The online version of this article contains supplementary material, which is available to authorized users.

Introduction

Leaves are the major factories using solar energy to produce essential components for plant growth and development. Leaf growth itself is a strictly controlled and complex process that is strongly influenced by environmental conditions such as light, water and nutrient availability. Interestingly, different studies have illustrated a link between photosynthesis, and thus CO_2 fixation and carbohydrate production, and the cellular processes during early leaf growth (Andriankaja et al. 2012; Lastdrager et al. 2014; Van Dingenen et al. 2016).

Leaves develop as primordia at the shoot apical meristem and subsequently grow by cell proliferation and cell expansion (Donnelly et al. 1999). These two cellular processes occur simultaneously during the so-called transition phase when cell expansion starts at the tip of the leaf and a cell division arrest front moves in a tip-to-base direction (Andriankaja et al. 2012). Establishment of the photosynthetic machinery and chloroplast differentiation is coupled with the onset of cell expansion during early leaf growth (Andriankaja et al. 2012). In accordance, when sink leaves receive enough sugars, chloroplast differentiation is postponed and cell proliferation is stimulated (Van Dingenen et al. 2016). *GLUCOSE-6-PHOSPHATE TRANSPORTER2* (*GPT2*) expression was found to play a key role in the promotion of cell proliferation as well as in the repression of plastome expression and chloroplast differentiation when seedlings are transferred to sucrose-containing medium (Van Dingenen et al. 2016). Sugars are used as substrates in respiration and important biosynthetic pathways, such as the oxidative pentose phosphate pathway, cell wall biosynthesis and starch biosynthesis, but they can also act as signaling molecules of the metabolic status, triggering diverse regulatory pathways (Rolland et al. 2006; Lastdrager et al. 2014; Sheen 2014). These metabolic signals are integrated in the growth regulatory network to enable an optimal plant growth and development in continuously changing environmental conditions.

Several proteins are involved in monitoring the cellular sugar status. One of these is HEXOKINASE1 (HXK1), a key glycolytic enzyme catalyzing the conversion of glucose to glucose-6-phosphate. The developmental arrest of Arabidopsis seedlings on high glucose concentrations (with repression of cotyledon expansion and chlorophyll synthesis) is exacerbated or mitigated by increasing or decreasing expression of HXK1, respectively (Jang et al. 1997). Consistently, the *glucose insensitive2* (*gin2*) mutant in the Landsberg *erecta* (L*er*) background was found to have reduced *HXK1* expression and impaired HXK1 protein accumulation (Moore et al. 2003). HXK1 signaling mediates glucose-induced repression of photosynthesis-related genes, such as *CHLOROPHYLL A/B BINDING PROTEIN* (*CAB*) and the small subunit of *RUBISCO* (*RBCS*) (Moore et al. 2003). The *gin2* mutant also exhibits more general growth defects such as small dark green leaves and a reduced root system when grown under high-light (high endogenous sugar) conditions (Moore et al. 2003). Based on the distance between neighboring trichomes, it has been proposed that the small *gin2* leaves result from reduced cell expansion. Complementation of the *gin2* mutant with catalytically inactive *HXK1* alleles resulted in the recovery of high glucose susceptibility, as well as leaf growth under high-light conditions, uncoupling its catalytic and regulatory functions (Moore et al. 2003). Remarkably, overexpressing *HXK1* in Arabidopsis and its orthologs in tomato and rice plants also results in growth defects, with reduced chlorophyll accumulation and photosynthesis, as well as early onset of senescence (Dai et al. 1999; Cho et al. 2006; Kelly et al. 2012). In tobacco, silencing of *NtHXK1* also leads to growth-inhibitory effects and leaf bleaching as a result of chloroplast degradation in source leaves and starch accumulation in sink leaves (Kim et al. 2013). These phenotypes indicate that, depending on the environmental conditions and developmental stage, HXK1 can be involved in growth-promoting as well as growth-inhibitory pathways. Taken together, these studies not only support a central role of HXK1 in sugar signaling during plant growth, but also

stress that a better understanding of how HXK1 monitors sugar status and the underlying cellular mechanisms of HXK1-mediated signaling and growth control is highly needed.

The predominant localization of HXK1 is on the outer membrane of mitochondria where it exerts its metabolic function in glycolysis (Heazlewood et al. 2004; Claeyssen and Rivoal 2007; Granot 2008). Several GFP-fusion studies in Arabidopsis, tomato, spinach and tobacco revealed that the N-terminal hydrophobic membrane anchor domain of HXK1 is required for the association with the mitochondria (Dai et al. 1999; Balasubramanian et al. 2007; Damari-Weissler et al. 2007; Kim et al. 2013). In addition, a small portion of the HXK1 proteins is localized in the nucleus where they have been reported to interact with the vacuolar H(+)-ATPase B1 (VHA-B1) and the 19S regulatory particle of the proteasome subunit RPT5B and directly regulate transcription (Cho et al. 2006). However, how this nuclear HXK1 complex is established exactly and whether additional HXK1-interacting protein partners are involved in the HXK1-mediated regulation of transcription is still not well understood.

Besides the HXK1 glucose sensor, the Target Of Rapamycin (TOR) protein kinase and the Sucrose Non-Fermenting1 (SNF1)-Related Kinase (SnRK1) are well-known conserved sugar signaling regulators involved in coordinating growth with environmental signals. Glucose-induced TOR activity mediates many different transcriptional responses to stimulate biosynthetic pathways and inhibit catabolic processes to sustain growth (Xiong and Sheen 2014). Conversely, the SnRK1 kinase plays a key role in detecting sugar and energy depletion. SnRK1 functions as a heterotrimeric complex with a catalytic α subunit and regulatory β and γ subunits and is also highly conserved in yeasts (Sucrose-Non fermenting1 or SNF1) and in mamals (AMP-activated kinase or AMPK) (Broeckx et al. 2016).

In this study, we characterized a *hxk1* mutant in the Col-0 background and studied the role of HXK1 in sucrose-regulated leaf growth. Furthermore, tandem affinity purification experiments identified the SnRK1 γ subunit-like protein KINγ (*AT3G48530*) containing four cystathionine β-synthase (CBS) domains as a novel HXK1-interacting protein.

Materials and methods

Growth conditions *in vitro* and in soil

For the soil experiments, plants were grown for 22 days at 21°C under a 16-h day (100 or 135 µmol m^{-2} s^{-1}) and 8-h night regime. For the *in vitro* experiments, seedlings were grown on half-strenght Murashige and Skoog (MS) medium (Murashige and Skoog 1962) under a 16-h day (50 µmol m^{-2} s^{-1}) and 8-h night regime. Seedlings were grown for nine days on nylon mesh of 20-µm pore size overlaying growth medium without sucrose. At 9 days after stratification (DAS), seedlings were transferred to plates containing control medium without sucrose or medium supplemented with 15 mM sucrose (Van Dingenen et al. 2016). In the high-glucose experiments, seedlings were grown for eight days on MS medium with 6% glucose. For the root waving experiments, seedlings were grown on vertical plates without sucrose or supplemented with 1% sucrose.

Transgenic lines and mutants

All experiments were performed on *Arabidopsis thaliana* (L.) Heyhn. ecotype Columbia (Col-0). The *hxk1* mutant was obtained from the SALK collection (SALK_018086). The *KINγ* mutant (SALK_074554) was described by

Ramon et al. (2013). The *pCYCB1;1::CYCB1;1-D-box:GUS* reporter line (Eloy et al. 2012) was crossed with *hxk1* to obtain homozygous *pCYCB1;1::CYCB1;1-D-box:GUS*/*hxk1* lines. The 35S::HXK1-GS^{green} and 35S::HXK1-GS^{rhino} constructs were made through Multisite Gateway cloning (Karimi et al. 2007a; Karimi et al. 2007b). 35S::HXK1-GS^{green} was introduced into the pH7m34GW-FAST vector (Shimada et al. 2010) and transformed into *Arabidopsis thaliana* Col-0 by floral dip using *Agrobacterium tumefaciens* strain C58C1 (pMP90) (Clough and Bent 1998).

Growth analysis

For the leaf area analysis, leaves were cleared in 100% ethanol, mounted on microscope slides in lactic acid, and photographed. Leaf areas were measured with the ImageJ software (http://rsb.info.nih.gov//ij/). Abaxial epidermal cells were drawn using a DMLB microscope (Leica) fitted with a drawing tube and a differential interference contrast objective. Drawings were scanned and analyzed using automated image analysis algorithms (Andriankaja et al. 2012). Subsequently, drawings were used to measure average cell area, from which the total pavement cell number was calculated. The stomatal index was defined as the percentage of stomata compared with all cells.

GUS ataining and analysis

Seedlings of two to three biological repeats were harvested at 12 and 14 DAS, incubated in heptane for 10 min and subsequently left to dry for 5 min. Then, they were submersed in 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-Gluc) buffer [100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)-HCl, 50 mM NaCl buffer (pH 7.0), 2 mM $K_3[Fe(CN)_6]$, and 4 mM X-Gluc], vacuum infiltrated for 10 min and incubated at 37°C overnight. Seedlings were cleared in 100% ethanol and then kept in 90% lactic acid. The third leaf was dissected, mounted on slides and photographed under a light microscope.

RNA extraction and expression analysis by qRT-PCR

Seedlings or shoots were frozen in liquid nitrogen and RNA was extracted using Trizol (Invitrogen) and the RNeasy Plant Mini Kit (Qiagen). DNase treatment was done on columns with RNase-free DNase I (Promega). The iScript cDNA synthesis kit (Bio-Rad) was used to prepare cDNA from 500 ng µg RNA and qRT-PCR was done on the LightCycler 480 with SYBR Green I Master (Roche) according to the manufacturer's instructions. Normalization was done against the average of the three housekeeping genes *AT1G13320, AT2G32170,* and *AT2G28390.*

Western blot

Protein extraction was done as described before (Van Leene et al. 2007). Western blotting was performed with primary rabbit anti-GFP antibodies (Santa Cruz; diluted 1:4000) for 1 h and secondary horseradish peroxidase-conjugated donkey anti-rabbit antibodies (1:10000) for 1 h. Proteins were detected by chemiluminescence (NEN Life Science Products).

Flow cytometry

Leaves were chopped with a razor blade in CyStain UV Precise P Nuclei extraction buffer (Partec) according to the manufacturer's instructions. Nuclei were analyzed with the CyFlow MB flow cytometer with FloMax software (Partec).

Subcellular localization and bimolecular fluorescence complementation (BiFC)

A double GFP-tag (dGFP) and the N-terminal and C-terminal parts of YFP (nYFP and cYFP) were fused to HXK1 and KINγ. Constructs were transiently (co-)expressed in freshly prepared leaf mesophyll protoplasts for 6 h (dGFP) and 16 h (n/cYFP) as described by Yoo et al. (2007). GFP fluorescence and bimolecular YFP fluorescence complementation were detected by confocal imaging.

Confocal imaging

Imaging of Arabidopsis seedlings and cell cultures was performed with an Olympus FV10 ASW confocal laser scanning microscope using a 20x lens (NA 0.75) or 60x water immersion lens (NA 1.2) with up to 3x digital zoom for some images. Imaging of leaf mesophyll protoplasts was performed with an Olympus FV1000 confocal laser scanning microscope using a 40x lens with up to 3x digital zoom.

Tandem affinity purification (TAP)

Cloning of transgenes fused with the GS^{rhino} tag (Van Leene et al. 2015) under control of the constitutive cauliflower mosaic virus 35S promoter and transformation of Arabidopsis cell suspension cultures (PSB-D) with direct selection in liquid medium were carried out as previously described (Van Leene et al. 2011). TAP experiments were performed with 100 mg of total protein extract from 3-day-old cell cultures continuously grown in the presence of sucrose or from 8-day-old sucrose-starved cell cultures with or without supplementation of 3% sucrose as input, as described in Van Leene et al. (2015). Protein interactors were identified by mass spectrometry using an LTQ Orbitrap Velos mass spectrometer. Proteins with at least two matched high-confident peptides were retained. Background proteins were filtered out based on frequency of occurrence of the co-purified proteins in a large dataset containing 543 TAP experiments using 115 different baits (Van Leene et al. 2015).

Transmission electron microscopy

Leaves were immersed in a fixative solution of 2.5% glutaraldehyde, 4% formaldehyde in 0.1 M Na-cacodylate buffer, placed in a vacuum oven for 30 min and then left rotating for 3 h at room temperature. This solution was later replaced by fresh fixative and samples were left rotating overnight at 4°C. After washing, samples were post-fixed in 1% OsO4 with $K_3Fe(CN)_6$ in 0.1 M Na-cacodylate buffer, pH 7.2. Samples were dehydrated through a graded ethanol series, including a bulk staining with 2% uranyl acetate at the 50% ethanol step, followed by embedding in Spurr's resin. In order to have a larger overview of the phenotype, semi-thin sections were first cut at 0.5 µm and stained with toluidine blue. Ultrathin sections of a gold interference color were cut using an ultra-microtome (Leica EM UC6), followed by post-staining with uranyl acetate and lead citrate in a Leica EM AC20 automated contrasting system and collected on Formvar-coated copper slot grids. Two leaves of control and sucrose-treated Col-0 and *hxk1* seedlings were viewed with a JEM 1010 transmission electron microscope (JEOL,

Tokyo, Japan), operating at 80 kV, using Image Plate Technology from Ditabis (Pforzheim, Germany). For each line (Col-0 and *hxk1*), two leaves were analyzed per condition (control and sucrose) and chloroplast size and number of 27 to 87 mesophyll cells of the tip (distal) and the base (proximal) of the leaf were measured.

Statistical analysis

All analyses were performed with SAS [Version 9.4 of the SAS System for windows 7 64bit. Copyright © 2002-2012 SAS Institute Inc. Cary, NC, USA (www.sas.com)].

All growth experiments involved one, two or three factors and consisted of three independent biological repeats. For the representation of the ratios, measurements of the sucrose-treated leaves were compared with the measurements of the control leaves of the same repeat. Averages were then taken over the three independent repeats and represented in the graphs with their standard error. When needed, raw measurements were log-transformed to stabilize the variance prior to statistical analysis; this is specified in the figure legends. For all growth experiments, a linear mixed model was fitted to the variable of interest with all main factors and their interaction, in case of two factors, as fixed effects using the mixed procedure. The biological repeat term was included in each model as a random factor to take into account the correlation between observations done at the same time. In the presence of a significant F-test (for the main effect in case of one factor, for the interaction term in the case of two factors), appropriate post-hoc tests were performed. Multiple testing correction was done according with Tukey adjustment. For the time course experiment, simple tests of effects were performed at each day separately with the plm procedure.

For chloroplast-related analysis, fixed effects were genotype, treatment, tip or base and all interaction effects. A random intercept model was fitted to the mesophyll area data and log transformed chloroplast data using the mixed procedure of SAS. The Kenward-Roger method was used for computing the denominator degrees of freedom for the tests of fixed effects. For mesophyll area, one random effect was included in the model to take into account the correlations between observations originating from the same leaf. For chloroplast area, two random effects were included in the model to take into account the correlations between observations originating from the same leaf, and originating from the same mesophyll cell within leaf. The fixed part of the full model was reduced until all remaining factors were significant at the 0.05 significance level. For the mesophyll area data, the three-way interaction term was significant at the 0.05 significance level, and thus the model was not reduced. All-pairwise comparisons were calculated and p-values were adjusted using the Tukey adjustment method as implemented in SAS. For the chloroplast area, only the tip x base (TB) interaction had a significant effect. Least-square means estimates for both levels of TB were calculated as well as the difference between tip and base. A generalized linear mixed-effect model was fitted to the number of chloroplasts with the glimmix procedure of SAS assuming a Poisson distribution and a log link function. The Satterthwaite method was used for computing the denominator degrees of freedom for the tests of fixed effects. A random effect was included for leaf to take into account the correlations between observations originating from the same leaf. The fixed part of the full model was reduced until all remaining factors were significant at the 0.05 significance level. For the number of chloroplasts, only treatment had a significant effect. Least-square means estimates for both levels of treatment were calculated as well as the difference.

Results

Characterization of a sugar-insensitive *hxk1* mutant in the Col-0 genetic background

To date, most studies on HXK1 in Arabidopsis used the *gin2* mutant, identified in a genetic screen for glucose-insensitive mutants in the L*er* background (Zhou et al. 1998; Moore et al. 2003). To study the role of HXK1 during early leaf development, we selected a T-DNA mutant from the SALK collection (SALK_018086) (Alonso et al. 2003) in the Col-0 background that is extensively used for leaf growth analyses (Gonzalez et al. 2012). This insertion mutant harbors a T-DNA at the end of the first intron of the *HXK1* gene (Supplementary Fig. 1a). Homozygous plants were selected and qRT-PCR analysis demonstrated the complete loss of *HXK1* expression (Supplementary Fig. 1b).

The sugar insensitivity of the *hxk1* mutant in the Col-0 background was verified in different growth assays. First, *hxk1* seedlings did not show a developmental arrest on MS medium supplemented with 6% glucose, whereas wild-type (Col-0) seedlings exhibited reduced cotyledon expansion and root growth, and anthocyanin accumulation under this growth condition (Fig. 1a) (Moore et al. 2003). Secondly, sugar responsiveness was assessed by the sucrose-induced waving response of primary roots (Oliva and Dunand 2007). Whereas the primary root of 10-day-old wild-type seedlings grown on vertical plates with MS media supplemented with 1% sucrose showed a typical waving pattern, sucrose addition did not result in a distinct waving pattern of the primary root of *hxk1* seedlings (Fig. 1b). In addition to this absence of waving, the *hxk1* mutant seedlings generally had a reduced root system when grown on MS with or without sucrose (Fig. 1b).

The shoot growth of the *hxk1* mutant was studied by growing plants in soil under a 16-h day regime with a light intensity of approximately 100 $\mu mol/m^{-2}s^{-1}$. Twenty-two days after stratification (DAS), *hxk1* mutants showed a significant decrease in rosette area of on average 77% compared with Col-0 plants ($P < 0.0001$; Fig. 1c). This growth defect confirms the phenotype previously described for the *gin2* mutant in higher light intensities (Moore et al. 2003). All individual rosette leaves were significantly smaller and the third leaf area of *hxk1* was only 25% of that of wild-type plants ($P < 0.0001$; Fig. 1d, e). This decrease in leaf size was due to a significant reduction in both (pavement) cell number (46%) and size (61%, $P < 0.0001$; Fig. 1e).

Taken together, we show that the *hxk1* mutant in the Col-0 background exhibits a decrease in shoot and root growth and insensitivity to sugars similar to the previously described *gin2* mutant in the L*er* background. Leaves of the *hxk1* mutant grown in soil were significantly smaller with both less and smaller pavement cells, suggesting a role for HXK1 in both cell division and cell expansion.

hxk1 mutants have more pavement cells in young leaves but undergo a faster onset of cell differentiation

To further investigate the underlying cellular mechanisms responsible for the reduced leaf size of *hxk1* plants and to study the potential role of HXK1 during sucrose-induced stimulation of early leaf growth, the *in vitro* experimental setup described in Van Dingenen et al. (2016) was used in all following experiments. In this setup, the sugar status can be altered during the cell proliferation phase of the third leaf, which allows to study the function of HXK1 in a developmental context. Seedlings were first grown on meshes covering MS medium without carbon source under low light conditions (50 $\mu mol/m^{-2}s^{-1}$) for nine days. At 9 DAS, seedlings were transferred to control

medium without sucrose or to 15 mM sucrose-supplemented medium after which rosette size, leaf area, pavement cell number and cell area of the third leaf were measured from 9 to 16 DAS and at the final time point of 21 DAS. Surprisingly, in the absence of sucrose, the average final rosette size was similar in the wild-type and *hxk1* mutant plants (Fig. 2a), which is in contrast with the observed growth defect of the *hxk1* mutant grown in soil (Fig. 1c). In accordance, no difference was observed in pavement cell area and cell number between *hxk1* and wild-type third leaves on control medium (Supplementary Fig. 2). However, during early development, from 10 until 14 DAS, the third leaf area of *hxk1* seedlings was significantly larger than that of wild type ($P < 0.05$; Fig. 2b). At 10 DAS, *hxk1* leaves were 43% larger with a significantly increased number of pavement cells that were slightly, but not significantly, smaller (69%, $P < 0.05$ and 13%, $P = 0.21$ respectively; Fig. 2c).

To further examine the underlying cellular effect, pavement cell number and size were determined daily from 9 until 16 DAS (Fig. 2d, e). At 9 DAS, *hxk1* leaf primordia already contained more cells than those of the wild type albeit not signifcant(17%, $P = 0.21$; Fig. 2d inset), and the cell number was significantly increased at 10 DAS to 14 DAS (Fig. 2d), whereas no difference in the average cell size was observed (Fig. 2e). The positive effect on total pavement cell number was further investigated in the homozygous *pCYCB1;1::CYCB1;1-D-box:GUS*/*hxk1* line obtained after the crossing of *hxk1* with the *pCYCB1;1::CYCB1;1-D-box:GUS* reporter line that allows the visualization of mitotically active cells. At 14 DAS, only a weak GUS staining could be detected for the *pCYCB1;1::CYCB1;1-D-box:GUS/hxk1* mutant line, whereas *pCYCB1;1::CYCB1;1-D-box:GUS* seedlings demonstrated a strong staining at the base of the third leaf (Fig. 2f). Also, the *pCYCB1;1::CYCB1;1-D-box:GUS*/*hxk1* leaves showed weaker GUS activity as well as a cell cycle arrest front of *pCYCB1;1::CYCB1;1-D-box:GUS*/*hxk1* leaves was closer to the leaf base compared with that of *pCYCB1;1::CYCB1;1-D-box:GUS* control leaves (Fig. 2f). These observations suggest that, at this time point, almost all cells of *hxk1* leaves showed a reduced cell division activity and/or stopped dividing entirely, whereas wild-type leave cells were still actively dividing at the base of the leaf. Similar GUS staining profiles were observed at 12 DAS (Supplementary Fig. 3). To further examine cellular differentiation, ploidy levels in the third leaves of *hxk1* and wild-type seedlings were determined by flow cytometry daily from 11 to 17 DAS. *hxk1* leaves had an increased endoreduplication index, i.e. the average number of endocycles a cell undergoes, between 11 and 13 DAS, indicating that *hxk1* cells have higher ploidy levels than wild-type cells (Supplementary Fig. 4). In accordance, decreased 2C levels and increased 4C levels were observed at the same time points (Supplementary Fig. 4).

In conclusion, early during development, leaves of *hxk1* seedlings grown *in vitro* without sucrose are larger as a result of more but not larger cells, although *hxk1* leaf cells stop dividing and start differentiating earlier than those of the wild type.

hxk1 seedlings are insensitive to sucrose-induced cell proliferation during early leaf development

We have previously shown that transfer of young seedlings to sucrose-containing medium stimulates cell proliferation and increases final leaf size in wild-type plants. To investigate whether HXK1 plays a role in this process, *hxk1* and wild-type seedlings were transferred to 15 mM sucrose-supplemented medium or control medium at 9 DAS and leaf area, pavement cell number and cell size of the third leaves were measured.

At 21 DAS, the third leaf area of wild-type plants transferred to sucrose was significantly increased by 43% ($P < 0.05$) compared with wild-type plants grown without sucrose, whereas the third leaf area of the *hxk1* plants was not significantly increased (11%, $P = 0.57$; Fig. 3a). Subsequently, a time-course leaf area measurement experiment was performed by harvesting the third leaf daily after transfer to sucrose from 10 until 21 DAS. In wild-type seedlings, the third leaf of plants transferred to sucrose was significantly larger than that of control plants starting from 12 DAS, corresponding to three days after transfer (37%, $P < 0.05$), and remained larger until 21 DAS (Fig. 3b). Also for the *hxk1* mutant, the third leaf size was significantly increased by sucrose at 12, 14, 16 and 18 DAS, but this effect was less pronounced than in wild-type plants (Fig. 3c). The increased leaf area of wild-type plants at 21 DAS was mainly due to a significantly higher number of cells (37%, $P < 0.05$; Fig. 3d, Supplementary Fig. 5). In the *hxk1* mutant, the cell number was not significantly increased (11%, $P = 0.70$; Fig. 3d and Supplementary Fig. 5). Average cell size of wild-type and *hxk1* leaves was unaffected by transfer to sucrose ($P > 0.05$). The stomatal index was slightly increased in wild-type leaves (8%, $P < 0.05$) but did not change in the *hxk1* mutant ($P > 0.05$) upon transfer to sucrose. The positive effect of sucrose on cell proliferation was already significant at 10 DAS (24 h after transfer) in wild-type seedlings (41%; $P < 0.05$; Fig. 3e), whereas for *hxk1* mutant leaves, transfer to sucrose did not significantly increase pavement cell number (16%; $P = 0.13$) at 10 DAS. Also at later time points, the positive effect on cell number observed in wild-type leaves was not found in *hxk1* mutant leaves, except at 16 DAS (41%, $P < 0.05$; Fig. 3e). In addition, similar to wild-type seedlings, no consistent effect in the average cell size was found between control and sucrose-transferred *hxk1* seedlings (10-16 DAS; Fig. 3f).

In conclusion, *hxk1* mutants are less sensitive to sucrose induction of cell proliferation than wild-type plants, showing no significant increase in final leaf size after transfer to sucrose.

hxk1 mutant leaves have larger chloroplasts upon transfer to sucrose

Expression of *GPT2,* encoding a chloroplastic glucose-6-phosphate/phosphate transporter*,* is necessary for the sucrose-induced stimulation of cell proliferation at 10 DAS (Van Dingenen et al. 2016). To examine whether *GPT2* expression was compromised in *hxk1* seedlings, transcript levels were determined by qRT-PCR analysis in shoots of seedlings 24 h after transfer (Supplementary Fig. 6). Interestingly, shoots of wild-type and *hxk1* seedlings had similar *GPT2* transcript levels when transferred to control medium, and also upon transfer to sucrose, *GPT2* expression was similarly upregulated in both lines.

As described previously (Van Dingenen et al. 2016), transfer of wild-type seedlings to sucrose-supplemented medium increases cell number and cells have fewer, smaller and less differentiated chloroplasts at 10 DAS. The effect of sucrose on *hxk1* seedling chloroplast number and size was examined by transmission electron microscopy. Transverse cross-sections of wild-type and *hxk1* leaves of seedlings transferred to control or sucrose-containing media were made two days after transfer (11 DAS). Mesophyll cell area, chloroplast number and chloroplast size were measured taking into account the differences between the tip and the base of the leaf. The average mesophyll cell size did not differ between wild-type and *hxk1* leaves transferred to control medium and cells were larger at the tip than at the base of the leaf ($P < 0.05$; Fig. 4a). Sucrose treatment led to a significantly decreased mesophyll cell area (26%, $P < 0.05$) in wild-type leaves at the tip but had no effect in *hxk1* leaves (Fig. 4a), confirming the observations made for epidermal cells. No clear difference in chloroplast ultrastructure and shape could be observed between wild-type and *hxk1* seedlings or sucrose and control conditions (Fig. 4b). However, chloroplasts

at the tip of sucrose-transferred *hxk1* leaves showed slightly increased grana stacking compared with all other conditions (Fig. 4b). For both seedlings and conditions, chloroplasts were larger at the tip than at the base of the leaves ($P < 0.05$; Fig. 4c). Furthermore, in *hxk1* leaves, transfer to sucrose resulted in approximately 50% larger chloroplasts, although this was not statistically significant due to large variation. Finally, chloroplast number per cell did not differ significantly between wild-type and *hxk1* seedlings in control conditions and transfer to sucrose resulted in significantly less chloroplasts in both seedlings (Fig. 4d).

In summary, transfer of seedlings to sucrose resulted in smaller mesophyll cells with less and smaller chloroplasts in wild-type leaves, whereas no difference in mesophyll cell size but larger chloroplasts were found in *hxk1* leaves after transfer to sucrose.

Expression of *35S::HXK1-GS^{green}* restores growth and sugar responsiveness in *hxk1* plants

To identify novel interaction partners of HXK1, TAP experiments were performed with the HXK1-GS^{rhino} tagged fusion protein. The functionality of the construct was tested by transforming *hxk1* plants with a similar vector, *35S::HXK1-GS^{green}*. These tags are derivatives of the TAP tag GS (Van Leene et al. 2008). GS^{rhino} consists of the protein-G (G) tag and the streptavidin-binding peptide (S) separated by rhinovirus 3C protease cleavage sites (Van Leene et al. 2015). In the GS^{green} TAP-tag, the protein-G tag in GS^{rhino} has been replaced by GFP, allowing also *in vivo* visualization of the bait protein. Both tags were C-terminally fused to HXK1 because the N-terminal domain contains a mitochondrion anchor domain (Balasubramanian et al. 2007). Both constructs were expressed under the control of the 35S promoter.

The abundance of the GS^{green}-tagged HXK1 protein was tested in 9-day-old seedlings by western blot analysis and showed to be high in two independent T2 transgenic lines (Supplementary Fig. 7a). One of the lines was selected for further upscaling and the overexpression of *HXK1* was confirmed in 10-day-old T3 seedlings with qRT-PCR analysis (Supplementary Fig. 7b). Next, the *hxk1* mutant complementation was verified. As described above, the average rosette size of the *hxk1* mutant was reduced when grown in soil under a light intensity of approximately 100 µmol/m^{-2}s^{-1}. Mutant plants expressing the *35S::HXK1-GS^{green}* vector showed a rosette growth similar to that of wild-type plants (Fig. 5a). To investigate the sugar responsiveness of the *35S::HXK1-GS^{green}*/*hxk1* line, the seedlings were grown on 6% glucose-containing medium. The transgenic line showed a hypersensitive response with almost complete inhibition of cotyledon expansion and chlorophyll accumulation, with high anthocyanin production and reduced root growth compared with *hxk1* mutant seedlings, which were insensitive to the high glucose concentrations (Fig. 5b). Finally, the root waving pattern of 10-day old seedlings grown on vertical plates with 1 % sucrose was checked. The typical waving pattern, which was absent in the *hxk1* mutant, was partially restored in the *hxk1* mutant expressing the *35::HXK1-GS^{green}* vector (Fig. 5c).

Next, the localization of the HXK1-GS^{green} fusion protein was verified in roots and cotyledons of 5-day-old *35S::HXK1-GS^{green}*/*hxk1* seedlings. Fluorescence was detected in both roots and cotyledons, with an apparent localization at the mitochondria, which was confirmed by co-localization with the mitochondria-specific Mito tracker red dye (Supplementary Fig. 8). Interestingly, in the cotyledons, the two signals of the HXK1-GS^{green} fusion protein and the Mito tracker red mainly merged in larger foci, as previously described (Balasubramanian et al. 2007), and that probably represent aggregated mitochondria.

In conclusion, expression of the GS-tagged HXK1 under control of the 35S promoter in the *hxk1* mutant background restores growth and sugar sensitivity and produces HXK1 proteins with normal mitochondrial localization. These results confirm (1) that the construct is functional, and (2) that the *hxk1* T-DNA mutant phenotype is indeed caused by HXK1 deficiency.

Identification of sucrose-responsive interaction partners of HXK1

To identify novel and sugar-dependent HXK1 interactors, HXK1 was used as bait in TAP experiments and consecutive mass spectrometry analyses. TAP experiments were done on Arabidopsis suspension cells expressing HXK1 C-terminally fused to the GS^{rhino} TAP tag (Van Leene et al. 2008) with two biological repeats from cultures grown in three different growth conditions. In the first condition, Arabidopsis suspension cells were continuously grown in the presence of 3% sucrose. In the second condition, cell cultures were sucrose-starved for 24 h. In the third condition, the sucrose-starved cell cultures were resupplied with 3% sucrose for 15 min. From the three different growth conditions, a total of 23 co-purified proteins could be retained after subtraction of specific background proteins based on the procedure described in the Material and methods (Supplementary Table 1) but only seven proteins were found in both biological repeats (Table 1). TAP performed on sucrose-starved cell cultures resulted in the isolation of six proteins, from which five were also found after re-supplementation of sucrose. Two of these five overlapping proteins are mitochondrial inner membrane proteins, the alternative oxidase 1A (AOX1A; AT3G22370) and a leucine zipper-EF-hand-containing transmembrane protein (LETM1; AT3G59820) (Zhang et al. 2012). A similar purification pattern was found for two plasma membrane proteins, a non-race-specific disease resistance (NDR)-like protein/tobacco hairpin-induced gene (HIN)-like protein (AT5G06320) and a senescence/dehydration-associated protein EARLY-RESPONSIVE TO DEHYDRATION7 (ERD7; AT2G17840). The fifth protein found to interact with HXK1 in both conditions was a histone H2A protein, HTA6 (AT5G59870). KINγ (AT3G48530) was the only protein isolated twice after sucrose starvation and not after re-supplementation of sucrose for 15 min. Only one protein was exclusively isolated after sucrose addition in both repeats, a quinone reductase family protein (AT4G36750).

Taken together, only two of the seven proteins pulled-down with HXK1-GS^{rhino} were isolated twice independently in specific growth conditions, a quinone reductase family protein and KINγ.

KINγ interacts with HXK1 and possibly affects its regulation of plant growth

From the TAP experiments, we found that KINγ (AT3G48530) is a possible HXK1 protein partner, putatively involved in sugar responses (Table 1). Based on its structure with a conserved Four CBS domain (FCD), KINγ was originally suggested to act as a regulatory γ subunit of the plant heterotrimeric SnRK1 complex (Gissot et al. 2006). However, KINγ is one of several more distantly related plant γ-like FCD proteins (belonging to the FCDIb family), while a hybrid βγ (FCDIa family) protein acts as the canonical SnRK1 complex γ subunit (Ramon et al. 2013; Emanuelle et al. 2015). The exact function of KINγ therefore remains unknown.

If HXK1 and KINγ interact or are part of the same complex, they should co-localize. According to the webtool SUBA3 (http://suba3.plantenergy.uwa.edu.au/), KINγ is predicted to be localized in the cytosol. Cytosolic localization of KINγ was confirmed for both C- and N-terminal KINγ fusion proteins in 8-days-old Arabidopsis

cell cultures (Supplementary Fig. 9) and in leaf mesophyll protoplasts transiently expressing a double GFP-tagged KINγ protein (Fig. 6a). GFP fluorescence of the HXK1-GS^{green} fusion protein was detected in small and large foci possibly representing mitochondria (Supplementary Fig. 8) and also in leaf mesophyll protoplasts, HXK1 was observed to be localized at mitochondria (Fig. 6a). No co-localization could be detected for HXK1-GS^{green} and KINγ-RFP fusion proteins, whereas the RFP-KINγ fusion protein co-localized with HXK1-GS^{green} in some cells (Supplementary Fig. 9). Interaction of HXK1 and KINγ at the mitochondria was confirmed with bimolecular fluorescence complementation assays using leaf mesophyll protoplasts co-expressing *HXK1* and *KINγ* with respectively a C-terminal nYFP- or cYFP-tag (Fig. 6b).

To explore whether KINγ is involved plant growth regulation, *kinγ* null mutant seedlings (SALK_074554) (Ramon et al. 2013) were grown in soil under a light intensity of approximately 100 µmol/m^{-2}s^{-1}. At 22 DAS, rosettes of *kinγ* mutant plants were slightly but significantly larger than wild-type plants (9%, $P < 0.05$; Supplementary Fig. 10a) suggesting a role of KINγ as a negative regulator of growth under these environmental conditions. To study whether KINγ is involved in the sucrose-induced leaf growth stimulation, *kinγ* seedlings were subjected to the experimental sucrose assay described above under a light intensity of 50 µmol/m^{-2}s^{-1} (Van Dingenen et al. 2016). At 21 DAS, the average third leaf area was significantly increased upon transfer to sucrose in both wild-type (60%, $P < 0.05$) and *kinγ* mutant plants (46%, $P < 0.05$). No difference in the effect on final third leaf size could be observed ($P = 0.74$; Supplementary Fig. 10b). To further study the possible genetic interaction between KINγ and HXK1 during plant growth, we created a *hxk1 kinγ* double mutant and measured rosette sizes of the single and double mutants grown in soil under a higher light intensity of approximately 135 µmol/m^{-2}s^{-1}. At 22 DAS, *hxk1* single mutants demonstrated a severe growth defect (Fig. 6c), which confirms the phenotype previously described for the *gin2* mutant in high light intensities (Moore et al. 2003). Rosettes of *kinγ* single mutants were again slightly larger (although not statistically significant) than wild-type plants (10%, P=0.06; Fig. 6c), whereas the rosettes of the *hxk1 kinγ* double knock-out plants demonstrated a very similar growth reduction as the *hxk1* single mutant with an average decrease of 93% compared with wild-type rosettes ($P < 0.001$; Fig. 6c).

In summary, we identified and confirmed KINγ as a novel HXK1 interactor. Whereas KINγ does not appear to have a role in the sucrose stimulation of leaf growth, the physical interaction, the slight increase in rosette size of the *kinγ* mutant, and the growth defect of the double mutant suggest that KINγ might act in the regulation of plant growth, together with HXK1, under specific environmental conditions.

Discussion

The *gin2/hxk1* mutant was selected from a large ethyl methanesulfonate-mutagenized seed collection in the L*er* genetic background as one of several glucose-insensitive mutants that do not show seedling growth arrest on 6% glucose medium (Zhou et al. 1998; Moore et al. 2003). Conversely, soil-grown *gin2* mutant plants do show an overall reduction in growth and delay in senescence in high light (300 µmol/m^{-2}s^{-1}) conditions, indicating that HXK1 is also required for growth promotion in response to increased endogenous sugar supply.

We found a similar glucose insensitive seedling phenotype for a *hxk1* T-DNA insertion mutant (SALK_018086) in the Col-0 background. Furthermore, loss of *HXK1* expression in the Col-0 background also results in a significant growth inhibition of soil-grown plants, even in the absence of high light. We show that the

role of HXK1 in the growth control is complex and that the reduced leaf size of soil-grown *hxk1* plants is due to both less and smaller cells. In order to explore in more detail whether and how HXK1 is important for the increase in leaf growth upon increased sugar supply, a previously described experimental *in vitro* setup with transfer of seedlings to sucrose was used (Van Dingenen et al. 2016). Under control growth conditions, i.e. a low light intensity of approximately 50 µmol/$m^{-2}s^{-1}$ and no exogenously supplied sugar, both final rosette size and third leaf area of the *hxk1* mutant were similar to those of wild-type plants. Therefore, these growth conditions can be used to monitor the role of HXK1 during early leaf development without drastic metabolic disturbance. Interestingly, differences in size between wild-type and *hxk1* leaves were found at early time points (9-14 DAS), whereas no difference could be detected at the mature stage (21 DAS) under these control conditions. At the early developmental stages, i.e. 9-10 DAS, leaves mainly grow by cell proliferation and act as sink tissues (Andriankaja et al. 2012). Leaves of *hxk1* mutants contained more cells compared with wild-type plants, suggesting that HXK1 plays a role in limiting cell proliferation in sink tissues to fine-tune growth. Increases in cell number are often accompanied by a delay in the transition to cell expansion (Gonzalez et al. 2012). However, *hxk1* leaves showed a decreased intensity and smaller region of *pCYCB1;1::CYCB1;1-D-box:GUS* marker gene expression at 12 and 14 DAS, indicating a reduction in mitotic cell cycle activity. This reduction is in contrast to the increased cell number observed at earlier time points (9-10 DAS). One possibility to explain this could be that the *hxk1* mutant already has more cells during leaf primordium formation. Recruitment of more cells to the shoot apical meristem has been demonstrated to be one of the mechanisms that can contribute to an increased leaf size (Eloy et al. 2012; Gonzalez et al. 2012). Furthermore, the increased endoreduplication index observed during the transition phase (12-13 DAS) further indicates that at least some cells of *hxk1* leaves on average stop dividing and are triggered to differentiate earlier than in wild-type plants. The onset of the transition to cell differentiation and expansion was found to be preceded by the establishment of the photosynthetic machinery (Andriankaja et al. 2012). It is well known that HXK1 represses nuclear-encoded photosynthesis-related gene expression, feedback-inhibiting photosynthesis (Jang et al. 1997; Moore et al. 2003). Because sugars are unable to repress the transcription of photosynthesis-related genes in the *gin2* mutant (Moore et al. 2003), it is possible that sink leaves undergo an accelerated transition to cell expansion. However, no difference in epidermal cell size and in chloroplast shape, size or ultrastructure was observed between wild-type and *hxk1* control leaves. These findings suggest that in sink cells, HXK1-mediated signaling does not affect the establishment of the photosynthetic machinery, which consequently could affect the onset of cell expansion.

At 9 DAS, when the third leaf was still fully proliferating, seedlings were transferred to sucrose-supplemented medium. In wild-type plants, these higher sucrose levels result in the repression of transcription of the plastome, a delay in chloroplast development, and the stimulation of cell proliferation via the sucrose-induced expression of *GPT2* (Van Dingenen et al. 2016). However, *GPT2* transcript levels were similar in wild-type and *hxk1* shoots grown in control conditions and *GPT2* was similarly induced by sucrose in both lines. Also in the L*er* background, sugar treatment did not result in a different response in *GPT2* expression between wild-type and *gin2* plants (Heinrichs et al. 2012). These findings indicate that HXK1 does not play a role in the regulation of *GPT2* expression. Furthermore, sucrose was found to repress chloroplast differentiation in wild-type sink leaves because smaller chloroplasts with less differentiated thylakoid membranes and starch granules have been observed (Van Dingenen et al. 2016). Contrastingly, in *hxk1* leaves, transfer to sucrose resulted in larger chloroplasts, particularly in the tip of the leaf. Higher sucrose levels in the cell might result in higher glucose and glucose-6-phosphate (G6P)

levels in both wild-type and *hxk1* leaves, which can then be imported in the chloroplasts via *GPT2*, resulting in a repression of plastome transcription. It has been described that the *gin2* mutant maintains half of the glucose phosphorylation capacity of wild-type plants, but shows increased glucose-6-phosphate (G6P) levels (Moore et al. 2003). G6P can be generated by other HXKs, such as HXK2 and HXK3 (Karve et al. 2008), or can be formed from F6P by phosphoglucoisomerase (Fettke and Fernie 2015). *hxk1*-silenced plants are known to induce nuclear-encoded photosynthesis gene expression independent of the presence of sugars (Jang et al. 1997). In addition, transfer to sucrose did not stimulate cell proliferation in the *hxk1* mutant, which suggests that the sucrose-induced repression of chloroplast differentiation through *GPT2* is overruled by the induction of photosynthesis through downregulation of *HXK1*.

Interestingly, pavement cell number was found to be increased by sucrose at later time points in the *hxk1* mutant. At these time points, most cellular divisions result from the stomatal lineage (Gonzalez et al. 2012). So, this late increase in cell number might point to an induction of asymmetric meristemoid division by sucrose. Meristemoids are stomatal precursor cells that divide asymmetrically, resulting in the formation of additional pavement cells during leaf development (Geisler et al. 2000). In wild-type plants, sucrose addition increases the stomatal index in leaves (Van Dingenen et al. 2016), but this was not observed in the *hxk1* mutant. Higher sucrose levels might trigger HXK1 signaling to generate stomata from meristemoids. Impairment in HXK1 signaling might result in more asymmetric divisions forming additional pavement cells instead of stomata at later time points.

To further elucidate how HXK1 exactly regulates leaf growth, we set out to identify novel HXK1 protein interactors involved in sugar-mediated signaling. Recently, it has been shown that glucose-bound and unbound HXK1 exhibit conformational differences explaining the dual functions of HXK1 and suggesting different protein-protein interactions depending on sugar availability (Feng et al. 2015). A total of seven different proteins were found to co-purify with tagged HXK1 in cell cultures. However, some of the putative interactors have a different predicted localization than the mitochondria-associated and nuclear HXK1. For example, two mitochondrial inner-membrane proteins, AOX1A and the LETM1-like protein, were found to interact with HXK1 when sucrose was supplemented to cell cultures. AOX1A is the major isoform of alternative oxidases in Arabidopsis involved in the alternative respiration pathway to reduce ROS and ATP production (Millar et al. 2011). Different stresses are known to induce *AOX1A* expression and a role of AOX1A in mitochondrial retrograde signaling has been demonstrated (Rasmusson et al. 2009; Van Aken et al. 2009). LETM1 has been described to act redundantly with LETM2 in maintaining mitochondrial function (Zhang et al. 2012). Co-purification of these two inner-membrane mitochondrial proteins is probably due to the use of a mild detergent to solubilize membranes in the TAP protocol, resulting in strong association between membrane proteins that normally do not co-localize (Van Leene et al. 2015). However, these interactions also hint at the main metabolic function of HXK1. Higher sucrose levels stimulate HXK1 to phosphorylate glucose and subsequently generate pyruvate in glycolysis to fuel mitochondrial respiration (Fernie et al. 2004).

One protein, KINγ, was reproducibly found to interact with HXK1 when cells were sucrose-starved. We could also confirm interaction between HXK1 and KINγ in the cytosol of leaf mesophyll protoplasts. KINγ was originally described as a putative regulatory ɣ subunit of the SnRK1 heterotrimeric complex based on its structure with a conserved FCD (Bouly et al. 1999; Ramon et al. 2013). In yeast SNF1 and animal AMPK, the ɣ subunit acts as the energy-sensing module, binding adenosine nucleotides (Xiao et al. 2011; Broeckx et al. 2016). *KINγ* expression is induced in the dark, suggesting a role in carbon starvation signaling, but it does not complement the

yeast *snf4* (γ subunit) mutant growth defect and does not take part in heterotrimeric SnRK1 complexes with α and β subunits (Bouly et al. 1999; Ramon et al. 2013; Emanuelle et al. 2015). Moreover, *kinγ* mutant plants were not affected in starvation- and SnRK1-induced gene expression responses. Instead, plants use a unique hybrid βγ protein as the canonical SnRK1 complex γ subunit (Ramon et al. 2013; Emanuelle et al. 2015). KINγ belongs to a separate monophyletic clade of FCD proteins (FCD-Ib) (Ramon et al. 2013) and its function is still unclear. Other plant FCD γ-like proteins (FCD-Ic) have been implicated in seed stress responses (Bradford et al. 2003; Rosnoblet et al. 2007; Bolingue et al. 2010) and reproductive development (Fang et al. 2011), but direct evidence for a link with SnRK1 signaling is still missing. In this study, we found a putative link between KINγ and HXK1 function. *kinγ* mutant seedlings were still sensitive to sucrose when grown *in vitro* and transfer to sucrose resulted in a similar increase in final leaf size as for wild-type plants, indicating that KINγ is not involved in sucrose stimulation of leaf growth. However, the rosette area of *kinγ* mutants was slightly larger than that of wild-type plants, whereas the *hxk1 kiny* double knock out mutant demonstrated a similar growth defect as the *hxk1* single mutant under higher light intensities. These phenotypes suggest that KINγ might act upstream of and negatively regulate HXK1 function. KINγ might be part of an additional sensing system, which is only active in specific conditions, hence the effect of the different light intensities. Mutant phenotypes then would only become apparent in these specific (stress) conditions or in other mutant backgrounds. Additional experiments are necessary to test the involvement of KINγ in HXK1-dependent growth regulation, the stress responsiveness of the *KINγ* single mutant, and to shed more light on the exact function of KINγ as a, possibly stress-responsive, growth regulator.

In conclusion, we show that HXK1 regulates both cell proliferation and cell expansion early during leaf development, and that HXK1 is important for sucrose-stimulated leaf growth, independent of *GPT2* induction or repression of chloroplast differentiation. In addition, we identified the FCD protein KINγ (AT3G48530) as a novel interacting protein of HXK1 function.

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Tables

Table 1 Proteins co-purified with HXK1 in cell cultures grown in three different growth conditions. TAPs were performed on *Arabidopsis* cell cultures continuously grown in the presence of sucrose (cell cultures), on cell cultures that were sucrose-starved for 24 h (sucr starvation) and on sucrose-starved cells resupplied with 3% sucrose for 15 min (3% sucr). The numbers indicate the number of experiments in which the protein was identified. TAPs were performed in two independent repeats.

			sucr starvation	**3% sucr**	**cell cultures**
Gene ID	**Name**	**Description**			
AT4G29130	HXK1	Hexokinase1	2	2	2
AT3G48530	KINγ	Four cystathionine β-synthase (CBS) domain protein	2		
AT5G59870	HTA6	Histone H2A 6	2	1	
AT4G36750		Quinone reductase family protein		2	
AT2G17840	ERD7	Senescence/dehydration-associated protein-related	1	2	
AT3G22370	AOX1A	alternative oxidase 1A	1	2	
AT3G59820	LETM1	LETM1-like protein	1	2	
AT5G06320	NHL3	NDR1/HIN1-like 3	1	2	

FIGURE LEGENDS

Fig. 1 *hxk1* plants are insensitive to sugars and exhibit growth defects. **a** Eight-day-old seedlings grown on MS medium supplemented with 6% glucose. **b** Ten-day-old seedlings grown on vertical plates without sucrose (-S) or with 1% sucrose (+S). **c-e** *hxk1* mutant and wild-type (Col-0) plants were grown in soil for 22 days after stratification (DAS) under a light intensity of approximately 100 µmol/$m^{-2}s^{-1}$. Rosette area (**c**) and individual leaf area (**d**) were measured at 22 DAS. Cot = cotyledons; Lx = leaf position x in the order of appearance on the rosette. **e** Ratio of the third leaf size, pavement cell area and total cell number of *hxk1* compared with wild-type. Values are means of three biological repeats with their SE. Rosette and leaf area was measured for 10 to 12 plants in each repeat. Cellular data are from three leaves in each repeat. *, $P < 0.0001$ compared with wild-type, mixed models.

Fig. 2 *hxk1* leaves have more cells and undergo faster differentiation early during leaf development. Wild-type (Col-0) and *hxk1* mutant seedlings were grown on medium without sucrose. **a** Rosette area at 21 DAS of wild-type and *hxk1* plants. **b** Third leaf area from 9 to 16 DAS of wild-type and *hxk1* seedlings. **c** Ratio of leaf area, pavement cell area and total cell number of *hxk1* leaves compared with wild-type at 10 DAS. **d, e** Pavement cell number (**d**) and cell area (**e**) in 9 to 16 DAS of wild-type and *hxk1* leaves. **f** GUS-stained third leaves at 14 DAS of *pCYCB1;1::CYCB1;1-D-box:GUS* in Col-0 and *hxk1* background seedlings and GUS intensity quantification along the leaf. GUS staining was quantified in a defined region from the base to the tip of each leaf as described by Vercruyssen et al. (2014). The red line indicates the cell cycle arrest front. Values from (**a**) and (**b**) are the means of three biological repeats with their SE. Rosette and leaf area were measured for on average 12 leaves in each repeat. Cellular parameters were measured for four to five leaves in each repeat. Values in (**c**) and (**e**) are the means of four to eight leaves with their SE. Values in (**f**) are the means of two repeats with their SE. GUS intensity was measured for eight to ten leaves in each repeat. *, $P < 0.05$ for log-transformed values in (**b**) to (**c**) for *hxk1* compared to wild-type, mixed models and *, $P < 0.05$ in (d) and (e) for *hxk1* compared to wild-type, Student's t-test.

Fig. 3 *hxk1* leaves are insensitive to sucrose early during leaf development. Wild-type (Col-0) and *hxk1* mutant seedlings were grown on medium without sucrose and, at 9 DAS, transferred to medium without (control) or supplemented with 15 mM sucrose (sucr). **a** Third leaf area at 21 DAS of wild-type and *hxk1* plants transferred to control and sucrose. **b** Leaf area from 10 DAS until 21 DAS of wild-type (**b)** and *hxk1* plants (**c**) transferred to control or sucrose-supplemented medium. The insets are close-ups of 12 and 13 DAS. **d** Ratio of pavement cell area, cell number and stomatal index of the third leaf of wild-type (black) and *hxk1* (blue) plants transferred to sucrose relative to the control, at 21 DAS. **e, f** Ratio of epidermal cell number (**e**) and cell area (**f**) from 10 until 16 DAS of the third leaf of wild-type and *hxk1* plants transferred to sucrose relative to the control conditions without sucrose. Values from (**a**) to (**d**) are the means of three biological repeats with their SE. Rosette and leaf area was measured for on average 12 leaves in each repeat. Cellular parameters were measured for four to five leaves in each repeat. Values in (**e**) and (**f**) are the means from four to nine leaves with their SE. *, $P < 0.05$ for log-transformed values in (**a**) for *hxk1* compared to wild-type and (**b**) to (**f**) for sucrose compared to control, mixed models.

Fig. 4 Mesophyll cell area, chloroplast size and chloroplast number in wild-type (Col-0) and *hxk1* leaves upon transfer to sucrose. Wild-type and *hxk1* leaves were grown on control medium without sucrose for nine days, and subsequently transferred to control or 15 mM sucrose (sucr)-supplemented media for two additional days. Average mesophyll cell area (**a**), average chloroplast size (**c**) and chloroplast number (**d**) in the tip and base of the third leaf of control and sucrose-treated wild-type (white=base and gray=tip) and *hxk1* (light green=base and dark green=tip) seedlings at 11 DAS. **b** Transmission electron micrographs of tip and base of the 11-day-old third leaves of wild-type and *hxk1* seedlings after transfer to control or 15 mM sucrose (sucr)-supplemented medium. The bar represents 1 µm. Values are means ± SE from 27-87 mesophyll cells of the tip and the base of two independent leaves. *, $P < 0.05$ for tip compared to base and a, $P < 0.05$ for sucrose compared to control leaves, mixed models.

Fig. 5 Complementation of *hxk1* mutation with the 35S::HXK1-GS^{green} fusion protein. **a** Twenty two-day-old wild-type, *hxk1* and *35S::HXK1-GS^{green}/hxk1* plants grown in soil under a light intensity of approximately 100 µmol/m^{-2}s^{-1}. **b** Eight-day-old wild-type, *hxk1* and *35S::HXK1-GS^{green}/hxk1* seedlings grown on vertical plates supplemented with 6% glucose. **c** Ten-day-old wild-type, *hxk1* and *35S::HXK1-GS^{green}/hxk1* seedlings grown on vertical plates with 1% sucrose.

Fig. 6 HXK1 and KINγ localization, interaction and phenotype. **a** Fluorescence microscopy of transiently overexpressed *HXK1-dGFP* and *KINγ-dGFP* in Arabidopsis leaf mesophyll protoplasts, 6 h after transfection. **b** Bimolecular fluorescence complementation by transient overexpression of nYFP- and cYFP-fused HXK1 and KINγ in Arabidopsis leaf mesophyll protoplasts 16 h after transfection (different focal planes). **c** Plants were grown in soil for 22 days under a light intensity of approximately 135 µmol m^{-2} s^{-1} and ratio of rosette area of single *hxk1*, *kinγ* and double *hxk1 kinγ* mutants compared with wild-type (Col-0) is given. Values are the means of three biological repeats with their SE. Rosette area was measured for seven to 12 rosettes in each repeat. *, $P < 0.001$, mixed models.